Proceedings of the
Fifth International Conference on
Plant Pathogenic Bacteria

Centro Internacional de Agricultura Tropical

1981

Proceedings of the
Fifth International Conference
on
Plant Pathogenic Bacteria

August 16-23, 1981 at CIAT, Cali, Colombia

Technical Editor
J. Carlos Lozano
CIAT Colombia, S.A.

Production Editor
Paul Gwin
Professor Emeritus
Agricultural Editor's Office
University of Missouri-Columbia

COMMITTEES

Local Organization Committee:
Chairman: J. C. Lozano
Secretary: R. Zeigler
Other members: F. J. Morales
                 J. I. Victoria
                 M. A. Pastor-Corrales
                 S. W. Ahn
                 J. Lenné

International Society for Plant Pathology, Bacterial Section:
Chairman: J. M. Young (New Zealand)
Other members: J. C. Lozano (Colombia)
                 S. Süle (Hungary)
                 A. K. Vidaver (United States)
                 M. Ridé (France)
                 K. Rudolph (Germany)

Planning Committee for next meeting, Beltsville, MD., U.S.A., August 1985:
Chairman: L. Sequeira
Other members: R. de L. D. Ribeiro
                 E. L. Civerolo
                 M. C. M. Perombelon
                 J. C. Lozano
                 N. J. Panopoulos

LIST OF PARTICIPANTS

Argentina

Gotuzzo, Esther Atlas de. Facultad de Agronomía de Buenos Aires. Av. San Martín 4453, Buenos Aires (1417), Argentina.

Zagory, Devon. Inta-Bella Vista, 3432 Bella Vista, Corrientes, Argentina.

Belgium

Maraite, Henry. Laboratoire de Phytopathologie, Place Croix du Sud 3, B 1348 Louvain-La-Neuve, Belgium.

Verhoyen, Michel. Centre D'Etudes des Phytobacterioses. Place Croix du Sud 3. B-1348 Louvain-La-Neuve, Belgium.

Brazil

Athayde, Jose Tadeu. EMCAPA-LAB, Fitopatología. Caixa Postal 391. 29.000-Vitoria Espirito Santo, Brazil.

Ribeiro, Raul de L. D. U.F.R.R.J. Km 47 Antiga Rod. 23460 Seropedica-Itaguai-R.J. Brazil

Robbs, Charles. Universidad Federal Rural Do Rio de Janeiro. 23.460 Seropedica (Itaguai). R. J. Brazil.

Canada

De Boer, Solke H. Agriculture Canada, 6660 N. W. Marine Drive. Vancouver, B.C. Canada V6T 1X2.

Dhavantari, B. N. Agriculture Canada, Res. Station. Harrow, Ontario. Canada, Nor 1GO.

Chile

Ciampi-Panno, Luigi. Instituto de Ecología. Universidad Austral de Chile. Casilla 567, Valdivia, Chile.

Colombia

Afanador, Lucía. CIAT, Apartado Aéreo 6713. Cali, Colombia, S.A.

Ahn, Sang-Won. CIAT, Apartado Aéreo 6713. Cali, Colombia. S.A.

Beebe, S.E. CIAT. Apartado Aéreo 6713, Cali, Colombia, S.A.

Bernal, Blanca. CIAT, Apartado Aéreo 6713. Cali, Colombia, S.A.

Bermudez, Alonso. CIAT, Apartado Aéreo 6713, Cali, Colombia, S.A.

Bravo, Nelson. Universidad Nacional, Facultad de Agronomía, Palmira, Colombia, S.A.

Buitrago, Hilda Lucía. GTZ, Investigador Cenicafé. Chinchiná, Colombia, S.A.

Buruchara, Robin. CIAT, Apartado Aéreo 6713. Cali, Colombia, S.A.

Japan

Goto, Masao. Shizuoka University. 836 Ohya, Shizuoka 422. Japan.

Makino, Takahiro. Chuen Plant Protection, Office of SchizuokaPrefecture. Chuen Nogyo Jimusho. Karwaramachi Iwata, 438 Japan.

Tsuyumu, Shinji. Shizuoka University. Faculty of Agriculture. 836 Ohya Shizuoka 422. Japan.

Kenya

Mukunya, Daniel. Dept. of Crop Sciences. University of Nairobi. Kenya.

Liberia

Bangura, Nathaniel. West Africa Rice Development St. P. O. Box 1019, Monrovia, Liberia.

Mexico

Fucikovsky. Leopold. Centro de Fitopatología. Colegio de Postgraduados. Chapingo, Mexico.

García de la Rosa, José. Pfiser S.A. de C. V. Pfizer. Apartado 19-212 Mexico 19, D.F. Mexico.

Jaimes, Fernando. Centro de Fitopatología. Colegio de Postgraduados. Chapingo, Mexico.

López Fuentes, Ma. Cristina. Centro de Fitopatología. Colegio de Postgraduados. Chapingo, México.

The Netherlands

Maas Greesteranus, Hendrik, Research Inst. for Plant Protection, (IPO). P. O. Box 6700 AA Wageningen. The Netherlands.

Nigeria

Ofuya, C. O. Christopher. School of Biological Sciences. University of Port Harcourt. Port-Harcourt, Rivers State. Nigeria.

Perú

Delgado, Martín. Universidad Nacional de Piura. Perú.

Elango, Fritz. International Potato Center. Apdo. Postal 5969. Lima, Perú.

French, Eduardo. International Potato Center. Apdo. Postal 5969. Lima, Perú.

Gutarra Vilchez, Lilian R. International Potato Center. Apdo. Postal 5969. Lima, Perú.

Martín, Carlos. International Potato Center. Apdo. Postal 5969. Lima, Perú.

Republic of China

Hsu, Shih-Tien. Dept. of Plant Pathology. National Chung Hsing Univ. Taichung, Taiwan. Rep. of China.

Lin, Chiien-Yih, Plant Protection Division of provincial Dept. of Agriculture and Forestry. 8, Kuan-Hwa Rd., Chung-Hsing New Village, Nantou, Taiwan. Rep. of China.

South Africa

Hattingh, Martín J. Dept. of Plant Pathology. Univ. of Stellenbosh. Stellenbosh 7600, South Africa.

Mildenhall, John Peter. Dept. of Genetics, Faculty of Agric. Univ. of Fort Hare. Alice 5700, South Africa.

Spain

Cambra, Mariano. Inst. Nacional de Inv. I.N.I.A. Dept. de Protección Vegetal. Crida 07-Apartado Oficial. Moncada, (Valencia), España.

López, Maria Milagros. Dept. Protección Vegetal Crida 07. Instituto Nal. de Inv. Agrarias (INIA). Apdo. Oficial. Moncada, Valencia, España.

Palazon, Ignacio. Centro de Investigaciones y Desarrollo Agrario del Ebro. C.R.I.A. 03. (Aula Dei). Zaragoza, España.

Switzerland

Egli, Thomas. Ciba-Geigy Ltd. R 1207 P. 02 Ch 4002. Basel, Switzerland.

Grim, Richard. Swiss Federal Research St. For Fruit Growing, Hort. & Agric. Ch 8820 Wadenswil. Switzerland.

United Kingdom

Bradbury, John F. Commonwealth Mycological Inst. Ferry Lane Kew, Surrey, TW93AF, England.

Garrett, Constance M. E. East Malling Research Station. Maidstone Kent, U. K. ME 19 6BJ.

Gunawardena, Kaludura Indra. East Malling Research Station, Maidstone, Kent, U.K. ME19 6BJ.

U.S.A.

Aruga, Sreeramulu. University of Hawaii, 3190 Maile Way Honolulu, Hawaii. U.S.A. 96822.

Alvarez, Ann. University of Hawaii, 3190 Maile Way, Honolulu, Hawaii. 96822.

Bartz, Jerry. University of Florida 1419-HSPP. Gainesville, Florida. 32611. U.S.A.

Beer, Steven V. Cornell University. Dept. of Plant Pathology. Ithaca, New York. 14853. U.S.A.

Civerolo, Edwin. Dept. of Agric. SEA-AR, NER. HSI, Fruit Laboratory. Room 120, B-004, BARC WEST. Beltsville, Maryland. 20705. U.S.A.

Chatterjee, Arun K. Kansas State University. Manhattan, Kansas. 66506. U.S.A.

Cook, Allyn. University of Florida. Dept. of Plant Pathology. Gainesville, Florida 32611. U.S.A.

Coplin, David L. Ohio Agric. Research Center. Dept. of Plant Pathology. OARDC. Wooster, Ohio. 44691. U.S.A.

Currier, Thomas. Kansas State University. Manhattan, Kansas. 66506. U.S.A.

Echándi, Eddie. North Carolina State University. Box 5397. Raleigh, North Carolina 27650. U.S.A.

Granada, Gustavo. Univ. of Wisconsin-Madison. Dept. of Plant Pathology. Madison Wisconsin 53705. U.S.A.

Hopkins, Donald L. University of Florida. Agricultural Res. Center. Leesburg, Florida 32748. U.S.A.

Kelman, Arthur. Dept. of Plant Pathology. University of Wisconsin-Madison. 1630 Linden Drive. Madison, Wisconsin 53706. U.S.A.

Mew, T. W. Dept. of Plant Pathology. North Carolina State University. Raleigh North Carolina 27650. U.S.A.

Miller, John W. Division of Plant Industry. P. O. Box 1269. Gainesville Florida 32062. U.S.A.

Mills, Dallice. Oregon State University. Dept. of Botany and Plant Path. Corvallis, Oregon 97331. U.S.A.

Moore, Larry W. Oregon State University. Dept. of Botany & Plant Path. Corvallis Oregon 97331. U.S.A.

Patil, Suresh. University of Hawaii. Maile Way 3190. Honolulu, Hawaii 96822. U.S.A.

Ries, Stephen. University of Illinois at Urbana-Champaign. N-519 Turner Hall. 1102 South Goodwin Ave. Urbana Illinois 61801. U.S.A.

Saettler, Alfred W. Dept. of Botany & Plant Path. Michigan State University. E. Lansing, Michigan 48824. U.S.A.

Sequeira, Luis. University of Wisconsin-Madison. Dept. of Plant Pathology. Madison Wisconsin 53706. U.S.A.

Stall, Robert. University of Florida. HS/PP Building. Gainesville, Florida 32611. U.S.A.

Stein, Jeffrey. Allied Corporation. P. O. Box 6, Solvay, New York 13209. U.S.A.

Thurston, David. Dept. of Plant Pathology. Cornell University. 334 Plant Science Building. Ithaca, New York 14853. U.S.A.

Vidaver, Anne. Dept. of Plant Pathology. University of Nebraska. Lincoln, Nebraska 68583. U.S.A.

Venezuela

Camino, José Miguel. ULCLA. Apartado Postal 400. Barquisimeto, Edo. Lara, Venezuela.

CONTENTS

Abstracts

Session II. New Developments in Microbial Identification and Taxonomy

Abstracts

Session III. Ecology of Bacterial Pathogens

xiii

Abstracts

Abstracts

Special Contributions

Discussion Group Reports

Introduction
to the Fifth International Conference
on Plant Pathogenic Bacteria

WELCOMING ADDRESS

José Carlos Lozano
Chairman, Organizing Committee

On behalf of the Organizing Committee and the Colombian Association of Plant Pathologists I wish to thank you for having accepted our invitation to participate in the 5th. International Conference of Plant Bacteriologists.

Three years ago, when you selected me to organize this Conference, I was aware of the tremendous task and responsibility handed to me. However, I accepted the privilege, considering the benefit for all of us in holding the Congress in this tropical environment. The benefits not only relate to the beautiful climate here, but also to gaining an acquaintance with some of the economic crops which many of you may not have seen before. Some are generally cultivated within a cultural and socioeconomic system of subsistance agriculture, while others are more typical of temperate zone agriculture. Often, when systems and theories are transplanted from temperate zones, biotic outbreaks occur which were probably uncommon in former ecologically balanced traditional systems. Some are becoming a general penomenon in both the modern and the traditional systems.

Simply, we are creating inbalances which are reflected in pest and pathogen outbreaks, with serious consequences for production. Many scientists consider that this is due to our lack of expertise or physical facilities, or both, but we must realize that the tropics offer an ecological situation distinct from the temperate zone, a situation that often requires different philosophical and conceptual approaches. However, the exchange of ideas, points of view, philosophy, etc., along with the knowledge of the area, undoubtedly will lead us to a better conceptual framework to handle these problems.

Our initial goal for the organization of the Conference was to get a general idea of the topics that you wish to consider. More than 1,000 letters were sent to you in this respect, and according to the responses received, 5 paper sessions and 10 discussion groups were organized. We also decided to share the responsibilities for such sessions and discussions. The persons nominated as chairmen of the discussion groups were asked to organize them according to their considerations.

From our point of view, one of the most important aspects of the Conference will be the lively and informal exchange of ideas during both the discussion groups and paper sessions. We want to be sure that all of you have ample time and opportunity to express your ideas, uncertainties, and points of view. If we leave the Conference feeling that we all have had the opportunity to communicate freely and thoroughly then we can consider that we have reached the goals and objectives outlined three years ago.

We are aware that some things may not go as smoothly as planned, so we hope you will be patient. However, please keep in mind that we in the Organizing Committee have done our best for the success of the Conference and for a pleasant stay at CIAT.

José Carlos Lozano
CIAT
Cali, Colombia

Challenges in Plant Pathogenic Bacteriology for the Year 2000

OPENING REMARKS

Anne Vidaver, Chairperson
Bacteriology Section
International Society for Plant Pathology (ISPP)

In opening remarks for the 5th International Conference on Plant Pathogenic Bacteria, I have chosen to speak on "Challenges in Plant Pathogenic Bacteriology for the Year 2000." Both mycoplasma-like and rickettsia-like organisms are included in these remarks. I frankly hope to stimulate controversy and at least some discussion. Your reactions will be valuable, especially where I am wrong and have overlooked important problems.

By definition, good plant health is the optimal functioning of a plant with freedom from disease and abnormality. One of the agents affecting plant health is bacteria. Diseases caused by bacteria present special challenges in isolation, identification, and control. While the monetary value of crops lost to bacterial diseases cannot be ascertained, there is general consensus, I believe, that we cannot sustain such losses and continue to feed the world. These problems are some of the challenges we face in the coming decades.

Isolation of Fastidious Prokaryotes

One of the first steps in studying bacterial diseases is isolation of the causative agent. We are accustomed to success with easily cultivable bacteria, but some prokaryotes are less easily cultured. It is certain, however, that further advances will be made in culturing rickettsia-like and mycoplasma-like organisms. It seems plausible that some plant components may be essential for such success. Also, few attempts have been made to manipulate the gaseous environment — the assumption being that aerobic conditions are essential. Yet anaerobes and microaerophilic animal pathogens are well known. There is accumulating evidence for plant pathogenic anaerobes such as the clostridia. There may be others whose growth or pathogenicity is inhibited by oxygen. The majority of plant pathologists do not work with anaerobic microorganisms and, in my experience, are reluctant to do so (that includes me!)

Diagnosis and Identification

The challenges in diagnosis are those of early detection of disease and specificity. Early diagnosis makes it easier to limit the spread of disease; physical containment, roguing, etc. can be used with greater success. But our current procedures can be described as primitive when compared to diagnosis of human and animal diseases. More sensitive tests are

required. If detectable levels of bacteria could be decreased to less than 10^3 CFU/gm fresh weight, the current level of sensitivity for fluorescent antibody detection, control measures could be instituted much earlier. Such sensitivity of detection might be achieved by treating plant tissues in various ways to release bacteria or specific bacterial components. Such materials might be tested directly for the presence of bacteria or concentrated before testing.

Specificity of identification is likely to be improved in various ways. These include methods involving selective media for certain pathogens, serological tests of various types, metabolic tests, and gel electrophoretic analyses of proteins and nucleic acids. Specificity is likely to be enhanced by increased knowledge of pathogenic determinants, i.e. compounds associated with the ability to cause disease. Isolation and identification of such determinants are likely to increase the specificity of antisera for diagnostic purposes. As both investigator time and crops become more valuable, automation of identification tests will become desirable, if not necessary. Automation of identification tests, or at least quicker identification methods have been very successful in medical microbiology. Plant pathologists working with large numbers of samples would find such methods particularly useful. In the future it may become common to have resident or regional laboratories to service large farms, orchards or plantations, analogous to a hospital's diagnostic laboratory. Consulting services may fill this role; some have begun to provide diagnostic services for bacterial diseases in the U.S. Such services have not generally provided laboratory tests, however, but this may change.

Interactions of Bacteria With Plants and Other Microbes

Bacterial interactions with plants are understood at only a primitive level. The greatest challenge, it seems to me, is to determine what occurs in the early stages of infection and to separate out cause and effect. If early stages can be detected and understood, it may be possible to interfere with the early steps in an infection. Crown gall is the only disease in which early stages of infection are reasonably well understood. Such early steps can be surprisingly rapid. We have found that with *Pseudomonas syringae* pv. *syringae* and *P. syringae* pv. *glycinea,* bacteriocins applied to leaf surfaces 5 to 10 minutes after spray inoculation have little or no effect on the course of lesion development, whereas spraying before inoculation dramatically decreases the number of lesions. These bacteria thus begin the process of infection very rapidly. To date, metabolic, ultrastructural, and physical methods of analyses have not been sensitive enough to detect early perturbations in plants. Microchemical and microphysical methods of analysis *in situ,* as used in some animal studies, may provide insight into such early interactions.

Several studies have shown that both bacterial and plant surface interactions are important in establishing infection. It would seem that the use of tissue culture and protoplast systems would yield information on early interactions. In these systems, both the chemical and physical properties of surfaces can be manipulated. Such studies may provide insight needed to interfere with early infection processes.

Another kind of interaction of bacteria with plants is represented by the phenomenon of induced resistance. In this case inoculations with avirulent or weakly pathogenic bacteria can elicit either localized or systemic resistance to pathogens inoculated later. Several such examples are known, involving several different bacteria and plants, suggesting that this is a wide-spread phenomenon. The mechanisms of such induced resistance are complex and more likely to be well understood in the near future. Nevertheless, the challenge is to find and isolate bacterial components or analogues which can be cheaply produced and applied in practice. Some crops, particularly perennials, would warrant substantial investment in this area of research.

Yet another kind of interaction of bacteria with plants might be termed constitutive resistance, in which the majority of cultivars of any crop are resistant to most microorganisms, whether bacteria or otherwise. In animals, many physical and biochemical factors contibuting to constitutive or inherent resistance are known and a continuing source of study, e.g. different blood fractions. In plants, the challenge is to identify such factors so that they can be analyzed for breeding purposes. If we knew how to control and transfer or elicit both constitutive and induced resistance, diseased plants would be a rarity.

Turning now to the interactions of bacteria with other microbes, such interactions provide tremendous challenges and opportunities. The interactions of most concern to plant pathologists are those in which other microbes act synergistically to enhance infection and those in which antagonism occurs. it is sometimes difficult to us to deal with interactions of plant pathogenic bacteria with one or more different microbial species. Enhancement of plant growth and yield by treatment of seed or transplants with beneficial bacteria is already a promising technique; it is not yet clear whether the benefit results from competition for nutrients or whether antagonism of root pathogens, bacterial or not, is occurring or some combination of such interactions. Being able to predict success with these bacterial enhancers is the challenge. In the case of fastidious prokaryotes that have a resident phase in insects these bacteria may be susceptible to antagonisms introduced into the insects.

Control

The ultimate aim of plant pathology is the control of plant disease. It is striking that our only means of control are, as yet, preventive. To my knowledge we have no means to cure infected plants; at best, they can undergo temporary remission of symptoms as long as treatment is continued. Are we limited to preventive control by plant structure and environment?

A relatively new type of control for plant pathogenic bacteria is biological. Biological control, defined as the use of one biological entity to affect and minimize damage caused by another living organism, is still in its infancy. The effectiveness of *Agrobacterium radiobacter* strain 84 for nearly world-wide control of crown gall in many nursery stocks has given impetus to the search for other bacteria effective in control of bacteria and fungi. Such controls will need to play a greater role in the future as traditionally available chemicals and antibiotics become either too expensive to use or

ineffective. For insects that harbor and transmit pathogens, biological control of the insect vector is likely to be accomplished because insects are usually subject to specific viral or bacterial diseases. The successes already achieved with insect control by polyhedrosis viruses and *Bacillus thuringensis* serve as models to emulate.

The use of antibiotics and chemicals for the control of bacterial diseases of plants has had a long and checkered career. In brief, it is fair to say that in two situations, fireblight and certain mycoplasma-like diseases, selective antibiotic use has been successful. Other compounds, e.g. copper derivaties, generally have given mixed results — successful use in certain areas by some investigators. The future for new antibiotics as sprays or seed treatment is not promising, given the regulatory and economic climate in many countries. Agents that have a gaseous phase have not been examined but might be effective in greenhouse crops. The prospects for systemic bactericides are not bright either; none have been marketed in the USA or elsewhere to my knowledge. My contacts with industry show either no interest in systemic bactericides or no success. The difficulty of systemic bactericide research can be illustrated by the example of the experimental chemical tech lof thalam, which is effective in plants against the rice pathogen *Xanthomonas oryzae*, but not *in vitro*. The compound may be altered *in situ* or else suitable conditions for *in vitro* assay have not been established. Yet this is an area in which essentially no research is being done and which might be productive. The need is there.

An unexplored possibility for control is the potential use of selective metabolic inhibitors for diseases in which temperature appears to be a critical factor. We are all familiar with bacterial diseases that are associated with certain temperature regimes. For example. *Pseudomonas syringae* pv. *phaseolicola* is considered a cool weather disease while *Xanthomonas campestris* pv. *phaseoli* is considered a warm weather disease. We don't know whether major metabolic changes occur in the bacteria, but it is probable that they do. Temperature can markedly alter the ratio of the hexose monophosphate to the Entner-Doudoroff pathways for glucose catabolism in *Pseudomonas fluorescens*, a close taxonomic relative of *P. syringae*. Thus, it may be possible to find metabolic inhibitors, perhaps already on the market, that might be effective chemical control agents for some bacteria, providing they have minimal adverse effects on plants. The need for new types of control agents has been pointed out many times; this need will intensify as agricultural products become more expensive.

Genetic Engineering

One of the most exciting areas of basic and applied modern science is genetic engineering, or the *in vitro* construction of viable microorganisms with characteristics obtained from other microorganisms, plants, or even from animals. A program for using the *Agrobacterium tumefaciens* tumor-inducing Ti plasmid as a vector or carrier to transfer desirable characters between different plant species, such as production of storage proteins, is well under way and documented in many scientific and popular press articles. There certainly are many technical difficulties ahead, but this is a challenging area of immense promise.

Genetic engineering also has potential for introducing genes responsible for plant resistance to infection into plants or into bacteria that could produce products of resistance genes, or into potential biological control agents. The *A. tumefaciens* plasmid or some other plasmid might be used to analyze such genes. The first step is to insert a known marker, such as for antibiotic resistance, if it is not already present, into the cloning plasmid. Then, after endonuclease treatment of plant DNA and subsequent ligation into the cloning plasmid, selection for antibiotic resistance would be performed in *Agrobacterium* or in another bacterium that could support replication of the cloning plasmid. If present, this antibiotic marker would ensure that the plasmid had been taken up by the bacterium.

The second stage, identification of plant resistance genes is more difficult. Where "resistance" genes produce a diffusable agent, they might be detected by transferring the plasmid-carrying bacterium to plates seeded with a pathogen of interest, e. g. *P. syringae* pv. *phaseolicola* and looking for growth inhibition. It would seem critical to have a low test population, since high populations of bacteria, whether in susceptible or resistant plants, appear to behave abnormally with respect to disease development. If such a screening method worked, then the resistance gene(s) could be identified, or even if not, they could still be used for production of novel control agents, analogous to antibiotic production. Resistance genes might also be transferred into a saprophyte that readily colonizes the plant needing protection. Several investigators already are examining saprophytic bacteria that are good colonizers of various crop plants.

A more general test for transfer of resistance genes would require that the putative genes be transferred from the plasmid in the bacterium to the plant cell itself. There is the strong possibility for such transfer with the *Agrobacterium* plasmid system, for dicotyledenous plants. It will be more difficult to consider such trasnfer for monocots. But, if resistance genes could be transferred and assayed for, such procedures would revolutionize plant breeding.

These efforts, taken together, would supplement plant breeding efforts and enhance the useful lifetime of cultivars with desirable agronomic traits. The research proposed would be more difficult with plants carrying multiple genes, for resistance, but that needs to be determined. For example, for purpose of recombinant DNA techniques, it may be feasible to link such genes, even if they are not linked in the plant genome.

Conversely, single genes for such properties as bacteriocin production, might be linked together by recombinant DNA techniques. Such a procedure offers the possibility of producing an antagonist superior to wildtype strains harboring genes for individual bacteriocins.

The fact that most plants are resistant to bacterial and other infections means that inherent resistance is common. Use of recombinant DNA techniques may lead to the discovery of the basis of this phenomenon. This would likely lead to the discovery of new biological principles, since almost all general principles in biology have been derived from animal and microbial studies.

Useful Pathogens

We are so accustomed to considering bacterial plant pathogens as undesirable that it is difficult to turn one's thinking around and ask whether their unique properties can be manipulated to advantage. The multiple uses of xanthan gum from *X. campestris* are reasonably well known. More recently, extraction of commercially useful specific restriction endonucleases has occurred, and more can be expected. Toxins, bacteriocins, or bacteriophages might be useful in human medicine. Agrocin 84, the bacteriocin produced by *Agrobacterium radiobacter* strain 84, is a substituted adenine nucleotide similar to the cancer drug in current use, ara-C. If it could be produced in quantity, it might be tested for similar activity. The ice-nucleating property of *P. syringae* pv. *syringae* and some saprophytes is well established. If the material determining this activity could be isolated cheaply or synthesized, it might replace costly silver-iodide for cloud seeding purposes. Finally, all of us are familiar with the plant degrading enzymes of many phytopathogenic bacteria; these might be useful for biomass conversion. Such enzymes are now obtained principally from other microorganisms for industrial purposes. Plant pathogens may be a new and cheaper source, and provide novel enzymes.

The possibilities discussed her are only a few; all of you undoubtedly could suggest other examples.

The Future

I have commented so far on exciting challenges ahead. Now, in closing, let me comment on the future in general remarks of concern. No one can accurately predict the future because of the complexity of today's world. It is certain that agricultural methods will change, as well as the crops and the varieties that are grown. Cooperation with other disciplines will ebb and flow. I am concerned that bacteriologists, including those working with the fastidious prokaryotes, often are not getting training in modern techniques of microbiology and biochemistry. Such techniques, in my view, are necessary to complement our studies in traditional plant pathology. More needs to be done to incorporate techniques for studying microbial functions and genetics into our programs of teaching and research.

Without such principles and techniques, the pace of meeting the challenges I have outlined here will be much too slow. And in concert with other scientific organizations, universities and national and international institutes, must be seen as a vital, integral part of the agricultural scene. They must be allowed not only to succeed, but also to fail. Without the freedom to fail, which is minimized by industry and certain government programs, agriculture and other sciences will be restricted to trying only what is safe. Our science thus would suffer and be unable to meet the multiple challenges of the future.

If you basically agree with what I have said, we must all speak out; the generation would expect it of us. Thank you.

Anne Vidaver
University of Nebraska

Session I

Reports of Bacterial Disease
on Economic Crops

Arthur Kelman, Chairman

Bacterial Blights of Beans
(Phaseolus vulgaris L.)
in Kenya

D.M. Mukunya
P.M. Muthangya
J.P. Esele

University of Nairobi
Nairobi, Kenya

Abstract

Pseudomonas phaseolicola (Burk.) Dows., *Xanthomonas phaseoli* (Smith) Dows., and *X. phaseoli* var. *fuscans* (Burk) Dows. were isolated from blighted bean plants collected during an extensive survey in small scale farms in Kenya. The blights were severe and widely distributed in all major bean growing areas. The symptoms of the three blights were similar, with leaves and pods showing water-soaked spots in all cases. Halo blighted beans sometimes showed characteristic halos, but common and fuscous blights showed irregular necrotic patches surrounded by yellow to brown margins. Brick-red longitudinal lesions were found on stems with water soaking at the nodes. Seed samples were tested for the presence of the seed-born bacterial pathogens; *X. phaseoli* ocurred more frequently in bean samples than *P. phaseolicola*. Percent incidence per sample, however, was higher for *P. phaseolicola* (20-36%) wherever it was found than for *X. phaseoli*, which ranged from 8 to 24%, and 32% in only one sample. The implication of farmers using their own seed for planting is discussed in the light of the high seed infection and the widespread occurrence of these blights in the field.

Introduction

Beans *(Phaseolus vulgaris* L.) are the most important pulse crop in Kenya (1,5). Yields are generally low with a national average below 500 kg/ha (5). The major problems in bean production include uneven rainfall distribution, poor cultural practices, and destruction by pests and diseases.

Bacterial diseases of beans are becoming severe. Although not well studied in Kenya, bean diseases caused by phytopathogenic bacteria have been observed and are one of the major causes of yield fluctuations (1, 5, 6, 7). The three diseases discussed in this paper are halo blight, caused by *Pseudomonas phaseolicola* (Burk.) Dows.; common blight, by *Xanthomonas phaseoli* (Smith) Dows.; and fuscus blight, by *X. phaseoli* var. *fuscans* (Burk.) Dows. Distribution, incidence, and severity of these diseases are noted from survey samples collected from different small farms in different bean growing districts of Kenya.

Materials and Methods

Surveys

A survey was conducted in the major bean growing areas during the short rainy season from October to December 1980. Farms were picked at random on specified routes on a map. Beans were examined for presence of bacterial blight symptoms. Suspected samples of leaves, stems, pods or whole plants were preserved for further re-examination and isolation in the laboratory of the Faculty of Agriculture, Kabete. Infected tissues were cut into small pieces, and placed in a drop of sterile distilled water for a few minutes. The ooze was streaked on nutrient agar plates (NA). Morphological, cultural, and biochemical characteristics of the pure cultures were determined using methods of Dye (4) and Cowan (3).

A similar survey was conducted in which seeds collected from small farms in the same bean growing districts were tested for the presence of seed borne pathogenic bacteria. Five samples of 10 seeds each were selected randomly from each seed lot. Seeds were first surface sterilized in a 1% solution of sodium hypochlorite for 4 minutes and then dried with blotting paper. Next, they were ground finely and suspended in distilled water at 25 g/100 ml. Aliquots of the suspension were incubated on plates at 27°C. After 24 to 48 h incubation, different bacterial colonies were examined and isolated by streaking them on fresh NA plates.

To determine the percent incidence of each bacterium per seed lot, individual seeds were first surface sterilized in 1% solution of sodium hypochlorite for 4 minutes and incubated overnight in 10 ml of sterile distilled water in test tubes. Loops of the resulting bacterial suspensions were streaked on NA plates and the resulting bacterial colonies restreaked on fresh NA plates to obtain pure cultures. Identification of phytopathogenic bacteria obtained was completed by procedures noted above.

Pathogenicity Tests

Two tests were carried out on the pathogenicity of the different bacterial isolates and 24 to 48-h old bacterial cultures were used.

Seedling inoculation test. Seeds of "Canadian Wonder" cultivar were germinated in soil in pots in a greenhouse; after 10 to 14 days seedlings were inoculated with a suspension of 1×10^8 bacteria/ml by pricking in the suspension at the primary leaf node with sterile needles. Alternatively, potted plants were dusted with carborundum and inoculated by rubbing the bacterial suspension on the leaves, gently. Control seedlings were treated in a similar fashion with distilled water.

Seed inoculation test. The seeds were surface sterilized as previously described and soaked overnight in distilled water. Then the seed coats were removed aseptically and the seeds were dipped in a bacterial suspension. The inoculated seeds were incubated at 27°C in Petri dishes with moistened blotting paper. Five inoculated seeds were placed per plate and with four plates/isolate. One check plate per isolate consisted of five seeds dipped in sterile distilled water.

Results

Field Survey

A total of 54 small farms were examined in the five provinces where beans are grown. Forty-one fields were sampled in Central and Eastern Provinces, the two major bean growing areas, and 13 fields were sampled in the other provinces where beans are grown in a much smaller scale.

Halo blight was easier to distinguish in the field by typical halo symptoms on leaves and creamish white ooze on pods and stems. Common and fuscous blights were more difficult to distinguish since their symptoms varied. On the leaves necrotic lesions developed, with yellow to brown margins. Some leaves showed necrotic lesions at the edges, particularly in severely infected plants. Stems had brick red necrotic lesions and pods had greasy water-soaked lesions. In all cases, diagnosis of the disease was confirmed by isolation of the respective pathogen in the laboratory. *X. phaseoli*, the cause of common blight, produced yellow colonies on NA, whereas *X. phaseoli* var. *fuscans*, the cause of fuscous blight, produced yellow colonies accompanied by browning of the medium. *Pseudomonas phaseolicola*, the cause of halo blight, produced creamish white colonies on nutrient agar medium. All cultures with the characteristics of these pathogens were tested for pathogenicity and found to cause disease in cultivar "Canadian Wonder" plants in the green-house.

Bacterial blights were distributed at varying degrees of severity in all the provinces surveyed (Table 1). Halo blight tended to be more severe than common blight in Central Province. Of 19 fields surveyed in Central Province, all except one had halo blight and all except two had common blight and fuscous blight. In Eastern Province, common blight and fuscous blight were more severe than halo blight. High incidences of halo blight were found in Western, Nyanza, and Rift Valley Provinces. Common blight and fuscous blight tended to show low incidences in Western and Nyanza but high incidences in fields sampled in Rift Valley Province.

Seed Survey

A total of 16 seed samples were collected from farms in Central and Eastern Provinces, the two major bean growing provinces (Table 2). *P. phaseolicola* or *X. phaseoli* or both were isolated from all the samples. Percent incidence of *P. phaseolicola* in seed was high in the samples where it was isolated, ranging from 19 to 36%. *X. phaseoli* had lower incidences ranging from 9 to 24%, except in one case at Manyatta, Eastern Province where it was as high as 32%.

Discussion

In Kenya, bacterial blights of beans were found to be widely distributed. Halo blight had the highest incidence in many bean growing districts. Common and fuscous blights were prevalent in all areas, but reached epidemic proportions in a few areas. Previous surveys by Schronherr and Mbugua (2) have shown that bacterial diseases were widely distributed in Eastern Province, with Meru and Kitui districts having incidences of 59% and 19%.

Table 1.   Incidence of bacterial blights of beans on small scale farms in Kenya.

Province	Total	Incidence[a]											
		Halo Blight				Common Blight				Fuscous Blight			
		None	Low	Moderate	High	None	Low	Moderate	High	None	Low	Moderate	High
Central	19	1	3	2	13	2	7	2	8	2	7	1	7
Eastern	22	2	11	2	7	3	8	2	9	0	9	4	9
Western	3	1	0	0	2	1	1	0	1	1	1	0	1
Nyanza	6	1	2	0	3	1	5	0	0	1	5	0	0
Rift Valley	4	1	1	0	2	2	0	0	2	1	0	1	2

[a] None = no disease incidence;   low = incidence less than $10^{o}/o$;   moderate = incidence $10\text{-}30^{o}/o$;   high = incidence greater than $30^{o}/o$.

Table 2. Incidence of seed-borne bacterial pathogens in farmers' seed collected from Central and Eastern Provinces, Kenya.

Location	%o incidence	
	Pseudomonas	*Xanthomonas*
Central Province		
Dagoretti	20	12
Gatundu	—	11
Kikuyu	20	8
Kandara	—	16
Mathira	22	24
Ngariama	30	16
SabaSaba	21	12
Makuyu	23	12
Mutira	—	15
Eastern Province		
Manyatta	36	32
Siakago	24	8
Muchonoke	25	13
Runyenjes	27	14
Kirua	19	9
Kabare	23	—
Kibirichia	34	—

In many farms where the three diseases occurred, their interaction seemed to cause complete leaf necrosis, resulting in severe defoliation and wilting of beans. The most common land races found in farmers' fields were "Canadian Wonder," "Mwezi moja," and "Monel." These were in all cases severely infected.

The results of the seed survey have shown that *X. phaseoli* and *P. phaseolicola* have a high incidence in farmer seeds. *X. phaseoli* is more common than *P. phaseolicola*, especially in Central Province. When present, *P. phaseolicola* was more prevalent than *X. phaseoli*.

It is apparent that bacterial pathogens are present at a high frequency in seed obtained from small scale farmers in Kenya. Most farmers plant their own seed from the previous season (5). Since seeds are the major source of bacterial inoculum, the high prevalence of bacterial blights in farmers' fields in Kenya can be correlated with the practice of farmers using their own infected seed. Therefore, farmers should be encouraged to buy certified seed for planting to avoid bacterial blight incidences. This is possible since Kenya has an organized bean certification and distribution system through the Kenya Hortiseed Company.

Literature Cited

1. Acland, J.D. 1971. East African Crops. Longman. London. 252 p.
2. Schronherr, S. and E.S. Mbugua. 1976. Bean production in Kenya's Central and Eastern Provinces. Occasional paper No. 23. I.D.S. University of Nairobi 69 p
3. Cowan, S T and K.J. Steel. 1974. Manual for the Identification of Medical bacteria Cambridge University Press. 238 pp.
4. Dye, D.W 1962 The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N.Z.J. Sci. 5:393-416.
5. Mukunya, D.M. and S.O. Keya. 1975. *Phaseolus* bean production in East Africa Fac Agric University of Nairobi. 71 p.
6. Nattrass, R M. 1961. Host list of Kenya fungi and bacteria. Myco. paper No. 81 C.A.B
7. Ondieki, J J 1973. Host list of Kenya Fungi and Bacteria (A ten-year supplement 1961-1970). E. Afr. Agric. For. J. 38 (special issue).
8. Saettler, A W. 1971. Seedling injection as an aid in identifying bean blight bacteria Plant Dis. Reptr. 55:703-706.

Acknowledqments

Species of *Erwinia* Associated with Soft Rot Diseases of Plants in Taiwan

Shih-Tien Hsu
Kuo-Ching Tzeng

National Chung Hsing University
Taichung, Taiwan, Republic of China

Abstract

Erwinia carotovora subsp. *carotovora* (Ecc) and *E. chrysanthemi* (Echr) were identified in the 105 strains of soft-rot *Erwinia* isolated from rotted tissues of 16 hosts collected from various parts of Taiwan. Ecc could be isolated more frequently from different hosts and localities than Echr, and appeared to be the predominant bacterium associated with bacterial soft rot diseases of plants in Taiwan. Fifty-two strains of Ecc and 11 strains of Echr from different host origins were compared for their phenotypic characteristics. The two bacteria shared many characters, but differed in 14 characters. All strains of Echr collected could be separated into two phenotypic subdivisions (II and IV) according to Dickey's classification. Most strains of Echr produced white to grayish white colonies on nutrient agar medium, but some strains from Welsh onion were gray or deep gray. Most strains of Ecc also produced typical white to cream colonies on yeast extract-dextrose-calcium carbonate medium, but two strains from Chinese chives formed yellow colonies. Strains of Ecc and Echr induced soft rot on a wide range of plants; however, strains of Echr (in subdivision IV) possessed stronger rotting ability on several plant tissues than other strains of Echr and all strains of Ecc. The strains of Echr (in subdivision IV) behaved similarly in their pathogenic properties, on the hosts tested.

Introduction

Bacterial soft rot diseases are among the most important and widespread of bacterial plant disease problems in Taiwan. They occur on a wide variety of vegetables and other plants and are particularly destructive on cabbage, Chinese cabbage, radish, and potato. However, the diseases have not been sufficiently investigated. Little information is known on the bacteria responsible for the diseases that occur on various crops in Taiwan. Although several bacteria in other genera are able to induce soft rots, bacteria in the genus *Erwinia* are considered to be the most prevalent (16). In Taiwan, a number of soft rot diseases were reported to be caused by *E. carotovora* subsp. *corotovora* (*E. carotovora* or E. aroideae) (Ecc) (17). The etiology of soft rot of many other plants remains to be determined.

9

This paper describes the species of *Erwinia* associated with the soft rot diseases of various crops and the phenotypic characteristics and pathogenicity of the soft-rot *Erwinia* species found in Taiwan.

Materials and Methods

Soft rotted tissues of various plants were collected from fields or markets at different parts of Taiwan. The diseased tissues were washed with running tap water, and small pieces of the tissues from advancing margin of the rot were suspended in sterile distilled water to give a slight turbidity. Aliquots of the suspensions were streaked on nutrient agar (NA) plates. *Erwinia*-like colonies formed on the plates were selected and purified by selecting single colonies from repeated platings. The bacterial strains obtained were maintained in sterile distilled water in screw cap tubes at room temperature. The stock cultures were streaked on NA plates and incubated for 48 hr at 30°C. A single colony from each culture was then transferred to NA or yeast extract-dextrose-calcium carbonate (YDC) medium (6) and incubated for 2 to 7 days before it was used for various tests. All tests were made at 24 to 30°C unless otherwise stated.

Colonial characteristics and pigment production were examined on NA and YDC media. Flagella were stained by the methods of Mayfield and Inniss (14) or examined with an electron microscope by negative staining. Motility was examined by phase contrast microscopy.

Acid production from lactose, maltose, trehalose, and $\mathcal{L}$-methyl-D-glucoside, indole production, growth in 6% NaCl, lecithinase and phosphatase tests, and reducing substances from sucrose were determined according to Graham's methods (8). Erythromycin sensitivity was tested on NA plates containing erythromycin (50 ng/ml). Acid production from other carbohydrates and related carbon sources, gas from glucose, growth at 36°C, utilization of organic acids, hydrogen sulfide production from sodium thiosulfate, acetoin production, action on B.C.P. milk, growth factor requirement, and catalase, methyl red, and urease tests were performed by the methods of Dye (6). Gram reaction, gelatin liquefaction, hydrolysis of starch and nitrate reduction were determined by methods described in Manual of Microbiological Methods (19). Hydrolysis of casein and phenylalanine deaminase were tested by methods described in Diagnostic Microbiology (1). Dextrose utilization was determined by the method of Hugh and Leifson (11). Pectate degradation was tested on modified Cuppels and Kelman's medium (15). Lipolytic activity was determined on Sierra's medium (18). Kovac's method was used to detect oxidase (12).

The ability of the bacterial strains to induce soft rot was tested in the laboratory on detached plant parts of various plants of unknown cultivars obtained from markets and was also tested in a greenhouse on whole plants grown in pots. A water suspension containing approximately 5×10^8 cells/ml for each strain was used as an inoculum. Cells removed from 48-hr YDC slant cultures were also used in some tests. Sections of plant parts were gently pricked with a fine needle on the surface and inoculated by adding a drop of each bacterial suspension onto the pricked area. They

were then placed in moistened petri dishes and incubated at 24 to 30°C. Observations on rotting of the tissues were made 48-72 hr after inoculation.

Chrysanthemum (*Chrysantemum morifolium* Ramat. 'World I') plants were obtained from a commercial field. Stems were cut about 20 cm below the top of the plants and were inserted into test tubes containing sterile distilled water. Inoculation was done by wounding stem cuttings at 5 cm below the tip with a fine needle and then rubbing the wounded area gently with a cotton ball previously soaked in the bacterial suspension. The inoculated cuttings were enclosed with plastic bags and placed at room temperature. Examination for the amount of external and internal tissue breakdown was made 48 hr after inoculation.

Welsh onion (*Allium fistulosum* L.), shallot (*A. ascalonicum* L.), garlic (*A. sativum* L.), and Chinese chives (*A. tuberosum* Rottler) plants were grown in pots in a greenhouse. Plants were inoculated when they were 20 to 25 cm high. The basal part, 1 to 2 cm above soil line of the plant was punctured with a sterile needle and then rubbed with a cotton ball previously soaked in the bacterial suspension. The inoculated plants were covered with plastic bags and placed in the greenhouse. Rotting and collapsing of the plants were recorded at 24-48 hr after inoculation.

Inoculation of corn (*Zea mays* L. 'Tainan No. 5') was made on embryos of corn seeds, sections of the stalks, and whole plants. Embryos were removed from fresh corn seeds, surface-disinfected with ethyl alcohol, and then rinsed three times with sterile distilled water. Ten embryos were placed on the moistened tissue paper in a petri dish and inoculated by adding a drop of the bacterial suspension on the embryo surface. The petri dishes were incubated at 30°C. The number of embryos rotted was read at 48 hr after inoculation.

Stalks of 3-week old corn plants were cut into 2.5 to 3.0 cm sections. Five sections were placed in the bottom dish of a small petri plate (6 cm dia.). The small dish was then placed in another larger petri plate (9 cm dia.) in which sterile distilled water was added. The stalk sections were inoculated by puncturing of the stalk surface with a needle and rubbing the wounded area with cells removed from a 48-hr YDC slant culture. The large petri dishes were sealed with Scotch tape and incubated at 27°C. The stalk was examined for decay 48 hr after inoculation. The whorl inoculation method described by Hartman and Kelman (9) was used to inoculate the 3 to 4-week-old corn plants with the bacterial strains. The inoculated plants were kept in a greenhouse (24 to 32°C). Disease readings were recorded 1 week after inoculation.

Results and Discussion

Isolation and Identification

In total, 105 strains of bacteria were isolated from rotted tissues of 16 hosts that were collected from various localities in Taiwan (Table 1). All strains induced soft rot on slices of potato tubers, and were Gram-negative, oxidase negative, facultatively anaerobic rods that were motile with peritrichous flagella, and fermented glucose. Therefore, all strains were considered to be members of soft rot *Erwinia* group (2). The 105 strains

Table 1. Species of *Erwinia* associated with bacterial soft rots of plants in Taiwan.

Host	Number of *Erwinia* strains isolated	Number of strains identified as:	
		E. carotovora subsp. *carotovora*	*E. chrysanthemi*
Cabbage (*Brassica oleracea* L. var. *capitata* DC.)	8	8	0
Carrot (*Daucus carota* L.)	2	0	2[a]
Cauliflower (*Brassica oleracea* L. var. *botrytis* L.)	1	1	0
Celery (*Apium graveolens* L. var. *dulce* Pers.)	9	5	4
Chinese cabbage (*Brassica pekinensis* (Lour.) Rupr.)	24	24	0
Chinese chives (*Allium tuberosum* Rottler)	3	3[a]	0
Corn (*Zea mays* L.)	13	11[b]	2
Cyclamen (*Cyclamen persicum* Mill.)	1	1	0
Eggplant (*Solanum melongena* L.)	1	1[a]	0
Mustard (*Brassica juncea* Cosson)	1	1	0
Potato (*Solanum tuberosum* L.)	14	13	1
Radish (*Raphanus sativus* L.)	8	8	0
Rhyncostylis (*Rhyncostylis gigantea*)	1	0	1
Sweet pepper (*Capsicum annuum* L.)	3	3[a]	0
Tomato (*Lycopersicon esculentum* Mill.)	3	3	0
Welsh onion (*Allium fistulosum* L.)	13	1[a]	12

[a] Strain(s) isolated from samples obtained from markets.

[b] The strains did not induce stalk rot or soft rot on corn when artificially inoculated.

were differentiated into species or subspecies of the soft rot erwinias according to Graham's diagnostic tests (8). Only Ecc and Echr were found to be associated with the soft rot diseases in Taiwan (Table 1). In most soft rot samples, only one species or subspecies of *Erwinia* was isolated. Ecc could be isolated more frequently from different hosts and localities than Echr and was the predominant soft rot Erwinia. Echr, however, seemed to be the primary pathogen responsible for certain soft rots in the fields. Strains isolated from all Welsh onion samples collected from different fields were identified as Echr. Although Ecc and Echr were isolated from rotted stalk tissues of corn, stalk rot of corn was produced following artificial inoculation only by Echr. Echr has been reported as the causal organism of bacterial stalk rot of corn in other countries (9, 10). The association of *E. carotovora* subsp. *carotovora* with the rotted corn tissues may be due to the contamination from soils or other sources, or the bacterium may occur as a secondary invader in the tissues.

Phenotypic Characteristics

Sixty-three strains including 52 of Ecc and 11 of Echr from various hosts and localities were selected for comparative physiological and biochemical tests. The results showed that strains of Ecc and Echr were similar in many physiological and biochemical reactions (Table 2); these were relatively similar to those described by other investigators (2, 3, 7, 8). However, both bacteria could be differentiated by some phenotypic characteristics (Table 3). According to Dickey (3) and Dickey and Victoria (5), strains of Echr could be separated into 6 infrasubspecific subdivisions based on physiological properties. The subdivision of Taiwan strains of Echr was studied by their methods (3, 5). Two subdivisions, II and IV, were identified among strains from Taiwan (Table 4). Although more strains will have to be collected and studied, the results reported herein were in agreement with their observations in that strains originally isolated from a specific host belonged to the same subdivision (3, 5).

All strains of Ecc and most strains of Echr produced white to graysh white colonies on NA medium, but some strains of *E. chrysanthemi* from Welsh onion were gray or deep gray. On YDC medium, most strains of Ecc produced white to cream colonies, but two strains obtained from Chinese chive that possessed typical characteristics of Ecc formed yellow colonies. The two strains also produced yellow-pigmented colonies on NA supplemented with 1% glucose, but not on NA without addition of glucose. A similar yellow strain of Ecc has also been isolated recently from Chinese cabbage in Taiwan by Liao (13). Production of the yellow pigment was affected by temperatures (13). Temperatures at 34°C or higher suppressed pigmentation. These Taiwanese strains of *E. carotovora* that produced yellow colonies on certain media have not been described previously in other areas.

Pathogenicity Tests

Strains of Ecc and Echr usually induced soft rot extensively 48 to 72 hr after inoculation on storage tissue slices of carrot, potato, radish, sweet potato, taro, fruit sections of balsam pear, cucumber, and eggplant, and leaf sections of fennel, garland chrysanthemum, spinach, cabbage, and

Table 2. Phenotypic characteristics common for all or most of 63 strains of soft-rot *Erwinia (E. carotovora* subsp. *carotovora* and *E. chrysanthemi)* isolated from Taiwan.

Test	Reaction	Test	Reaction
Catalase	+	Acetoin production	+
Oxidase	−	Nitrate reduction	+
OF test	F	Phenylalanine deaminase	−
Motility	+	Urease	−
Pectate degradation	+	Casein hydrolysis	+
Gelatin liquefaction	+	Starch hydrolysis	−
Growth at 36° C	+	Lipolytic activity	−
Growth factor requirement	−	Acid from carbohydrates	$(+, -)^a$
H_2S from sodium thiosulfate	+	Utilization of organic acids	$(+, -)^b$

[a]Produced acid from D(+)—cellobiose, D(−)—fructose, D(+)—galactose, D(−)—mannitol, D(+)—mannose, ∝C—D(+)—raffinose, L(+)—rhamnose, D(−)—ribose, D(+)—xylose, and slightly from meso-inositol, but not from, adonitol, dulcitol, maltose, ∝—methyl-D—glucoside, and D(−)—sorbitol.

[b]Utilized sodium acetate, sodium citrate, sodium formate, potassium gluconate, fumaric acid, lactic acid, malic acid, and succinic acid, but not sodium benzoate, sodium oxalate, and sodium propionate.

Table 3. Differences in phenotypic characteristics between *Erwinia carotovora* subsp. *carotovora* (Ecc) and *Erwinia chrysanthemi* (Echr) isolated from plants in Taiwan.

Test	Reaction[a]	
	Ecc(52 strains)	Echr(11 strains)
Lecithinase	−	+
Phosphatase	−	+
Indole production	V	+
Methyl red test	+	V
Sensitivity to erythromycin	−	+
Action on B.C.P. milk	Acid, curd	Curd
Reducing substances from sucrose	−	V
Acid from lactose	+	(+)
Acid from D(+) trehalose	+	−
Gas from glucose	−	+
Utilization of sodium malonate	−	+
Utilization of sodium tartrate	−	+
Growth in 6o/o NaCl	+	−
Blue pigment on YDC medium	−	V

a +, positive; −, negative; V, variable; (+), delayed positive.

Table 4. Phenotypic subdivisions of strains of *Erwinia chrysanthemi* isolated from various plants in Taiwan.

Original host	No. of strains collected	No. of strains in subdivision[a]					
		I	II	III	IV	V	VI
Carrot	2	0	0	0	2	0	0
Celery	4	0	4	0	0	0	0
Corn	2	0	0	0	2	0	0
Potato	1	0	1	0	0	0	0
Rhyncostylis	1	0	0	0	1	0	0
Welsh onion	12	0	0	0	12	0	0

[a] Based on the classification of Dickey (3) and Dickey and Victoria (5).

Chinese cabbage, but did not cause rotting on tuberous rhizomes of ginger and the basal parts of edible bamboo shoots. However, difference in virulence or pathogenicity was observed when detached bulb scales of onion and detached embryos of corn seed were inoculated (Tables 5 and 6). Strains of Echr isolated from carrot, corn, Rhyncostylis and Welsh onion showed more rotting ability on the bulb scales of onion than strains of Echr from celery and potato and all strains of Ecc tested.

On detached embryos of corn seeds, all Ecc strains, with the exception of two Chinese cabbage strains, were unable to produce a soft rot reaction, whereas strains of Echr from carrot, corn, Rhyncostylis, and Welsh onion rotted more than 80% of the embryos. These were also more virulent than those of *E. chrysanthemi* from celery and potato. The reaction of corn seed embryos on the soft rot *Erwinia* species paralleled the reaction of the stalk sections; that is, virulence of strains to the embryos was correlated to their virulence to the stalks. It would appear, therefore, that reaction of the embryos may be used as an indication of the virulence of the soft rot *Erwinia* species to corn stalks.

When corn plants were inoculated by the whorl inoculation method (9), strains of Echr from corn and Welsh onion produced stalk rot, whereas celery strains of Echr and all strains of Ecc tested did not (Table 7). The similar differential reactions observed on onion bulb scales were also obtained when basal parts of Welsh onion, shallot, garlic, and Chinese chive plants were inoculated with strains of both bacteria. Variation in virulence was noted for the strains of Ecc and for strains of Echr on stem cuttings of chrysanthemum (Table 7). Strains of Ecc from the same or different hosts may cause different degees of tissues breakdown.

The results of inoculation tests described above revealed that both soft rot bacteria could induce soft rot on tissues of many kinds of plants; however, certain strains of Echr were more virulent than all strains of Ecc to some plant tissues. This may account for the apparent etiology of bacterial soft rot of Welsh onion in the fields observed in this study. Isolations revealed that only Echr could be isolated from the affected

Table 5. Reaction of detached bulb scales of onion (*Allium cepa* L.) to soft-rot *Erwinia* from Taiwan 48 hr after inoculation.

Erwinia species	Original host	Number of strains tested	Reaction[a]
E. carotovora subsp. *carotovora*	Cabbage	8	L,M
	Chinese cabbage	20	L,M
	Corn	1	L
	Potato	11	L,M
	Radish	8	L,M
E. chrysanthemi	Carrot	2	M
	Celery	4	L,M
	Corn	2	H
	Potato	1	L
	Rhyncostylis	1	H
	Welsh onion	12	H

a Reactions. L = Water-soaked only or rotted only at the inoculation site;
M = Rotted area over the inoculation site but not over half of bulb scale; and
H = Rotted area over half of bulb scale.

Table 6. Reaction of detached embryos of corn seeds to *Erwinia carotovora* subsp. *carotovora* and *E. chrysanthemi* 48 hr after inoculation.

Erwinia	Original host	Number of strains tested	Percentage of embryos rotted[a]
E. carotovora subsp. *carotovora*	Cabbage	3	0
	Chinese cabbage	5	20.4
	Corn	3	0
	Potato	3	0
	Radish	2	0
E. chrysanthemi	Carrot	2	81.6
	Celery	4	38.3
	Corn	2	99.2
	Potato	1	48.3
	Rhyncostylis	1	100.0
	Welsh onion	12	94.0

a Average o/o for the strains from 3-5 experiments. Ten embryos were inoculated for each strain experiment.

Table 7. Reaction of cuttings of chrysanthemum and corn plants to strains of soft-rot *Erwinia* from Taiwan.

Erwinia strains	Reaction on	
	Chrysanthemum[a]	Corn[b]
E. carotovora subsp. *carotovora* strains from:		
Cabbage	L,M,H	—
Chinese cabbage	L,M,H	—
Corn	M,H	—
Potato	L,M,H	—
Radish	L,M	—
E. chrysanthemi strains from:		
Celery	L	—
Corn	H	+
Welsh onion	M,H	+

[a]Reactions 48 hr after inoculation:
  L = Length of stem tissue brokendown less than 1 cm, or non-rotted;
  M = Length of stem tissue brokendown within 1-4 cm.; and
  H = Length of stem tissue brokendown more than 4 cm.
[b]Reactions 1 week after inoculation:
  + = stalk rot was produced; — = stalk rot was not produced.

tissues of Welsh onion in the field samples, although Ecc was consistently isolated from other vegetables cultivated adjacent to it. Difference in virulence among strains of Ecc to many hosts was relatively small. On the contrary, strains of Echr differed greatly in virulence to some hosts.

Generally, strains in infrasubspecific subdivision IV possessed higher rotting potential than those in subdivision II. Since number of strains collected and number of hosts from which strains of Echr were isolated were not very large, any relation of phenotypic subdivisions with their pathogenic properties must await more investigation. However, a correlation between phenotypic subdivisions and plant reaction groups was not generally observed (4).

Literature Cited

1. Bailey, W. R. and E. G. Scott. 1974. Diagnostic Microbiology, 4th ed., The C. V. Mosby Company, Saint Louis, U.S.A. 366 pp.
2. Buchanan, R.E., and N. E. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology. 8th ed. The Williams and Wilkins Co., Baltimore, MD, U.S.A. 1246 pp.

3. Dickey, R. S. 1979. *Erwinia chrysanthemi:* A comparative study of phenotypic properties of strains from several hosts and other Erwinia species. Phytopathology 69:324-329.

4. Dickey, R.S. 1981. *Erwinia chrysanthemi:* Relation of eight plant species to strains from several hosts and to strains of other *Erwinia* species. Phytopathology 71:23-29.

5. Dickey, R.S., and J. I. Victoria. 1980. Taxonomy and amended description of strains of *Erwinia* isolated from *Musa paradisiaca* Linnaeus. Int. J. Syst. Bacterial. 30 129-134.

6. Dye, D. W. 1968. A taxonomic study of the genus *Erwinia*. I. The *"amylovora"* group. N.Z.J. Sci. 11:590-607.

7. Dye, D.W. 1969. A taxonomic study of the genus *Erwinia*. II. The *"carotovora"* group. N.Z.J. Sci. 12:81-97.

8. Graham, D.C. 1972. Identification of soft rot coliform bacteria. Pp. 273-279. In H. P. Mass-Geesteranus; ed. Proc. 3rd. int. Conf. of Plant Pathogenic Bacteria Wageningen, The Netherlands. 365 pp.

9. Hartman, J. R., and A. Kelman. 1973. An improved method for the inoculation of corn with *Erwinia* spp. Phytopatology 63:658-663.

10. Hoppe, P. E., and A. Kelman. 1969. Bacterial top and stalk rot disease of corn in Wisconsin. Plant Dis. Reptr. 53:66-70.

11. Hugh, R. and E. Leifson, 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram-negative bacteria. J. Bacteriol. 6:24-26.

12. Kovacs, N. 1965. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178:703.

13. Liao, S. W. 1979. Study of the yellow strains of *Erwinia carotovora* var. *carotovora*. M.S. thesis, National Taiwan University, Taipei, Taiwan. 108 pp.

14. Mayfield, C. I., and W. E. Inniss. 1977. A rapid simple method for staining bacterial flagella. Can. J. Microbiol. 23:1311-1313.

15 O'Neill, R., and C. Logan. 1975. A comparison of various selective isolation media for their efficiency in the diagnosis and enumeration of soft rot coliform bacteria. J. Appl. Bacteriol. 38:139-146.

16. Perombelon M. C. M., and A. Kelman. 1980. Ecology of the soft rot erwinias. Ann. Rev. Phytopathol. 18:361-387.

17. Republic of China Plant Protection Society. 1979. List of Plant Diseases in Taiwan. 404 pp.

18. Sierra, G. 1957. A simple method for the detection of lipolytic activity of microorganisms and some observations on the influence of the contact between cells and fatty substrates. Antonie van Leeuwenhoek J. Microbiol. 23:15-22.

19. Society of American Bacteriologists. 1957. Manuel of Microbiological Methods. McGraw-Hill Book Co., New York. 315 pp.

Observations on Stalk and Leaf Necrosis of Onion Caused by
Erwinia herbicola

E. L. Mansvelt, A. Harding-Goodman, M. J. Hattingh

University of Stellenbosch
Stellenbosch, South Africa

Abstract

Stalk and leaf necrosis of onion in the Cape Province of South Africa is caused by a strain of *Erwinia herbicola*. The disease is characterized by rapid necrosis of seed stalks accompanied by breakdown of chlorophyll. Large numbers of bacteria embedded in an electron dense matrix were found tightly packed in the intercellular spaces of flower stalk parenchyma. Smaller numbers of bacteria, apparently free from surrounding electron dense matrix, were present in the xylem.

Introduction

Stalk and leaf necrosis of onion (*Allium cepa* L.) in the Cape Province of South Africa is a new disease caused by a strain of *Erwinia herbicola* (Löhnis) Dye (1). The disease was characterized by a rapid necrosis of flower stalks. Leaves were also affected but to a lesser extent. Other prominent features of the disease on stalks were the rapid breakdown of chlorophyll, the lack of breakdown where green tissue was covered by leaf sheaths or bracts, and the clear "islands" which remained in the necrotic areas. The borders between diseased and green tissue were sharply defined.

In view of these unusual symptoms it was decided to investigate the histopathology of diseased stalks.

Materials and Methods

Flower stalk tissue from uninfected onion plants (cultivar Caladon Globe) and infected tissue from plants inoculated as described previously (1) with *E. herbicola* strain SUH were fixed in 6% glutaraldehyde in 0.05 M sodium cacodylate buffer. After it was washed in buffer the material was post-fixed in 2% osmium tetroxide, dehydrated in a cold ethanol series, and embedded in Spurr's (4) resin.

Sections were cut on a LKB ultramicrotome using glass knives. Thick sections for light microscopy were stained for 2 min in a 1 : 1 mixture of 1% Azure II in distilled water and 1% methylene blue in a 1% aqueous solution

of sodium borate (2). Thin sections were stained with uranyl acetate and Reynolds' (3) lead citrate and examined in a Siemens electron microscope.

Results and Discussion

Thick sections seen under the light microscope showed the presence of large numbers of bacteria in the many intercellular spaces of the spongy parenchyma (Figs. 1 and 2). These intercellular spaces appeared to have undergone considerable expansion under the pressure of the large numbers of bacteria present and the accumulation of an electron dense matrix which surrounded these bacterial cells (Fig. 3). Bacteria were also observed, but less frequently, intracellularly, in some of the smaller cells of the spongy parenchyma (Fig. 4). These bacterial cells also appeared to be surrounded by the electron dense matrix.

During preliminary investigations, bacterial cells, apparently free from surrounding electron dense matrix, were present in small numbers in the xylem of vascular bundles (Fig. 5). This evidence, together with the characteristic rapid spread of the disease, supports the concept that the bacteria are dispersed via the vascular system through the plant. However, the manner in which the bacterial cells spread from the vascular bundles to surrounding parenchyma tissue has not been determined. Why the
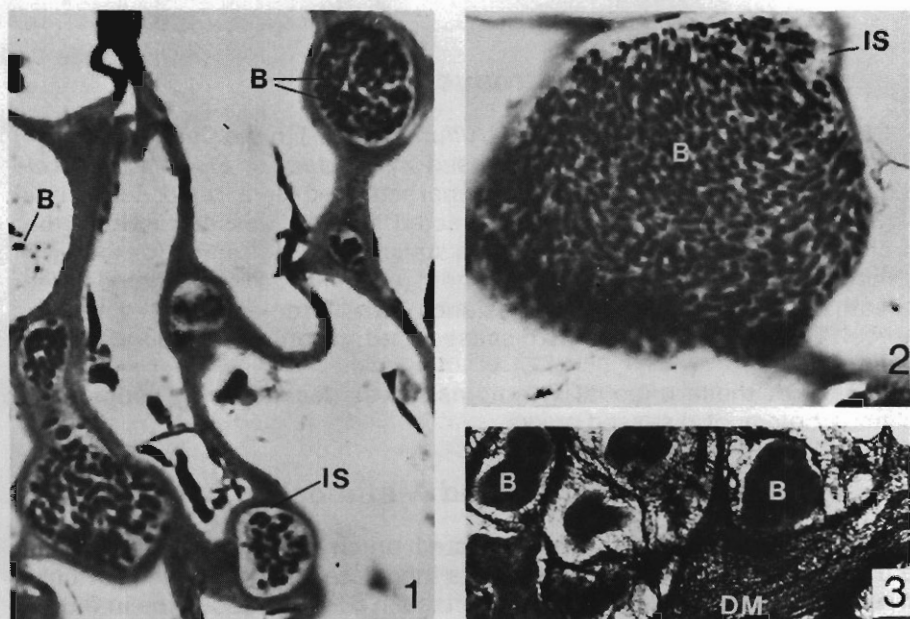
Figs. 1-5. Micrographs of sections of flower stalk infected with *Erwinia herbicola* strain SUH. 1 and 2—Light micrographs of longitudinal sections. Expanded intercellular spaces are filled with bacteria. Some bacteria occur intracellularly (x2,100). 3—Electron micrograph showing intracellular bacteria embedded in electron dense matrix (x14,000).

20
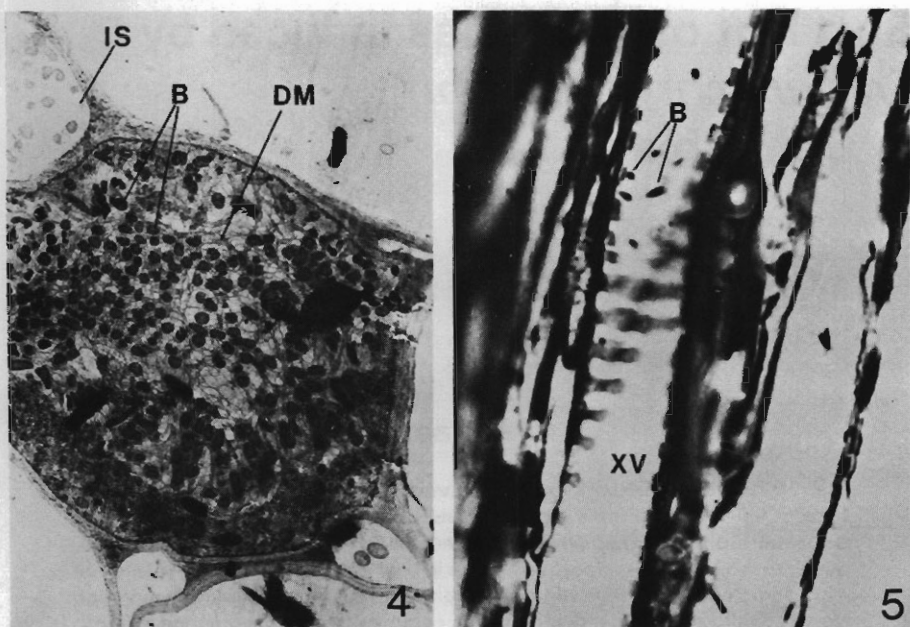
Figs. 4-5. Electron micrograph of intracellular bacteria embedded in electron dense matrix (x2, 170). 5—Light micrograph of longitudinal section showing bacteria in xylem (x2,100).

B. bacteria: DM electrom dense matrix; IS intercellular space, XV xylem.

chloroplasts, which are completely disrupted and eliminated in lesion tissue mesophyll cells, are unaffected in that part of the plant where green tissue is covered by leaf sheaths or flower bracts is also unexplained as yet.

It is proposed that the bacteria may spread throughout the spongy parenchyma of the resultant lesion by progressive multiplication of bacteria within the vast network of intercellular spaces. These bacteria, which are facultative anaerobes, produce copious amounts of slime when grown *in vitro.* They are, therefore, ideally suited to a prolonged existence in a micro-environment of the dense matrix which is possibly of bacterial origin and low in oxygen content.

Literature Cited

1. Hattingh, M.J. and D. F. Walters. 1981. Stalk and leaf necrosis of onion caused by *Erwinia herbicola.* Plant Dis. 64:
2. Moll, J. N. 1974. Citrus greening disease. Ph.D. thesis, University of Natal, Pietermaritzburg, South Africa. 43 pp.
3. Reynolds, E.S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. 17:208-213.
4. Spurr, A.R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.

Soft Rot of Tomatoes Induced by
Erwinia chrysanthemi
in Colombia

J. I. Victoria, G. A. Granada

Instituto Colombiano Agropecuario
Palmira, Colombia

Abstract

In 1978 a bacterial soft rot disease was observed in tomato crops in the Cauca Valley of Colombia. It occasionally produced losses of up to 60% of the total crop. External symptoms were observed at the flowering stage, consisting of small watersoaked lesions in leaves, petioles, and stem. Internal symptoms consisted of rotting of the pith of both petioles and stem, which became hollow. Vascular discoloration occurred. As a consequence, wilting, premature yellowing, and death occurred in all affected plants. A motile, Gram-negative, catalase-positive, oxidase negative, fermentative, and pectinolytic bacterium was isolated from affected stems and identified as *Erwinia chrysanthemi* pv. *zeae* (Sabet 1954) Young *et al.*, 1978. Koch's postulates were successfully completed with several isolates of the bacterium.

Introduction

Tomato (*Licopersicon esculentum* Mill.) is one of the most important horticultural crops grown in the Cauca Valley of Colombia. This crop is affected by several diseases that reduce its yield significantly. In 1977 a new disease, different in symptomatology from the others reported for this area, was observed. It occasionally produced losses up to 60% of the total crop.

The main symptoms consisted of small watersoaking lesions in leaves, petioles, branches, and stems. These lesions enlarge and may affect most aerial parts of plants. Pith of petioles, branches, and stems develop soft rot, leaving these parts hollow. The vascular system is decolored. Finally, affected plants suffer wilting, premature yellowing, and die.

Materials and Methods

Cultures and Pathogenicity

Samples of affected plants from several places were collected and examined for presence of microorganism. Isolations for fungi and bacteria were made on PDA and TZC media, respectively. Microorganisms that

developed on these media were purified and inoculated on healthy tomato plants, var. Chonto, to test for pathogenicity. When a positive reaction was obtained, the organism was reisolated and characterized according to methodology recommended by Schaad (5), Dye (3, 4), and Dickey (1).

Morphological and Cultural Properties

Cell morphology was observed by the Hucker modification of Gram stain and flagella were stained by the Bailey Method modified by Fisher and Conn (6). Cultural characteristics and pigment production were determined on NA and YDC media (3, 4, 6).

Physiological and Biochemical Properties

The methods used for determining the physiological and biochemical characters of the cultures have been described by several authors (1, 3, 4, 5).

Results

Cultures and Pathogenicity

Several microorganisms were isolated but only a bacterium was found frequently in infected samples which, when purified and inoculated in healthy tomato plants, reproduced the typical symptoms of the disease. Several replications of the inoculation and reisolation indicated that the cause of the soft rot of tomatoes was a bacterium.

Sixteen cultures of the pathogenic bacterium were characterized.

The bacterium isolated from *L. esculentum* was a Gram negative, non-sporeforming rod which occurred singly or in pairs. Cells from a 24 h old nutrient agar culture had average dimensions of 0.7 nm by 1.65 nm. The cells were motile and peritrichous: four to six flagella were commonly observed. The colonies on NA after 24 h at 27°C were convex, slightly to moderately irregular and undulate, pale creamcolored, and butyrous; on YDC some cultures produced a blue pigment.

The phenotypic characteristics for which all cultures from *L. esculentum* were positive include the following: facultatively anaerobic, pectate degradation, potato soft rot, gas production from glucose, catalase production, phosphatase production, indole production, nitrate reduction, growth at 36 and 39°C, sensitivity to erythromycin (15 ng), H_2S from sodium thiosulfate, KCN tolerance, production of lecithinase, gelatin liquefaction, acetoin production, utilization of sodium malonate and sodium tartrate, and acid from D(+) xylose, D(-) arabinose, D(+) melibiose, D(+) raffinose, D(-) manitol, D(-) sorbitol, and lactose.

The phenotypic characteristics for which all bacterial cultures from *L. esculentum* were negative include the following: cytochrome oxidase production; gluconate oxidation, and acid from Inulin, ∝ methyl-d-glucoside, trehalose, and maltose.

Discussion

Since the bacterial isolates pathogenic to tomato plants are Gram negative, non-spore forming rods, motile by peritrichous flagella,

facultatively anaerobic, degrade pectate, induce soft rotting of potatoes, produce catalase, reduce nitrate, and do not produce cytochrome oxidase, they could be considered as belonging to *Erwinia* genus, specifically the soft rot group of *Erwinia* species.

The problems inherent to the taxonomy of the soft rot group of *Erwinia* species have been reviewed by several authors (1, 2, 3, 4). The 16 cultures from *L. esculentum* used in this study were very similar to *E. chrysanthemi* strains when compared with phenotypically similar *Erwinia* species previously reported by some authours as shown in Table 1 (1, 2).

Strains of *E. chrysanthemi* have been isolated and identified from several host plants. Some of those strains have been distinguished in six groups by several physiological properties: gelatin liquefaction, KCN tolerance, growth at 39°C, lecithinase production, acid from D(-) arabinose, D(-) manitol, D(+) raffinose, D(-) sorbitol, inulin, and utilization of sodium tartrate. Comparison of phenotypic characteristics of *Erwinia* strains from *L. esculentum* with those strains of *E. chrysanthemi* isolated from other hosts indicated that the tomato strains' characteristics are very similar to the phenotypic subdivision IV (Table 2). It has been proposed that each phenotypic group be considered as a pathovar (1), where group IV has been named *E. chrysanthemi* pv. *zeae*. According to the above, the tomato strain found in the Cauca Valley (Colombia) could be designated as *E. chrysanthemi* pv. *zeae*. It must be pointed out that Dickey (1) placed an *Erwinia* strain from *L. esculentum* in subdivision V.

Table 1. Relationship between *Erwinia* strains from *L. esculentum* and phenotypically similar *Erwinia* species and subspecies.[a]

Characteristic	*Erwinia* strains from *L. esculentum*	*E. chrysanthemi*	*E. carotovora* subsp. *carotovora*	*E. carotovora* subsp. *atroseptica*	*E. cypripedii*	*E. rhapontici*
Pectate degradation	+[a]	+	+	+	—	—
Phosphatase production	+	+	—	—	V	V
Growth at 36°C	+	+	+	—	+	V
Indole production	+	+	—	—	—	—
Acetoin production	+	+	+	+	—	+
Susceptibility to erythromycin	+	+	—	—	+	+
Gas from glucose	+	+	V	V	+	—
Utilization of malonate	+	+	—	—	V	+
Acid from:						
αMethyl-d-glucoside	—	—	—	+	—	V
Trehalose	—	—	+	+	+	+
Maltose	—	—	V	+	+	+
Lactose	+	V	+	+	—	+

[a]The phenotypic results for the *Erwinia* species and their subspecies are derived from some strains included in the present study and from published data (1, 2).
[b]Symbols: +, 80 o/o or more strains positive; —, 80o/o or more strains negative; V, 21 to 79o/o strains positive.

Table 2. Comparison of some phenotypic characteristics of *Erwinia* strains from *L. esculentum* with those of strains of *E. chrysanthemi* isolated from other hosts.

Characteristic	Subdivision[a,b]						*Erwinia* strains from *L. esculentum*
	I	II	III	IV	V	VI	
Acid from:							
D (−) mannitol	+	+	+	+	+	−	+
D (−) sorbitol	+	+	+	+	+	−	+
D (−) arabinose	+	−	−	+	−	+	+
D (+) raffinose	−	+	+	+	V	+	+
Inulin	−	−	+	−	+	−	−
Utilization of sodium tartrate	+	+	−	+	+	+	+
KCN tolerance	+	+	−	V	−	−	+
Lecithinase	+	+	+	+	+	−	+
Gelatin liquefaction	+	+	+	+	+	−	+
Growth at 39°C	V	+	+	V	−	+	+

[a] I = *E. chrysanthemi* pv. *dieffenbachiae*, II = pv. *parthenii*, III = pv. *chrysanthemi*, IV = pv. *zeae*, V = pv. *dianthicola* and VI = pv. *paradisiaca* as suggested by Dickey (1) and Dickey and Victoria (2).

[b] Symbols: +, 80o/o or more strains positive; −, 80o/o or more strains negative; V, 21 to 79o/o strains positive.

Literature Cited

1. Dickey, R. S. 1979. *Erwinia chrysanthemi:* A comparative study of phenotypic properties of strains from several hosts and other *Erwinia* species. Phytopathology 69: 324-329.
2. Dickey, R. S. and J. I. Victoria. 1980. Taxonomy and amended description of strains of *Erwinia* isolated from *Musa paradisiaca* Lennaeus. Int. J. Syst. Bact. 30.129-134.
3. Dye, D. W. 1968. A taxonomic study of the genus *Erwinia*. I. The "amylovora" group N. Z. J. Sci. 11.590-607.
4. Dye, D. W. 1969. A taxonomic study of the genus *Erwinia*. II. The "carotovora" group N. Z. J. Sci. 12:81-97.
5. Schaad, N. W. (ed.). 1980. Laboratory guide for identification of plant pathogenic bacteria. The American Phytopathological Soc. St. Paul, Minnesota. 72 pp.
6. Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill Book Co. 315 pp.

Bacterial Diseases of Agave and Cactus in Mexico

L. Fucikovsky
J. F. Jaimes

Centro de Fitopatología
Colegio de Postgraduados
Chapingo, Mexico

Abstract

A soft rot of fleshy leaves of agave, *Agave atrovirens*, and cactus, *Opuntia* spp, was found in several areas of Mexico in 1980. Twenty to 56 per cent of the agave plants in some fields were infected. The agave slowly dies of central internal rot and does not produce honey water. Up to 46 per cent of cactus plants were infected in one area. The cladodes were partially or completely rotted or had fallen to the ground after the abscission zone was affected. Under greenhouse conditions the abscission took place 6, 7, and 8 days after local inoculation and development of decay in the cladode and was directly related to the distance between the inoculation point and the abscission zone. In both plants the bacteria appears to be introduced by insects. The bacteria have been partially characterized and can be assigned to the soft-rotting Erwinia group.

Introduction

Bacterial soft rot has been observed recently in Mexico on different cultivated plants such as sunflower, rape, carrot, potato, chrysanthemum, agave, and cactus (2, 3, 4, 5, 7). More recently damage to agave and cactus has been noted. These plants originated in Mexico. Agave was in cultivation before the Aztecs came to the central regions around the year 1000 A.D. and, at present, it is being cultivated on approximately 30,000 ha (10). The cactus plant, especially the *Opuntia* spp., the symbol on the Mexican flag, is grown widely in Mexico. Both cactus and agave are xerophytes of great importance in some areas where erosion of soil is a problem. *Agave atrovirens* (Kariv.) provides the farmer with honey-water and pulque rich in vitamin C and other compounds and the young fleshy cladodes and sweet fruits of the *Opuntia* species are popular food for many people.

Bacterial diseases of cactus and agave have not been investigated previously in Mexico, but they now appear to warrant it due to their increasing economic importance. Alcorn has studied soft rotting isolates from cacti and has identified *Erwinia chrysanthemi, E. carotovora* var. *atroseptica,* probably *E. carotovora* var. *carotovora,* and two additional groups distinct from the others (1).

27

Materials and Methods

Disease severity was estimated by counting the diseased plants in rows taken at random on three plantations of agave 5 to 6 years of age, totaling about 30 ha. Similar disease estimation was done for the cactus of approximately 4 to 5 years of age totaling about 15 ha.

The isolation of bacteria was carried out on CPG agar without tetrazolium (8). After colony development and purification, the isolates were tested for ability to macerate tissue by inoculation of potato slices. These isolates were then inoculated on potted agave and cactus plants in the greenhouse and plants growing outdoors; Koch's postulates were completed. The plants were inoculated by injecting 1 ml of bacterial suspension (10^8 cells/ml) into the parenchyma tissue of both plants. In case of *Opuntia*, the terminal cladodes were inoculated at different distances from the abscission zone to study the role of these bacteria on the induction of abscission of cladodes.

Cultural, morphological, and biochemical properties of the bacteria were also studied following the procedures of Graham (6).

Results

Disease Estimation

The incidence of the agave disease was estimated to be 38% on the average with variation between 20 and 56%. In the case of *Opuntia* spp, the disease incidence was estimated at 46%.

Symptoms

Agave. The main symptom observed was a slow death, apparently caused by an internal dark brown-black rot of the fleshy leaves (Fig. 1). A dark brown-black necrotic area appears on the outside of the leaves and affects internal parts down to the heart of the plants, with gradual collapse of the leaves (Fig. 2). The plant at this stage does not produce any honey-water and the rotted tissue has a repulsive odor.

Artificially inoculated leaves showed a rapid internal rot, which in 6 days produced a typical necrotic lesion with red exudate on the outside surface of the leaves (Fig. 3, 4).

Opuntia spp.

The soft rot of the cladodes has been observed throughout the year; the cladodes become soft, slimy, and dark with yellowish red exudates (Fig. 5). The soft rot is limited to the parenchyma tissue; the cuticle and vascular strands remain intact. Also an unpleasant odor is readily detected. In winter months many of the cladodes fall to the ground where they finally decay completely. Artificial inoculations with pure cultures produced not only quickly rotted cladodes, but also abscission from the mother plant; a distinctive shrimp or fish odor is a characteristic of decayed tissue. The abscission was related directly to time and the distance of inoculation of the cladode from the point of attachment to the plant. (Table 1).

Fig. 1. Diseased agave plant showing rot of leaves.
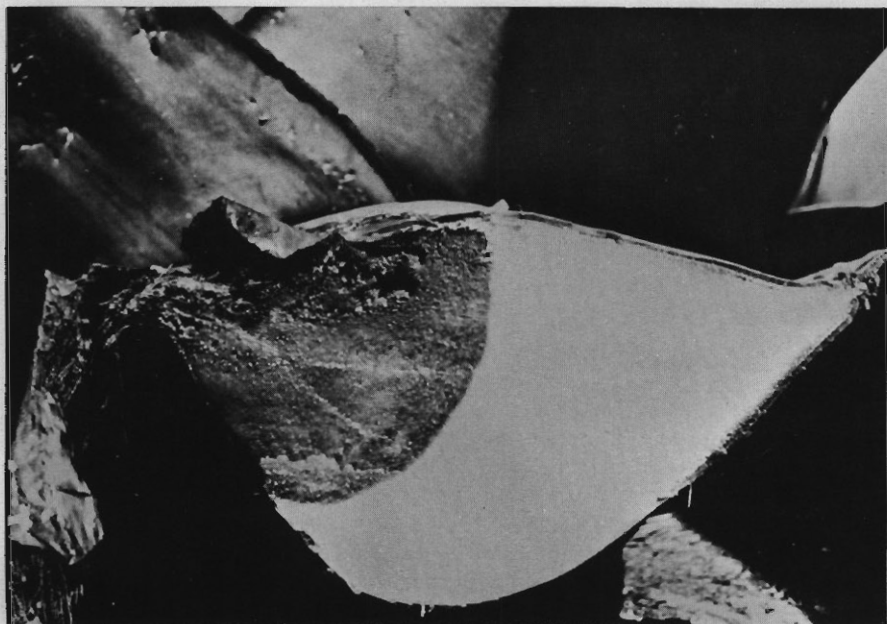
Fig. 2. Old leaf of agave with necrotic area (natural infection).

Fig. 3. Cross section of an agave leaf artificially inoculated.

Fig. 4. Exudate production from an inoculated area of agave leaf. Color of exudate is red.
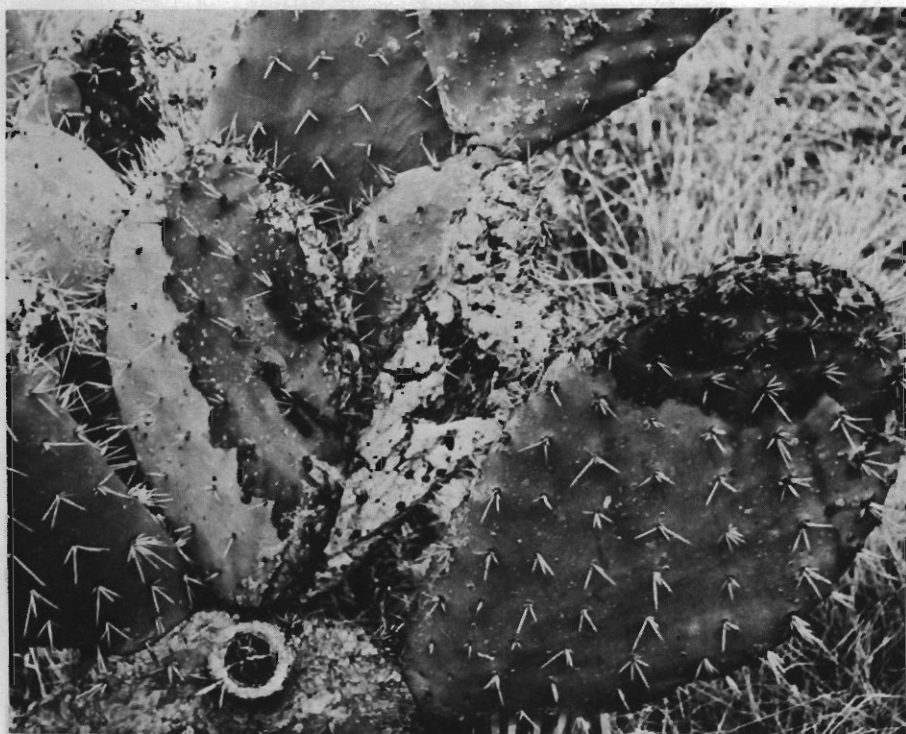
Fig. 5. Soft rot of cladodes of *Opuntia* spp. in the field (note round abscission zone, lower left).

The parts of the cladodes that were not decayed lost their normal green color, turning yellow. In both plants larvae have been observed inside the affected tissues, with initial perforations present on the outside of the cuticle. Because of the thick cuticle of both plants and absence of other points of ingress the bacteria may be introduced into the tissue via wounds made by insects.

Properties of Bacteria

The isolates have many common characteristics; creamy pale colonies on CGP medium after 3 days, Gram negative, rod-shaped motile with peritrichous flagella, fermentative, reduce nitrates to nitrites, pectolytic activity on polypectate, decay potato slices with a yellowish-green growth, utilize malonate, liquify gelatin, catalase positive, and oxidase negative. They do not produce gas from D-glucose or nitrates, blue or fluorescent pigment on B-King medium, indol phenylalanine deaminase, or oxidize gluconate. After 3 to 8 days they varied in respect to the production of acid from various carbon sources, phosphatase production, growth in 5% NaCl, utilization of citrate, and production of reducing substances from sucrose (Table 2).

Table 1. Abscission of cladodes following inoculation.

Distance of inoculation point (cm) from abscission zone	Days after inoculation
10	6
20	7
30	8

Table 2. Biochemical characteristics of soft-rotting bacteria from agave (A) and cactus (C), compared to different erwinias.

Test	$A_1A_2A_3A_4$				$C_1C_2C_3$			Erwinias[a]			
								E.c.	E.a.	E.ch.	E.s.
Acid from											
Lactose	+	+	+	+	−	+	+	+	+	−	+
Maltose	+	+	+	+	−	+	+	−	+	−	+
Thehalose	+	+	+	+	−	−	−	+	+	−	+
Melibiose	+	+	+	+	−	+	+	+	+	v[b]	−
Inulin	+	+	+	+	−	+	+	−	−	v	v
Ethanol	+	+	−	−	−	−	−	−	−	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	−	−	−	
Dulcitol											
Melezitose	−	−	−	−	−	−	−	−	−	−	−
Raffinose	+	+	+	+	+	+	+	+	+	v	
Phosphatase	−	+	+	+	+	−	−	−	−	+	+
Reducing substances											
from sucrose	+	+	+	−	−	+	−	−	+	v	
Utilization of citrate	+	+	+	+	−	+	+	+	+	+	−
Growth in 5o/o NaCl	+	+	+	+	+	−	+	+	+	+	+

[a]E.c. Erwinia carotovora subsp. carotovora; E.a. = E.c. subsp. atroseptica; E.ch. = E.ch. pv. chrysanthemi; E.s. = Erwinia from sugarbeet. Reactions noted are based on reports in the literature on these species.
[b]v = variable.

Discussion

Although bacterial soft rot caused by *Erwinia* species in various plants has been reported from Mexico previously, the bacteria that cause soft rot in agave and cactus have not been studied. Graham (6) notes that *E. carnegieana* from giant cactus is *E. carotovora*. Johnson and Hitchcock, mentioned by Leach (9), described a bacterial disease of prickly pear (*Opuntia* spp); the pathogen involved closely resembled *E. carotovora*. This is the first report of the bacterial disease of prickly pear (*Opuntia* spp.) in Mexico; however, it is quite possible that this disease has been endemic in cactus plants for a long time and simply was not noticed.

The response of the young cladodes to artificial inoculation in the present study was of interest, because the abscission of the cladode protects the plant from further spread of the infection. The cause of the abscission is unknown, but pectolytic enzymes or hormones may be involved at the abscission zone.

On the basis of motility, peritrichous flagellation, fermentative metabolism, reduction of nitrates to nitrites and other characteristics studied, all the isolates from agave and cactus plants belong to the coliform soft-rot group. The group from agave appears to be most similar to the *Erwinia* isolated from rotted sugarbeet in California (11) (Table 3). Of the *Opuntia* group pathogens the isolate C_1 is most similar to *E. chrysanthemi* subsp. *chrysanthemi* or *Erwinia* from sugarbeet, the isolate C_2 is most similar to *E. carotovora* subsp. *atroseptica* and the C_3 isolate most similar to *E. carotovora* subsp. *carotovora* or *atroseptica*. However, both groups differ from these bacteria and possibly could form other distinct subgroups within the soft rotting *Erwinia*. More investigation of these bacteria is needed.

Table 3. Number of differences of the biochemical properties studied of soft-rotting bacteria from agave (A) and cactus (C) compared to each of the 4 erwinias.

	A_1	A_2	A_3	A_4	C_1	C_2	C_3
Erwinia carotovora subsp. *carotovora*	5	6	5	4	6	6	4
E. c. subsp. *atroseptica*	3	4	3	4	8	4	4
E. ch. pv. *chrysanthemi*	6	5	6	6	4	7	6
Erwinia from sugarbeet	3	2	3	3	4	6	5

Literature Cited

1. Alcorn, S. M. 1979. Information obtained from computer (Servicio de Consulta a Banco de Información). Colegio de Post graduados, Chapingo, Mex.
2. Cartin, L. F. and L. Fucikovsky. 1980. Distribución de la pierna negra de la papa en México y la supervivencia de las bacterias causales. Agrociencia 39.59-66.

3. Fucikovsky, L. 1979. Bacterial disease of rape and carrot in México. Absts. Phytopathology 69, 8, 915.

4. Fucikovsky, L., J. Galindo and Ma. de Lourdes de la I. de Bauer. 1979. Pudrición blanda del capítulo del girasol. Agrociencia 34:31-44.

5. Fucikovsky, L., M. Rodriguez, and L. Cartín. 1978. Soft rot bacteria from plants and insects. In Proc. 4th Int. Conf. Plant Path. Bact. Angers; 603-606.

6. Graham, D.C. 1972. Identification of soft rot coliform bacteria. In Proc. 3rd. Inter. Conf. Plant Path. Bacteria, Ed. Maas Gestaranus, Centre. Agric. Pub. Doc., Wageningen 273-279.

7. Jaimes, F. 1979. Estudio de la marchitez bacteriana del crisantemo (*Chrysanthemum morifolium* Ram.) en el Estado de México. Tésis de Maestría, C.P. Chapingo, México. 54 p.

8. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

9. Leach, J. G. 1940. Insect transmission of plant disease. Mc Graw Hill Book Co., Inc. N.Y. 615 p.

10. Pérez, S. P. 1980. Problemas fitosanitarios del maguey (*Agave atrovirens*) en la Mesa Central. Tésis de Licenciatura U. A. CH. Chapingo, Méx. 51 p.

11. Thompson, S. V., M. N. Schroth, F. J. Hills, E. D. Whitney, and D. C. Hildebrand. 1977. Bacterial vascular necrosis and rot of sugarbeet; general description and etiology. Phytopathology 67:1183-1189.

Bacterial Leaf Spot and Dieback of *Centrosema* spp.

J.M. Lenné, C. Torres G
CIAT, Cali, Colombia

J. I. Victoria
Instituto Colombiano Agropecuario (ICA)
Palmira, Colombia

Abstract

A severe leaf spot and dieback of young growth of accessions of the promising tropical forage legumes *Centrosema brasilianum, C. plumieri, C. pubescens, C. virginianum,* and *Centrosema* spp. was observed at various evaluation sites in Colombia during 1980 and 1981. The causal organism was identified as *Pseudomonas* sp. Kock's postulates were successfully completed with 18 isolates of the bacterium. Seed of three accessions of *Centrosema* spp. were infected with the bacterium at levels of 8 to 34%. Further studies are being made to identify the species, to determine the host range of the bacterium, and to select resistant accessions of *Centrosema* spp. As this disease has not been reported previously on *Centrosema* spp., care must be taken to prevent its introduction to other countries where *Centrosema* spp. are promising tropical forage legumes.

Introduction

Centrosema species, especially *C. pubescens* Benth., have shown considerable promise as tropical forage legumes in Australia (3, 7), Central and South America (4), and southern Florida (5). Lesser known species, including *C. brasilianum* (L) Benth., *C. plumieri*(Turp. ex Pers.) Benth., and *C. virginianum* (L.) Benth., are presently under evaluation as forage legumes.

In 1980 and 1981, a previously unreported leaf spot and dieback of young growth was detected on accessions of *C. brasilianum, C. plumieri, C. pubescens, C. virginianum* and *Centrosema* spp. at various evaluation sites in Colombia. At the CIAT Research Station, Santander de Quilichao, Cauca, Colombia, leaf spotting and dieback considerably reduced the yield of promising accessions, including CIAT 5112, 5118 and 5278. A bacterium was consistently isolated from affected plants. The objective of this research was to identify the causal bacterium and to confirm its pathogenicity to *Centrosema* spp.

Materials and Methods

Isolates

Eighteen isolates were collected from affected plants of *Centrosema* spp. at Santander de Quilichao, Colombia. Isolates were grown on Difco nutrient agar (NA) and tetrazolium chloride medium (TZC) at 28°C. All isolates were maintained on NA + $CaCO_3$ at 24°C (2).

Morphology and Cultural Characters

Cell morphology was observed using the Hucker modification of Gram stain (6) and the Firher and Conn modification of Bayley's method (6) was used to observe flagella. Cultural characters and pigment production were determined on NA, TZC, and King's B Medium (6).

Biochemical and Physiological Properties

The methods used to determine biochemical and physiological properties of isolates are described in the Manual of Microbiological Methods (6).

Pathogenicity

Inocula for pathogenicity tests were grown in Petri plates of NA for 48 h at 28°C. Cells were suspended in sterile distilled water at concentrations of 10^6 cells/ml. Four-week old plants of *Centrosema* spp. accessions CIAT 438, 5064 and 5118 were used. Inoculation methods included (1) wounding by cutting leaves with a blade or by puncture with a needle carrying bacterial suspensions, (2) spraying with bacterial suspensions followed by incubation in moisture chambers for 48 h, and (3) injection of bacterial suspensions into the veins of plants. Controls were treated with sterile distilled water using the same methods. Plants were rated for disease reaction 8 to 10 days after inoculation. Reisolations were made from each inoculated plant and Kock's postulates were completed.

Seed Tests

The presence of bacteria in seed was tested on NA with seed of *Centrosema* spp. accessions CIAT 5112, 5118, and 5278. Seed were surface sterilized in 1% sodium hypochlorite solution, washed in sterile distilled water, placed on NA, and incubated at 28°C for 48 h. All bacteria that grew from seeds were compared with isolates from affected plants of *Centrosema* spp.

Results

Bacterial Characteristics

All isolates were Gram-negative, non-spore forming rods with a size range of 0.5 to 1.0 x 1.5 to 4.0 nm. The cells were motile with two flagella. Colonies on NA after 24 h at 28°C were convex with regular borders, cream-colored and butyrous. They averaged 2 to 2.5 mm in diameter and produced a florescent pigment.

All isolates were aerobic, oxidase-positive, catalase-positive, formed levan, and were florescent on King's B Medium (Table 1). They did not grow in nutrient broth at 41°C (Table 1).

Table 1. Characters used to differentiate commonly isolated plant pathogenic bacteria.[a]

Characters	Isolates from					
	Centrosema spp.	*Pseudomonas*	*Corynebac-terium*	*Agrobac-terium*	*Erwinia*	*Xanthomonas*
Grown on Common Media	+	+	+	+	+	+
Gram Stain	—	—	+	—	—	—
Yellow or Orange Colonies on NA, YDC or NBY Media	—	—	+	—	V⁻	+
Florescent Pigment on KB	+	V[b]	—	—	—	—
Anaerobic Growth	—	—	—	—	+	—

a   From: Laboratory Guide for Identification of Plant Pathogenic Bacteria. Ed. by N. W. Schaad.
b   V = Variable Result.

Symptomatology

Symptoms were initially manifest as wilting of young leaves and terminals and chlorotic spotting of mature leaves. Young leaves and terminals became partially or completely necrotic and dieback developed. On mature leaves, chlorotic spots became necrotic and were of varying size and shape. Leaves were often crinkled and distorted.

Pathogenicity

All isolates caused wilting, dieback, and necrotic spotting of four-week old plants of *Centrosema* spp. The bacterium was readily reisolated from affected plants and Kock's postulates were successfully completed with 18 isolates.

Seed Tests

The bacterium was found in seed of accessions of *Centrosema* spp. CIAT 5112, 5118, and 5278; levels of seed infection ranged from 8 to 34%.

Discussion

The causal organism was identified as a species of Pseudomonas on the basis of its morphological, cultural, biochemical, and physiological properties. Further tests are in progress to identify the species.

Although *Centrosema* spp. have been under forage evaluation in tropical areas for the past 15 years (3, 5, 7), no reports of this disease have been made previously. As *Centrosema* is native to the Americas (5), most probably this bacterial disease evolved with the host species that it presently affects. Cross-inoculation studies are in progress to determine the host range of the bacterium and, particularly, whether it is pathogenic to other leguminous crops such as beans.

As the bacterium is seed-borne, care must be taken to prevent its spread to other areas where *Centrosema* spp. are important tropical forage legumes.

Field screening of *Centrosema* spp. for resistance to bacterial leaf spot and dieback is continuing in Colombia. To date, the promising *C. macrocarpum* has shown resistance to this disease.

Literature Cited

1. Annual Report, 1980. Tropical Pastures Program, Centro Internacional de Agricultura Tropical, CIAT, Cali, Colombia.
2. Baigent, N.L., J.E. De Vay, and M.P. Starr. 1963. Bacteriophages of *Pseudomonas syringae*. N. Z. J. Sci. 6:75-100.
3. Grof, B. 1970. Yield attributes of some species and ecotypes of *Centrosema* in north Queensland. Queensland J. Agric. Anim. Sci. 27:238-242.
4. Hutton, E.M. 1979. Importance of legumes in cattle pastures of tropical Latin America. Oct. Seminar, CIAT, Cali, Colombia.
5. Kretschmer, A.E. Jr. 1977. Growth and adaptability of *Centrosema* species in South Florida. Soil Crop Sci. Soc. Florida Proc. 36:164-168.
6. Society of American Bacteriologists. 1957. Manual of Microbiological Methods. McGraw-Hill Book Co., Inc. New York.
7. Teitzel, J.K. and R. L. Burt. 1976. *Centrosema pubescens* in Australia Trop. Grassld. 10:5-14.

Bacterial Pod Rot of *Leucaena Leucocephala* Caused by *Pseudomonas Fluorescens* Biotype 2

J. M. Lenné, C. Torres G.
CIAT, Cali, Colombia

J. I. Victoria
Instituto Colombiano Agropecuario (ICA)
Palmira, Colombia

Abstract

During 1980 and 1981, a severe pod rot was found on the forage browse legume *Leucaena leucocephala* in Belize, Brazil, Colombia, Mexico and Panama. The causal bacterium was identified as *Pseudomonas fluorescens* Biotype 2. Kock's postulates were successfully completed with four isolates. The constant association of lesions with insect feeding holes indicates that the bacterium is insect-borne. Cross-inoculation studies showed that *L. diversifolia* and *L. shannoni* are not as susceptible to the disease as *L. esculenta*, *L. leucocephala* and *L. pulverulenta*. Further studies are needed to understand the insect-bacterium association and to quantify the effect of the bacterium on seed production. This is the first report of bacterial pod rot of *L. leucocephala*.

Introduction

Leucaena leucocephala (Lam.) de Wit. is a deep rooting tree or browse legume native to the subhumid and humid tropics of Central America (6). Its value as a high quality protein forage in association with a grass or as a "protein bank" for supplementing native pastures (4) is being investigated in tropical Latin America (2, 4), Australia (5), Hawaii, and other countries (1, 5). Few diseases have been reported on *L. leucocephala* in the past (7).

In February 1980, a pod rot was found for the first time on *L. leucocephala* in its native habitat in Mexico and at forage evaluation sites in Belize and Panama. Later, the same disease was observed in Colombia and Brazil on promising cultivars of *L. leucocephala* - Cunningham and Peru. A bacterium was consistently isolated from rotted pods and seeds. The objective of this study was to identify the bacterium and determine its pathogenicity to *Leucaena leucocephala* and related species.

Materials and Methods

Isolates

Four isolates were obtained from diseased pods of *L. leucocephala* at Palmira, Colombia. Isolates were grown on Difco nutrient agar (NA) and tetrazolium chloride medium (TZC) at 28°C. All isolates were maintained on Na + CaCO3 at 24°C (3).

Morphology and Cultural Characters

The Hucker modification of Gram stain was used to observe cell morphology (8). The Fisher and Conn modification of Bayley's method (8) was used to observe flagella. Cultural characters and pigment production were determined on NA, TZC, and King's B. Medium (8).

Biochemical and Physical Properties

The methods used to determine biochemical and physiological properties of the isolate are described in the Manual of Microbiological Methods (8).

Pathogenicity

Inocula for pathogenicity tests were grown in Petri plates of NA at 28°C for 48 h. Cells were suspended in sterile distilled water at concentration of 10^6 cells/ml. Well developed green pods of accessions of *L. leucocephala, L. escuelenta* (Moc. & Seese) Benth., *L. pulverulenta* (Schlecht) Benth., *L. shannoni* Donn and *L. diversifolia* (Schlecht) Benth. were inoculated by injection. Controls were inoculated with sterile distilled water using the same method. Pods were rated for disease reaction 10 days after inoculation. Re-isolations were made from affected pods and Kock's postulates completed.

Results

Bacteria Characteristics

All isolates were Gram negative, non-spore forming rods with a size range of 0.5 to 1.0 x 1.5 to 4.0 nm. The cells were motile with two flagella. Colonies on nutrient agar after 24 h at 28°C were convex with regular borders, creamy white, butyrous, 2 to 2.5 mm in diameter and produced a green florescent pigment.

All isolates were aerobic, oxidase-positive, catalase positive, arginine dihydrolase-positive, formed levan, caused soft rot potato, reduced nitrates to nitrites, utilized meso-inositol, trehalose, ethanol, propylene glucol, sorbitol, propionate, benzoic acid, L-arabinose, D-arabinose, β alanine, L-valine and lipase (using Tween 80 as a substrate), 2-ketogluconate-positive, produced acid from sucrose and hydrolyzed both starch and gelatin (Tables 1 and 2).

The isolates did not accumulate poly-β-hydroxybutyrate, did not produce a hypersensitive reaction when injected into tobacco and did not grow in nutrient broth at 41°C (Tables 1 and 2).

Table 1. Characters used to differentiate commonly isolated plant pathogenic-bacteria.[a]

Characters	Isolates from *Leucaena leucocephala*	*Pseudomonas*	*Corynebac-terium*	*Agrobac-terium*	*Erwinia*	*Xanthomonas*
Growth on Common Media	+	+	+	+	+	+
Gram Stain	–	–	+	–	–	–
Yellow or Orange Colonies on NA, YDC and NBY Media	–	–	+	–	V⁻	+
Florescent Pigment on KB	+	V+b	–	–	–	–
Anaerobic Growth	–	–	–	–	+	–

a   From: Laboratory Guide for Identification of Plant Pathogenic Bacteria. Ed. by N. W. Schaad.
b   V = Variable Result.

Table 2. Fluorescent species of *Pseudomonas*[a] and comparison with isolates from *Leucaena leucocephala*.

CHARACTERS	Isolates from *Leucaena leucocephala*	*P. florescens*	*P. aeruginosa*	*P. toloasii*	*P. agaricae*	*P. cichorii*	*P. viridiflava*	*P. syringae*
Oxidase	+	+	+	+	+	+	—	—
Arginine Dihydrolase	+	+	+	ND[c]	ND	—	—	—
Nitrate to N2	+	+	+	ND	ND	—	—	—
Growth at 41oC	—	—	+	ND	ND	—	—	—
Utilization for Growth	—	—	+	ND	ND	—	—	—
Mannitol	+	+	+	+	+	+	+	V
Sorbitol	+	+	—	—	—	+	+	V
Trehalose	+	+	—	—	—	—	—	
Sucrose	+	+	—	—	—	—	—	V
D—Arabinose	V[b]	—	—	ND	ND	—	—	—

a   From: Laboratory Guide for Identification of Plant Pathogenic Bacteria. Ed. by N. W. Schaad.
b   V = Variable Result.
c   ND = Not Determined.

Symptomatology

First, water-soaked lesions surrounding insect feeding holes appeared on well developed green pods in the seed-filling stage. Lesions expanded and became necrotic as seeds began to rot. Under humid conditions, there was a general rotting of the pod and bacteria oozed from insect feeding holes. Pods often fell prematurely. Few seed were recovered from affected pods.

Pathogenicity

All isolates caused pod rot of *L. leucocephala* when inoculated by injection. Bacteria were readily re-isolated from affected pods and Koch's postulates were successfully completed with four isolates. Cross inoculation studies with four other species of *Leucaena* showed that all species were susceptible to pod rot; however, *L. diversifolia* and *L. shannoni* were more resistant than *L. esculenta* and *L. pulverulenta*.

Discussion

On the basis of morphology, cultural characters, biochemical, and physiological properties, the causal bacterium is identified as *Pseudomonas florescens* Biotype 2. This is the first report of this disease on *L. leucocephala*.

The development and association of lesions with insect feeding holes under natural conditions indicates that the bacterium may be insect-borne, probably by a Heteroptera of the family Pentatomidae (Dr. Mario Calderón, personal communication). Further work is needed to understand this insect-bacterium association.

Because the bacterium is associated with seeds and pods, it could be easily transferred with them. Appropriate quarantine restrictions should be enforced to prevent introduction of the bacterium to tropical forage evaluation regions in Australia, Hawaii and other countries where *Leucaena* spp. are presently under intensive evaluation (4, 5).

Further work is needed to quantify the effect of pod rot on seed production of *L. leucocephala*. The resistance of *L. diversifolia* and *L. shannoni* may be of value in future breeding programs.

Literature Cited

1. Acad. Sci. Washington, D. C. 1977. *Leucaena,* promising forage and tree crop for the tropics. pp. 22-39.
2. Annual Report 1978. Centro Internacional de Agricultura Tropical, CIAT, Cali, Colombia.
3. Baigent, N.L., J. E. De Vay, and M.P. Starr. 1963. Bacteriophages of *Pseudomonas syringae.* N. Z. J. Sci. 6:75-100.
4. Brewbaker, J.L. and E. M. Hutton. 1979. Leucaena-versatile tropical tree legume. In: "New Agricultural Crops", G.A. Ritchie (Ed.) Amer. Assn., Adv. Sci. Press. Boulder, Colorado. pp. 107-259.
5. Jones, R.J. 1979. The value of *Leucaena leucocephala* as a feed for ruminants in the tropics. World Anim. Rev. 31:13-23.

6. Le Houerou, H. N. 1978. The role of shrubs and trees in the management of natural grazing lands (with particular reference to protein production). In: Proc. 8th. Annu. World For. Congr., Jakarta, Indonesia. p.13.
7. Lenné, J.M. 1979. Camptomeris leaf spot on the tropical forage legume *Leucaena leucocephala*. Austr. Plant Path. 8:7.
8. Society of American Bacteriologist. 1957. Manual of Microbiological Methods. McGraw-Hill Book Co., Inc., New York.

Bacterial Speck of Tomato [*Pseudomonas tomato* (Okabe) Alstatt] in Greece

Characterization of Isolates by Physiological Features and Production of Chlorosis-Inducing Toxin.

A. Mavridis
K. Rudolph

Institut für Pflanzenpathologie
    und Pflanzenschutz
Universitat, Gottingen
Federal Republic of Germany

Abstract

Pseudomonas tomato was isolated from naturally infected tomato plants collected from different provinces of Greece during the years 1978 and 1979. The disease occurred predominantly on leaves and stems but rarely on fruit. Greenhouse grown tomatoes (under plastic cover) were much more affected than field tomatoes. Early infections in greenhouses led to serious economic losses.

Results of the LOPAT tests revealed that all the isolates fell into group Ia of *Pseudomonas* (Lelliott *et al.* 1966). Both mucoid isolates liquefied gelatin but did not produce lipase with Tween 80, whereas the non mucoid ones did not liquefy gelatin but produced lipase.

A chlorosis-inducing toxin could be obtained and purified from liquid cultures on *P. tomato* as well as from infected tomato leaves. The toxin was not specific to tomato, as leaves from many other plant species were likewise affected. An antibiotic effect towards fungi or bacteria could not be demonstrated. The toxin has been characterized as an acidic compound with a molecular weight under 500 daltons.

Introduction

Survey of bacterial diseases of tomato in Greece was undertaken during the years 1978 and 1979. Bacterial isolates were obtained from different locations and characterized by physiological tests and determination of pathogenicity. These studies with *Pseudomonas tomato* are reported in this paper.

Materials and Methods

The selective medium D 4 for *Pseudomonas* (7) was used for the isolation of the pathogen from naturally infected plants.

The isolates were identified according to morphological and physiological criteria. Pathogenicity of the isolates was tested by inoculation of tomato leaves with suspensions of ca. 10^7 bacterial cells/ml (20).

Isolates with fluorescence on medium B of King *et al.* (8) were tested for the LOPAT-criteria according to Lelliott *et al.* (9) Gelatinase and lipase activity were determined as described by Misaghi and Grogan (11). To differentiate between mucoid and non mucoid isolates, the bacteria were streaked on YDC-agar (1% yeast extract, 2% dextrose, 2% calcium carbonate).

Toxin production was studied by cultivating the bacteria in shake cultures. The synthetic media of Watanabe (25) modified by Rudolph *et al.* (18), and Woolley *et al.* (27) were compared with a complex medium (1% peptone, 0.3% yeast extract, and 0.5% NaCl). The bacteria were grown for 5 days in 1 l-Erlenmeyer flasks containing 500 ml nutrient solution. A rotating shaker at 100 rpm and an incubation temperature of 19.5°C were used. After centrifugation (20 min at 6000 g) and passage through Seitz-filters, the supernatant was further purified as described under Results.

Column chromatography was performed in cooled glass columns (4°C). A constant flow rate was maintained with peristaltic pumps (17).

The toxin content was determined by a semiquantitative toxin-assay. Droplets of 20 nl were pipetted onto the surface of leaves of tomato and tobacco (cultivar "white Burley"). The plants were kept in a greenhouse at 20 to 25°C and 50 to 70% RH. To determine toxin production *in vivo*, tomato leaves were harvested 5 days after inoculation and homogenized in water. The homogenate was processed for toxin determination in the same way as the supernatant from *in vitro* cultures.

Results

Tomatoes with typical symptoms of bacterial speck (26) were found in nearly all the surveyed areas of Greece. Typical symptoms of the leaves were small, dark-brown to black spots of 2 to 3 mm diameter, surrounded by yellow halos. Small necrotic spots on stems were also observed. Tiny dark spots appeared on the fruit.

The disease occurred mainly on tomatoes in greenhouses or under plastic cover and caused considerable economic loss. Early infections under these conditions seemed to favor the disease, probably because of the high humidity and temperature.

Inoculation of tomato plants with the Greek strains of the bacterium caused the following symptoms: necrotic spots of 2 to 3 mm diameter surrounded by chlorotic halos on the leaves, small necrotic lesions on stems, and tiny, convex, dark brown spots on the fruit.

Pathogenic bacteria could be isolated from all the surveyed areas, i.e., from the south (Crete and Peleponnes), central Greece (Volos and Prevesa), the north (Thessaloniki), and the north east (Xanthi). Thus, the disease seems to be prevalent all over the country.

The strains were fluorescent on King's medium; they had the characteristic of group Ia of *Pseudomonas* according to the LOPAT-criteria

Table 1. Physiological characters of *Pseudomonas tomato* isolates from Greece.

Test	Number of isolates	
	mucoid 18	non-mucoid 23
Levan production (L)	+	+
Oxidase activity (O)	—	—
Potato soft-rot (P)	—	—
Arginine dihydrolase (A)	—	—
Tobacco reaction (T)	+	+
Lipase activity	— (14)	+ (19)
Gelatinase activity	+ (16)	—

+ = positive reaction
— = negative reaction

of Lelliott *et al.* (9). (Table 1). Mucoid and non-mucoid isolates were obtained, and this differentiation correlated with the reaction for lipase and gelatinase activity. Thus, 14 of the 18 mucoid strains were negative for lipase, and 16 were positive for gelatinase. In contrast, from the 23 non-mucoid isolates, 19 were positive for lipase, and all were negative for gelatinase (Table 1).

A chlorosis-inducing toxin could be obtained from culture filtrates of the bacteria. The symptoms induced by this toxin on a tomato leaf are shown in Fig. 1a. The toxin was produced in both synthetic media but not in the complex medium. The toxin was purified by the following procedures: The sterile supernatant from the culture medium was precipitated 3 times with 70, 86, and 91% ethanol, respectively. The toxin remained in the supernatant. This solution was concentrated 20-fold and the pH was adjusted to 3.0 with 0.1 N HCl. The toxin was transferred into chloroform by 3 extractions of the acidified solution. The toxin-containing chloroform was alkalized with 1 N NH_3 to pH 10.0. At this pH the toxin could be extracted with water. The toxin-containing water-phase was concentrated 100-fold in a rotorvap at 36°C and then purified further by column chromatography on Bio-Gel P6 and eluted with 0.015 N NH_3. A second column chromatography was then carried out on Sephadex G-15, and a third on Sephadex G-10, where the toxin came together with the void volume. Table 2 summarizes the three toxin separations with molecular sieve chromatography.

The toxin was then further purified by ion exchange chromatography. The cation exchange resin Dowex 50 x 4 did not absorb the toxin, which could thus be eluted with water and separated from other contaminating compounds. On the anion exchange resin Dowex 2 x 8 the toxin was
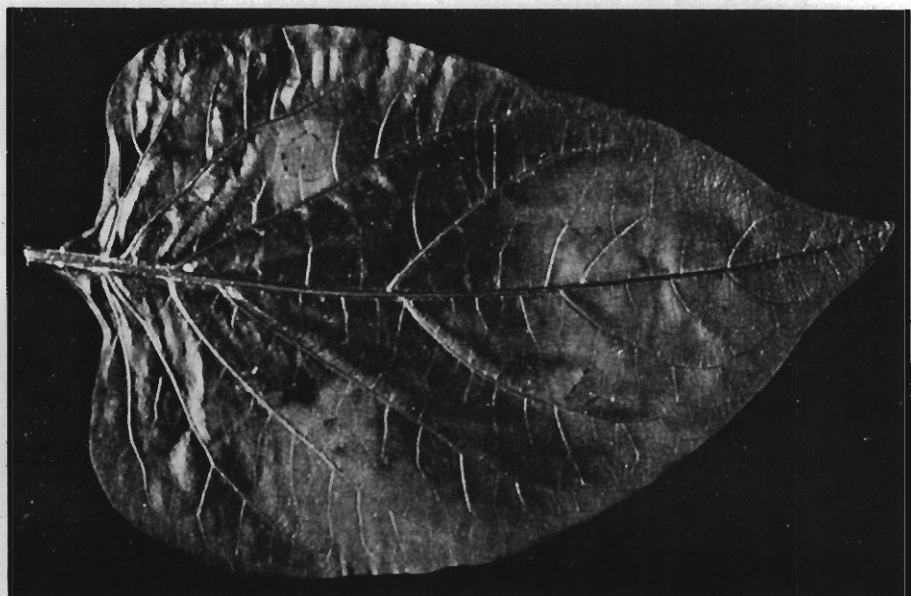
Fig. 1. Chlorosis induction by the toxin from *Pseudomonas tomato* on leaves of (a) tomato one week after treatment and (b) pepper one week after treatment. The circles on the halos were made by a marker and show the size of the toxin-containing droplet applied.

Table 2. Position of active fractions of the toxin from *Pseudomonas tomato* after molecular sieve chromatography on different materials.

	Position (ml eluant)	
	Void volume[a]	Active fraction[b]
Bio-Gel P-6	160	240
Sephadex G-15	220	300
Sephadex G-10	120	125

[a] determined with Dextran blue 2000
[b] determined by biotest on tobacco

strongly adsorbed, but could be eluted with 3 N acetic acid. The last step in toxin purification consisted of thin-layer chromatography (TLC) on silica gel F_{254} with the solvent system: chloroform: 96% ethanol: water (60:30:2), pH 9.5. The toxin suppressed the fluorescence of the TLC-plates and was thus visible under UV-light at the R_f-value of 0.69.

The toxin affected all green plants which have been tested. Thus it can be classified as a nonspecific toxin. Toxin-caused symptoms on a pepper-leaf are shown in Fig. 1b. Toxin-activity was also visible in the tissues surrounding the originally applied droplet, the size of which is indicated by a marker in Fig. 1. Fig. 2a shows toxin-induced chlorosis on a tobacco leaf 4 days after treatment. The first symptoms became visible on tobacco within 2 days after toxin application, whereas, on tomato, the symptoms appeared later, becoming visible 7 days after toxin application. Tobacco showed the highest sensitivity to the toxin among all the tested plant species. One week after toxin application the center of the chlorotic spots became necrotic; Fig. 2b, shows a tobacco leaf 10 days after toxin application. When the toxin was infiltrated into the petiole, a systemic effect on tobacco leaves could be observed.

Possible antimicrobial properties of the toxin, were tested with the following bacteria and fungi: *Escherichia coli, Pseudomonas syringae, P. fluorescens, P. tomato, P. phaseolicola, Xanthomonas pelargonii, Geotrichum candidum, Cladosporium herbarum, Botrytis cinerea, Alternaria brassicae, Aspergillus niger.* None of these organisms was inhibited by the toxin.

The following additional characteristics of the toxin were established:

The toxin passes the ultrafilter Amicon UM-05; the molecular weight should therefore be below 500 daltons. The toxin is ninhydrine negative, contains no phosphorus, and is stable to heat and extreme pH-values.

A toxin preparation could be isolated from infected leaves that showed the same features as the toxin from *in vitro* cultures, whereas a similar extract from healthy leaves did not possess toxin activity.
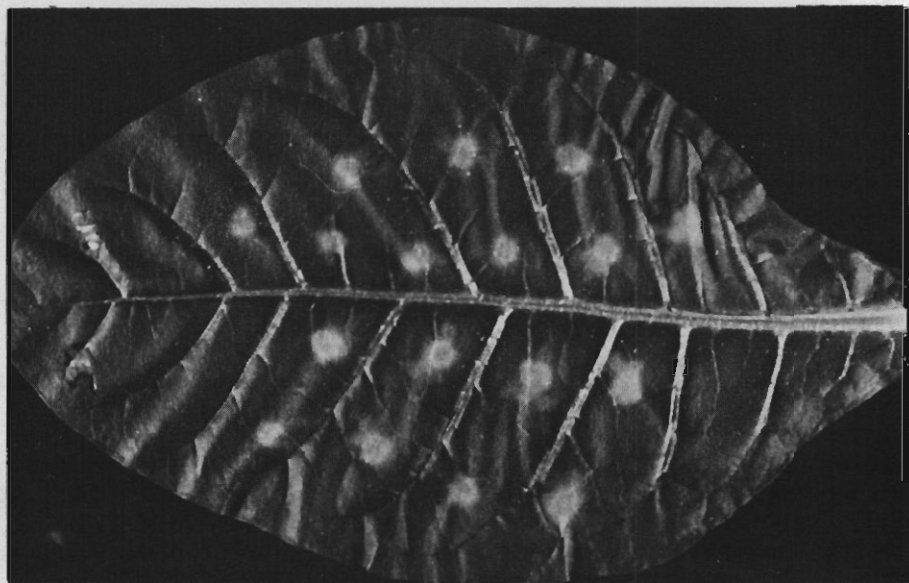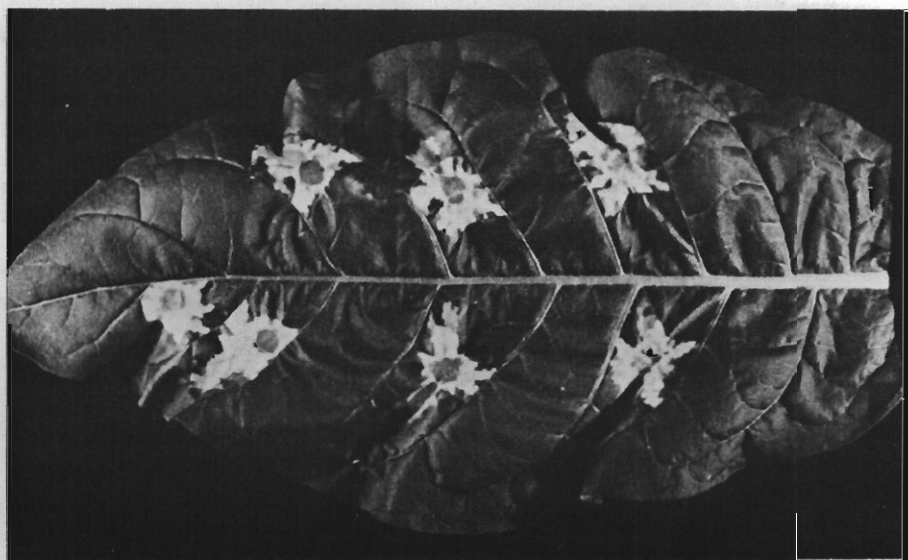
Fig. 2. Chlorosis induction by the toxin from *Pseudomonas tomato* on leaves of (a) tobacco four days after treatment, and (b) tobacco 10 days after treatment.

Discussion

Although bacterial speck of tomato was described in 1933 (13), there have been only a few reports of its occurrence in Europe (1, 3, 10, 24).

Economic losses due to this disease have been reported in the USA (19), Australia (2), and New Zealand (26). This paper gives the first description of the disease *P. tomato* in Greece, although its occurrence there has been known for many years (Panagopoulos, personal communication).

The results of the LOPAT-tests (9) clearly place this species into the group Ia of *Pseudomonas* and the specific symptoms induced on tomato identify the strains as *Pseudomonas tomato* (Okabe) Alstatt or *Pseudomonas syringae* pv. *tomato* Young *et al.* (28).

Varying results for gelatinase and lipase, which have been reported by other workers for *P. tomato* (9), may be due to the fact that their strains were not separated into mucoid and non-mucoid variants. Two or three strains that were studied by Misaghi and Grogan (11) did not show levan production, and thus behaved differently than the whole group Ia of *Pseudomonas*.

We could not reproduce the results of Okon *et al.* (14), who found a necrosis-inducing toxin in *P. tomato* cultures grown in yeast-peptone broth. Most of our raw culture filtrates induced necrotic spots that were surrounded by yellow halos. However, similar symptoms were also produced by uninoculated culture media. The necrosis-inducing principle could be excluded by the purification procedure.

Garber and Shaeffer (4) and Sinden and Durbin (21) reported that the culture filtrate from *P. tomato* contained toxic substances that caused an unspecific chlorosis. Due to its heat stability, it was likely that the structure was similar to that of the toxin from *P. phaseolicola*. However, Garber and Shaeffer (4) reported two active spots after chromatography in propanol: water (2:1) with R_f values of 0.10 to 0.20 and 0.85 to 0.90. When we tried this solvent system with TLC on cellulose, the toxin traveled with the front zone.

The toxin described here differs from other toxins from similar pseudomonads primarily in its negative reaction to ninhydrin in contrast to phaseolotoxin, tabtoxin or syringomycin (6, 12, 15, 23). Unlike phaseolotoxin or syringomycin it does not inhibit *E. coli* (22) or *Geotrichum candidum* (5).

The lipophilic or hydrophilic nature of the toxin in its dependence on pH, as well as its adsorption on the anion exchange resin, strongly suggests an acidic nature. Whether or not the toxin plays a role during pathogenesis is not known. Its non-host-specific nature excludes the toxin as a determinating factor in pathogenicity. Nevertheless, the toxin could contribute to the virulence of *P. tomato*, as has been shown for nonspecific toxins from similar bacterial pathogens (16).

Literature Cited

1. Burki, T. 1972 *Pseudomonas tomato* (Okabe) Alstatt, Erreger einer fur die Schweiz neuen Tomatenbakteriose. Schweiz. Landw. Forschg. 11:97-107.
2. Chambers, S.C. and R. P. Merriman. 1975. Perennation and control of *Pseudomonas tomato* in Victoria. Aust. J. Agric. Res. 26: 657-663.
3. Ciccarone, A. and A. Mezzetti. 1950. La "picchiettatura batterica del pomodoro" in Italia. Not. Mal. Piante. 10, 13.
4. Garber, E.D., and G. S. Shaeffer. 1963. Genetic and biochemical characteristics of six phytopathogenic species of *Pseudomonas*. Bot. Gaz. 124:324-328.
5. Gross, D.C., and J. E. DeVay. 1977. Production and purification of syringomycin, a phytotoxin produced by *Pseudomonas syringae*. Physiol. Plant Path. 11:13-28.
6. Gross, D.C., J. E. De Vay and F. H. Stadtman. 1977. Chemical properties of syringomycin and syringotoxin: toxigenic peptides produced by *Pseudomonas syringae*. J. Appl. Bacteriol. 43:453-463.
7. Kado, C.I. and M. G. Heskett. 1970. Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas* and *Xanthomonas*. Phytopathology 60:969-976.
8. King, E.O., M. K. Ward and D. E. Raney. 1954. Two simple media for the demonstration of pyocianin and fluorescin. J. Lab. Clin. Med. 44:301-307.
9. Lelliott, R.A., E. Billing and A.C. Hayward. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.
10. López, M.M., and C. Noval. 1980. Bacterial diseases of tomato in Spain. Proc. 5th Congr. Meditteranean Phytopathol. Union. 140-141.
11. Misaghi, I., and G. R. Grogan. 1969. Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent pseudomonads. Phytopathology 59:1436-1450.
12. Mitchell, R. E. 1976. Isolation and structure of a chlorosis-inducing toxin of *Pseudomonas phaseolicola*. Phytochemistry 15:1941-1947.
13. Okabe, N. 1933. Bacterial diseases of plants occurring in Formosa. II. Bacterial leaf spot of tomato. J. Soc. Trop. Agric. Taiwan 5:25-36.
14. Okon, Y., Y. Bashan and Y. Henis. 1978. Studies of bacterial speck of tomato caused by *Pseudomonas tomato*. Proc. 4th Int. Conf. Plant Path. Bact. Angers, pp.699-702.
15. Rasche, E., 1977. Untersuchungen zum Wirkungsmechanismus des *Pseudomonas phaseolicola*-Toxins. Diss. Univ. Göttingen.
16. Rudolph, K. 1976. Non-specific toxins. In: Heitefuss, R. and Williams, P.H. (Eds.), Encyclopedia of Plant Physiology, New Series, Vol. 4: Physiological Plant Pathology, 270-315. Springer-Verlag, Berlin-Heidelberg-New York.
17. Rudolph, K., and E. Rasche. 1981. Purification of the chlorosis-inducing toxin from *Pseudomonas phaseolicola* (Burkh.) Dowson. Phytopath. Z. 101:298-313.
18. Rudolph, K., E. Rasche and W. Zeller. 1978. Production of the chlorosis inducing toxin in liquid cultures of *Pseudomonas phaseolicola* (Burkh.) Dowson. Arch. Microbiol. 119:219-226.
19. Schneider, R.W., D. H. Hall and R. G. Grogan, 1975. Effect of bacterial speck on tomato yield and maturity. Proc. Amer. Phytopath. Soc. 2:118.
20. Schuster, M.L. 1955. A method for testing resistance of beans to bacterial blights. Phytopathology 45:519-520.

21. Sinden, S.L. and R. D. Durbin. 1969. Some comparisons of chlorosis-inducing *Pseudomonas* toxins. Phytopathology 59:249-250.

22. Staskawicz, B.J., and N. J. Panopulos. 1979. A rapid and sensitive microbiological assay for phaseolotoxin. Phytopathology 69:663-666.

23. Steward, W.W., 1971. Isolation and proof of structure of wildfire toxin. Nature 229 174-178.

24. Sutic, D. 1957. Bacteriosis of tomatoes. Zastita bilja, 6, Beograd, 66 pp.

25. Watanabe, M. 1963. Studies on the nutritional physiology of *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson. II. Effect of carbon and nitrogen sources on multiplication of bacteria. Ann. Phytopath. Soc. Jap. 28:201-208.

26. Wilkie, J. P., and D. W. Dye. 1974. *Pseudomonas tomato* in New Zealand. J. Agric. Res. 17:131-135.

27. Woolley, D.W., R. B. Pringle and A.C. Braun. 1952. Isolation of the phytopathogenic toxin of *Pseudomonas tabaci*, an antagonist of methionine. J. Biol. Chem. 197:409-417.

28. Young, J. M., D.W. Dye, J. F. Bradbury, C. G. Panagopoulos and C.F. Robbs, 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N. Z. J. Agric. Res. 21:153-157.

29. Zachos, D.G., and S. G. Georgopoulos. 1957. The bacterial canker of tomatoes in Greece. Ann. Inst. Phytopath. Benaki, N. S. 1:98-102

Acknowledgments

Bacterial Leaf Spot of Ornamental Triplaris Caused by
Pseudomonas andropogonis

C. F. Robbs
Universidade Federal Rural do Rio de Janeiro
Rio de Janeiro, Brazil

J. Rodrigues Neto
Instituto Biológico de Sao Paulo
Sao Paulo, Brazil

R. de L. D. Ribeiro, O. Kirmura
Universidade Federal Rural Do Rio de Janeiro
Rio de Janeiro, Brazil

Abstract

A bacterial leaf spot leading to severe defoliation was found at a nursery of *Triplaris filipensis* Cham. in Itaguaí county, Rio de Janeiro. The pathogen was a rod-shaped, Gram-negative bacterium with an atypical polar flagellum. Cells were characterized by sudanophilic inclusions. The organism was oxidase and arginine dihydrolase-negative. It did not utilize sucrose or hydrolize aesculin, and produced no fluorescent pigment on King's B medium and other media. On the basis of morphological and biochemical properties the bacterium was identified as *Pseudomonas andropogonis* (E. F. Smith) Stapp. Strains of the same species isolated from sorghum *(Sorghum vulgare* Pers.) including ATCC-23061, and coffee *(Coffea arabica* L.) were compared with the *Triplaris* pathogen. All the strains could infect sorghum, sweet corn *(Zea mays* L. var. *saccharata* (Sturtev.) Bailey), and black mucuna *(Stizolobium aterrimum* Piper & Tracy) by artificial inoculations. The sorghum strains were not pathogenic on *Triplaris* or coffee. The coffee strain did not infect *Triplaris* and vice-versa. None of the strains infected bougainvillea *(Bougainvillea spectabilis* Willd.), reported as a natural host of *P. andropogonis* in Africa. Results thus indicate the existence of pathogenic specialization among strains of *P. andropogonis*, as reported by other authors.

Introduction

Several species of the genus *Triplaris* are widely used as ornamental shade trees throughout the States of Rio de Janeiro and Sao Paulo, Brazil.

In 1978 a leaf spot was obsrved on *Tripolaris filipensis* Cham. at a nursery located in the county of Itaguaí, state of Rio de Janeiro. Lesions were initially dark green and watersoaked, becoming reddish to dark brown upon aging. They were circular or irregularly shaped, attaining 0.5 to 1.0

cm in diameter. Spotted leaves turned chlorotic and senesced prematurely, leading to severe defoliation.

Preliminary studies showed that the disease was caused by a Gram-negative, rod-shaped bacterium, containing refractible sudanophylic inclusions, motile by an atypical, thick polar flagellum, and negative for the oxidase reaction. The organism formed white colonies and did not produce fluorescent pigments on King's medium B (7). All these bacteriological properties are characteristic of the species *Pseudomonas andropogonis* (E.F. Smith) Stapp.

The purpose of this paper is to characterize the causal agent of the bacterial leaf spot of *T. filipensis* and to establish its relationship to *P. andropogonis* with respect to bacteriological characteristics and pathogenicity to other hosts.

Materials and Methods

Isolations

Leaf pieces bearing water-soaked lesions were surface-sterilized with a freshly prepared 0.5% solution of sodium hypochlorite for 3 min. After rinsing for 2 min. in sterile, distilled water, diseased tissues were removed, cut into smaller pieces and dipped in 0.1 ml of nutrient broth. After 30 min the suspension of bacteria was streaked on King's medium B and the plates were incubated for 5 days at 28°C. Individual colonies were selected and purified by streaking on new plates of the same medium. Stock cultures were maintained on King's B slants and stored at ± 5°C.

Bacterial Strains

The bacterial strains used in the study were: ENA-2485, ENA-2515, ENA-2540, and IB-155, all isolated from leaves of *T. filipensis* in Itaguaí, Rio de Janeiro; *P. andropogonis* IB-165 from *Coffea arabica* L. (9) in the State of Santa Catarina, and ENA-100 from sorghum (*Sorghum vulgare* Pers.) in Mogy-Mirim county, State of Sao Paulo, Brazil, and *P. andropogonis* NCPPB 934 (=ATCC 23061), originally isolated from sorghum in the U.S.A.

Morphological Properties

Cell morphology was determined from light microscope observations of Gram-stained preparations. The Gram staining method as modified by Hucker (Society of American Bacteriologists, 1957), was used. Flagella were observed by electron microscopy, using shadow-cast preparations. Sudanophilic inclusions were detected by Burdon's method (2) using cells grown on nutrient agar containing 0.5% beta-hydroxybutyrate. Colonies were examined for size, color, consistency, and morphology on yeast extract-dextrose-calcium carbonate agar (3).

Physiological and Biochemical Properties

Fluorescent pigment production was investigated on King's medium B after 3 days of incubation at 28°C, and examined under the UV light. The mode of glucose utilization, oxygen requirements, tolerance to sodium

chloride, utilization of asparagin as a sole source of carbon and nitrogen, catalase and urease formation, action on litmus milk, hydrogen sulphide and nitrite production, and starch, aesculin or gelatin hydrolysis were determined following the methods used by Dye (3). Formation of oxidase and arginine dihydrolase, action on egg yolk, levan production, potato soft rot induction, and the reaction of infiltrated tobbaco leaves were determined with the methods described by Lelliott *et al.* (8). For studying lipolysis of Tween 80 the medium of Sierra (12) was employed. Utilization of carbon compounds was determined by the methods described by Stanier *et al.* (13).

Pathogenicity

Inoculation tests were carried out by atomizing leaves immediately after they had been punctured with a hypodermic needle. A De Vilbiss atomizer was used. Inocula consisted of suspensions prepared from 24-h old cultures grown on King's B slants, and containing ca 10^6 colony forming units/ml. Inoculated plants were kept in moist chambers for 48 h then transferred to a screen house. Check plants were wounded similarly and sprayed with distilled sterile water. The following plant species were tested: sweet corn (*Zea mays* var. *saccharata* (Sturtev.) Bailey cv IAC Doce Cubano), sorghum (cv Sart), black mucuna (*Stizolobium aterrimum* Piper & Tracy cv Comum), coffee (cv Catuaí Amarelo), *T. filipensis,* and bougain-villea (*Bouganvillea spectabilis* Willd cv Vermelha). All potted plants were inoculated at the 3-5 leaf stage.

Results

All strains were Gram-negative, straight rods with rounded ends, occurring singly or in pairs. Electron microscopy revealed the presence of a single polar flagellum of unusual thickness which has been shown (4) to be sheathed. Sudanophylic inclusions were detected in all strains. The organisms were rather slow-growing on the media used, forming white, circular colonies, with entire margins, convex, butyrous, smooth, and characterized by a strongly viscid consistency as they grew older. All strains showed identical physiological and biochemical properties *in vitro.* No fluorescent pigments were produced. Oxygen was required for growth and a strictly oxidative metabolism of glucose was exhibited. Asparagin was utilized in all cases and growth was absent on media with 4% sodium chloride.

The bacteria were catalase-positive, oxidase-negative, and failed to produce levan colonies, to cause potato soft rot, and to reduce nitrates. Negative responses were obtained for production of arginine dihydrolase, hydrolysis of starch, gelatin or aesculin, and Tween 80 lipolysis. Results were also negative in the egg yolk and hydrogen sulphide tests. Litmus milk turned alkaline with curd formation.

Acid was produced from: arabinose, fructose, glucose, glycerol, mannitol, mannose, and sorbitol, within 10 days incubation, and from lactose within 14 days. The following substrates were not utilized: dextrin, cellobiose, dulcitol, maltose, erythritol, raffinose, rhamnose, sucrose, melibiose, salicin, D (-) tartrate, L(+) tartrate, meso-tartrate, benzoate, DL-

lactate, and oxalate. Variable reactions occurred with regard to the utilization of inositol (not utilized by strains ENA-2485, ENA-2540 and IB-165) and trehalose (utilized only by strains ENA-2485 and ENA-2515). All strains induced a hypersensitive reaction when infiltrated into tobacco leaves.

A comparative study of the pathogenicity of the strains (Table 1) revealed the following: (1) on sorghum and black mucuna, all strains produced water-soaked, expanding lesions around punctures, on 4th day after inoculation; (2) on sweet corn, the Triplaris and coffee strains were weakly pathogenic, producing discrete non-expanding, water-soaked lesions, whereas a typical compatible interaction was obtained with the sorghum strains; (3) on Triplaris and coffee, only the respective homologous strains were pathogenic; and (4) on bouganvillea all strains were non-pathogenic.

Discussion

The morphological, physiological, and biochemical characteristics of the causal agent of *T. filipensis* bacterial leaf spot indicated its relationship to *P. andropogonis*. Results are in agreement with those of other workers (1, 5, 6) who have studied strains of that species isolated from other hosts. *P. andropogonis* fits into group III of the subdivisions proposed by Sands *et al.* (11) for the phytopathogenic pseudomonads. This group included the non-fluorescent pseudomonads affecting gramineae; *P. andropogonis* is the only member with an atypical, sheathed flagellum (4) and a negative oxidase reaction, however.

Variability regarding the utilization of certain carbon sources was not correlated to the original hosts or pathogenicity of the strains. This variation has also been reported by other workers (5, 6) and has little or no taxonomic significance.

An important characteristic of *P. andropogonis* relates to its wide host range, when compared to the other pseudomonads affecting gramineae. Besides gramineae, species in other botanical families such as *Trifolium* spp. (6), *Bouganvillea* sp. (10) coffee (9), and now *T. filipensis* are recorded.

Pathogenic specialization within *P. andropogonis* previously suggested by Goto and Starr (5) has also been observed in this study. Thus, the coffee and the *Triplaris* strains did not cross-infect and were not pathogenic to *Bouganvillea spectabilis*, a natural host of the pathogen in Africa (10).

One of the bacterial strains from *T. filipensis* used in this investigation was deposited in the National Collection of Plant Pathogenic Bacteria, Harpenden, England, where it received the number 3132.

Table 1. Pathogenicity to selected hosts of strains of *Pseudomonas andropogonis* from Triplaris *(T. filipensis)*, sorghum, and coffee.

Strains	Original host	Response of test plants					
		Sorghum	Sweet corn	Triplaris	Black Mucuna	Coffee	Bougainvillea
ENA-2485	Triplaris	+[a]	±	+	+	−	−
ENA-2515	Triplaris	+	±	+	+	−	−
ENA-2540	Triplaris	+	±	+	+	−	−
IB-155	Triplaris	+	±	+	+	−	−
ENA-100	Sorghum	+	+	−	+	−	−
NCPPB-934	Sorghum	+	+	−	+	−	−
IB-165	Coffee	+	±	−	+	+	−

[a] + = pathogenic (typical, expanding lesions), − = non-pathogenic, ± = weakly pathogenic

Literature Cited

1. Allen, R.N., A. C. Hayward, W. J. Halliday and J. Fulcher. 1970. Bacterial blight of *Vicia sativa:* aetiology of the disease and identification of the pathogen. Aust. J. Biol. Sci. 23:597-606.
2. Burdon, K.L. 1946. Fatty material in bacteria and fungi revealed by staining dried fixed slide preparation. J. Bacteriol. 52:665-678.
3. Dye, D.W. 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N.Z.J. Sci. 5:393-416.
4. Fuerst, J.A. and A. C. Hayward. 1969. The sheathed flagellum of *Pseudomonas stizolobii.* J. Gen. Microbiol. 58:239-245.
5. Goto, M. and M.P. Starr. 1971. A comparative study of *Pseudomonas andropogonis, P. stizolobii,* and *P. albopreciptans.* Ann. Phytopath. Soc. Japan 37:233-241.
6. Hayward, A.C. 1972. A bacterial disease of clover in Hawaii. Plant Dis. Rep. 56:446-450.
7. King, E. O., M. K. Ward, and D. E. Raney, 1954. Two simple media for the demonstration of pyocyanin and fluorescent in J. Lab. Clin. Med. 44:301-307.
8. Lelliott, R.A., E. Billing, and A. C. Hayward, 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.
9. Rodrigues Neto, J., P. Figueredo, P.R. Mariotto, and C. F. Robbs. 1981. *Pseudomonas andropogonis* (Smith 1911) Stapp 1928, casual agent of "bacterial dark spot" in coffee leaves (*Coffea arabica* L.) Arq. Inst. Biol. S. Paulo (in press).
10. Rothwell, A., and A. C. Hayward. 1964. A bacterial disease of bouganvillea. Rhodesian J. Agr. Research 2:97-99.
11. Sands, D., M. N. Schroth, and D. C. Hildebrand. 1970. Taxonomy of phytopathogenic pseudomonads. J. Bacteriol. 25:133-137.
12. Sierra, G. A. 1957. A simple method for the detection of lypolytic activity of microorganisms. Antonie van Leeuwenhoek, J. Microbiol. Serol. 23:15-22.
13. Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. GEN. Microbiol. 43:159-271.

A Biovar of *Pseudomonas fluorescens* Pathogenic to *Allium sativum*

R. Samson

Institut National de Recherches Agronomiques
Angers, France

Abstract

The current status of *Pseudomonas fluorescens (sensu lato)* is that of an ordinary water and soil inhabitant. Phytopathologists, when isolating it from diseased plants, generally consider the bacterium a saprophytic contaminant. However, recent studies have implicated some *P. fluorescens* isolates as disease agents. This paper gives as an example a disease of garlic *(Allium sativum)*, called in France "café au lait," caused by a particular strain of *P. fluorescens*. The disease essentially results in a lateral rot of the plant in the field. Wet weather leads to soft rot of the whole plant. Three hundred fifty strains of *P. fluorescens* (Lelliott's groups IV and V) isolated from garlic, soil, and various plants were studied. The non pathogenic isolates were separated in many phenotypes that did not fit with Stanier's biotypes. On the other hand, all the 80 strains pathogenic to garlic could be classified as the same phenotype that is a subdivision of Stanier's biotype A. The most useful tests for definition of phenotypes within *P. fluorescens* are as follows: oxidase and arginine dihydrolase, growth at 41°C, levan formation, denitrification, gelatin, ethanol, trehalose, gluconate, serological agglutination and colistine sensitivity.

Introduction

Since 1976, a new bacterial disease of garlic *(Allium sativum)*, called "café au lait" disease, has appeared in the southwest of France, a traditional garlic growing area.

During the growing season, April to June, diseased plants show yellowing and wilting of one or two leaves; in addition, a brown rot of the pseudostem develops. The lesions appear initially at the base of the leaf sheath, generally on internal leaves. During periods of rain, diseased plants decay completely. However, bulb formation usually is not affected, and disease is restricted to the tunics that become torn and dark brown. Losses are incurred by growers when they have to market brown tunic garlic of low commercial quality.

No known phytopathogenic bacteria were obtained from the isolations from diseased plants, but many unknown bacteria, including numerous strains of *Pseudomonas fluorescens,* were isolated. All strains were

inoculated on garlic to check pathogenicity. Symptoms of garlic soft rot were reproduced with some of the isolates that were identified as *P. fluorescens*. However, strains pathogenic to garlic were only a portion of the *P. fluorescens* strains isolated; other strains did not elicit symptoms.

Bacteriological characteristics were studied to determine the differences between the pathogenic and nonpathogenic strains of *P. fluorescens*.

Materials and Methods

Bacteria

Studies were completed with 221 strains of *Pseudomonas fluorescens* isolated from "café au lait" garlic and from garlic field soil. These strains were compared with the following similar phytopathogenic species: *Pseudomonas marginalis* pv. *alfalfa* CNBP (Collection Nationale de Bacteries Phytopathogeues, INRA, France) 2039 (=NCPPB 2644), isolated from *Medicago sativa* by Shinde and Lukezic 1971; *P. marginalis* pv. *marginalis* CNBP 1387 (=ATCC 10844), isolated from *Cichorium iutybus* by Friedman 1949; *P. marginalis* pv. *pastinacea* CNBP 2038 (ATCC 13889), isolated from *Pastinaca* sp. by Burkholder 1959; *P. fluorescens* CNBP 1968, isolated from *Apium graveoleus* by Surico and Iacobellis 1978; *P. fluorescens* CNBP 1973, 1974, and 1975, isolated from *Foenicolum vulgare* by Surico and Iacobellis 1978; and *P. fluorescens* 26 II, isolated from *Psalliota bispora* by Olivier 1978.

Bacteriological Characters

Procedures followed were mainly those described by Lelliott *et al.* (2) and Stanier *et al.* (4). fluorescence on King's B, arginine dihydrolase according to Thornley, oxidase test, levan production, denitrification, and growth at 4°C and 41°C. The following carbon sources were added to a liquid mineral nutrient medium containing biomothymol blue to detect acidification or alcaliuisation: ethanol, trehalose, sucrose, adonitol, erythritol, sorbitol, inositol, and D(-) tartrate. The other physiological tests that were performed included: reducing substances from sucrose, egg yolk, gelatin according to Frazier, esculin hydrolysis, nitrate reduction, pectate liquefaction on polypectate (pH8) according to Hildebrand, and gluconate oxidation. Slide agglutination was detected with antisera prepared with strains pathogenic to garlic, one with heat-killed bacteria (n° 105), the other one with whole motile cells (n° 114). Sensitivity of the bacteria to colistin was tested with discs (50 ng). Hypersensitive reaction was completed on leaves of *Nicotiana tabacum* "Xanthi" plants.

Pathogenicity Tests

Potted or field plants of *Allium sativum* were inoculated by leaf infiltration with a suspension of 10^8 bacterial/ml. All bacteriological and pathological tests were repeated three times.

Results

All strains were fluorescent on King's B medium, produced no orange/blue pigment on King's A medium, grew at 4°C, did not grow at

41°C, and were oxidase and arginine dihydrolase positive. They all could be classified as strains of *P. fluorescens* (1).

Pathogenic Strains

All 85 pathogenic strains show the same characteristics (Table 1), with the exception of ability to liquefy polypectate. This was variable with the isolates. The majority are pectolytic, however. Nonpectolytic strains are stable because if they are reisolated from inoculated plants showing symptoms, all the colonies from such strains are also nonpectolytic. Thus, pectolytic activity, as demonstrated in vitro, is not a uniform characteristic for garlic strains. Hilbebrand's medium gave the most constant results, compared to potato slices and to Kaiser and Prunier's calcium pectinate. HR on tobacco is positive just after isolation. After two transfers, the cultures gave negative results.

Nonpathogenic Strains

Among 144 nonpathogenic strains, 11 were essentially similar to pathogenic isolates. The 133 remaining strains were distributed in about 60 different combinations of the tested characters. When the biotype classification of Stanier *et al.* (4) and Doudoroff and Palleroni (1) was followed for the species *P. fluorescens*, based on levan production and denitrification, nonpathogenic strains were as follows: (a) 22 percent belonged to biotype A or I, but only half were ethanol negative; strain 1973 was ethanol positive; (b) 20 percent belonged to biotype B or II, positive on any biochemical test (strains 1387, 2038, and 2039 were in this group); (c) 15 percent belong to biotype C or III, including strains 1974 and 1975; (d)46 percent belong to the miscellaneous group (1) or G biotype (4). Among them, 5 percent are *Pseudomonas putida*. Strains 1968 and 26 II are classed in G biotype.

Discussion

Pseudomonas fluorescens seems to be a very complex species composed of many different biovars. Strains pathogenic to garlic constitute one particular biovar. The 11 other isolates giving the same bacteriological pattern may be nonaggressive strains of that pathogenic biovar. Two tests seem to be specific to garlic strains: serological agglutination and colistine resistance. The presence of a specific serogroup within *P. fluorescens* is rather surprising, as well as evidence of colistine resistance since colistine is known to be particularly active on strains in the genus *Pseudomonas*.

Concerning the strains non pathogenic to garlic, they may represent a random sampling of *P. fluorescens* populations.

Garlic "café au lait" studies lead to just one *P. fluorescens* biovar. Discordant results exist in literature about potato macerating isolates of *P. fluorescens*. According to Sands and Hankin (9) they represent a continuum of phenotypes from *P. fluorescens* to *P. putida*. On the contrary, for Cuppels and Kelman (2), they generally belong to biotype II. Discordance may result from isolation methods, particularly when selective media are used.

Table 1. Differential characteristics of *Pseudomonas fluorescens* strains isolated from diseased garlic *(Allium sativum)* or garlic field soils.

Tests	Pathogenic strains		Nonpathogenic strains			
Levan production	+	+	+	+	–	–
Denitrification	–	–	–	+	+	–
Ethanol acidification	–	–				
Sucrose "	+	+				
Trehalose "	+	+				
Adonitol "	+	+				
Erythritol "	+	+	about			
Sorbitol "	+	+	–60 biovars			
Inositol "	+	+	(2 strains/biovar)			
D(–) tartrate alkalinisation	–	–				
Esculin hydrolysis	–	–				
Reducing substances from sucrose	+	+				
Gluconate oxidation	–	–				
Egg yolk	+	+				
Gelatin	+	+				
Nitrate reduction	–	–				
Polypectate (pH 8)	+/–	+				
Colistine disc 50 ng	R	R	generally sensitive			
Serological agglutination n^r 105 and 114	+	+	–			
Pathogenic to *Allium sativum*	+	–	–			
Number of strains	85	11	133			

Further work on the taxonomy of *P. fluorescens* is needed, particularly on isolates that elicit biological activity such as pathogenicity. Levan production and denitrification, if important, are not sufficient to give account of heterogeneity of the species. Sands and Rovira (10) added ethanol, trehalose, sorbitol, and gelatin utilization, and they classified their isolates according to GLDETS combinations. The present study indicates that more characters are needed and can help in the differentiation of strains in the whole species. When working on *P. fluorescens* isolates, one should choose a long list of discriminative biochemical tests, serological reactions, and antibiotic sensitivities in order to obtain an adequate data base for comparisons involving the strains.

Garlic pathogenic strains constitute one more example that

Pseudomonas fluorescens is not an ordinary saprophytic organism as it has been considered for years by many phytobacteriologists.

Literature Cited

1. Doudoroff, M., and N. J. Palleroni. 1974. Genus 1 Pseudomonas, p. 217-243 in: R. E. Buchanan and N. E. Gibbons (eds.) Bergey's manual of determinative bacteriology, 8th ed. Williams and Wilkins Co., Baltimore, 1246 pp.
2. Lelliott, R.A., E. Billing, and A. C. Hayward, 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads J. Appl. Bacteriol. 29 470-489
3. Sands, D.C., and A. D. Rovira. 1971. *Pseudomonas fluorescens* biotype G the dominant fluorescent pseudomonad in South Australia soils and wheat rhizospheres J. Appl. Bacteriol. 34:241-275.
4. Stainer, R.Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads a taxonomic study. J. Gen. Microbiol. 43:159-271.

Bacterial Brown Blotch, a Disease of Rice in Tropical America

J. C. Lozano, CIAT, Cali, Colombia
J. Victoria, Instituto Colombiano Agropecuario, Palmira, Colombia
A. C. Velasco, S. W. Ahn, CIAT, Cali, Colombia

Abstract

A bacterial disease of rice not described previously has been observed in Central and South America. Symptoms are characterized by spreading brown blotches on the leaves. Systemic invasion by the pathogen causes necrosis of the vascular system of the leaves, resulting in their partial or total browning. Under greenhouse conditions, lesion enlargement is generally slower than that of bacterial leaf blight. The pathogen grows on media containing different sources of carbohydrates. Colonies on Wakimoto and Yoshii medium are round, entire, and dark yellow, easily distinghuishable from those of *Xanthomonas campestris* pv. *oryzae* on the same medium. Although the organism has the general characteristics of members of the genus *Xanthomonas*, several physiological and biochemical characteristics are different from those reported for *X. campestris* pv. *oryzae*. Similarly, resistance to this organism is not correlated with resistance to the causal agent of bacterial blight of rice.

Introduction

In 1977, in the course of a survey for bacterial blight in the Americas (8) an atypical bacterial disease symptom was observed in several rice plantations of Central and South America (Panama, Colombia, Peru, Bolivia). Isolations from plants with these symptoms yielded bacterial colonies different from those of Xco on the same medium.

The following bacterial diseases of rice have been reported whose symptoms and causal agents are well defined (9):

1. Bacterial blight (*Xanthomonas campestris* pv. *oryzae*) (Xco) (20). On seedlings, it appears as tiny watersoaked spots at the margins of fully developed leaves. As the spots enlarge, the leaves turn yellow, dry rapidly, and wither. On leaf blades, lesions usually begin at the margin, a few cm from the tip, as watersoaked strips. Lesions enlarge, have a wavy margin and turn yellow within a few days. They cover the entire blade, turn white and later become greyish (9-10).

2. Bacterial leaf streak (*X. translucens* sp. *oryzicola*). Initially shows fine translucent streaks enlarging lengthwise over the larger veins. Old lesions become light brown and later, the entire leaves turn brown and die (9-10).

3. Bacterial stripe (*Pseudomonas panici*). The disease starts from the lower part of the leaf sheath, where water-soaked, dark green, longitudinal stripes are formed. The disease is present mostly on young plants (9).

4. Bacterial sheath rot (*P. oryzicola*). The disease develops before the ears emerge on leaves sheathing the panicles. On stems, it produces blurred spots which turn brown or black later (9).

This paper describes a fifth, previously unreported disease and its causal agent in the Americas. It is apparently different from the other four pathogens.

Materials and Methods

The pathogen was isolated on Wakimoto and Yoshii medium (9) by streaking a bacterial suspension that was obtained by suspending several leaf pieces in sterile water for 30 min. Forty-eight hours later after incubation at 28°C, pure cultures were obtained by selecting typical colonies and restreaking on the same medium. Cultures were grown for pathogenicity and biochemical tests at 28°C for 48 h.

Inoculations were made on 60-day-old rice plants by: a) the leaf-clipping inoculation technique of dipping a sterilized scissors in a bacterial suspension and cutting the leaves 4-5 cm from the tip (9); b) cutting the roots and then dipping them into the bacterial suspension; and c) pricking the leaf-blades with sterile needles smeared with the bacterial suspension. Root-inoculated plants were planted in sterile sandy soil and watered daily with sterile water for two weeks after first symptom expression. For these inoculations, bacterial suspensions were adjusted to concentrations of approximately 1×10^9 cells/ml.

Cultural, physiological, and biochemical tests were completed following the procedures previously used to characterize Xco (9, 10, 12). Strains of Xco (1185 and 1186) of the CIAT bacterial collection served as checks throughout the study. Pigments were extracted from 36 h cultures following Starr and Stephen's (18) procedures. Ten 2-month-old plants were inoculated per genotype and strain by the leaf clipping inoculation method. Readings were taken 4 weeks after inoculation.

Varietal reaction was recorded based on Ou's 1 to 10 scale for evaluation for resistance to Xco (11).

Results and Discussion

Five days after leaf clipping and pricking inoculations, initial symptoms appeared as tiny brown spots spreading from the inoculated point. These spots enlarged and coalesced, spreading as brown blotches; in hand sections, vascular necrosis was evident 15 days after inoculation. Fifteen days later, partial or total blight with brownish borders is present resembling those symptoms induced by Xco in advanced stages of infection (Fig. 1). In plants inoculated via roots, leaf symptoms appeared 7 days after inoculation. Initially, they appeared on leaf tips as tiny brown spots that enlarged to form blotches and then the syndrome described above. Roots
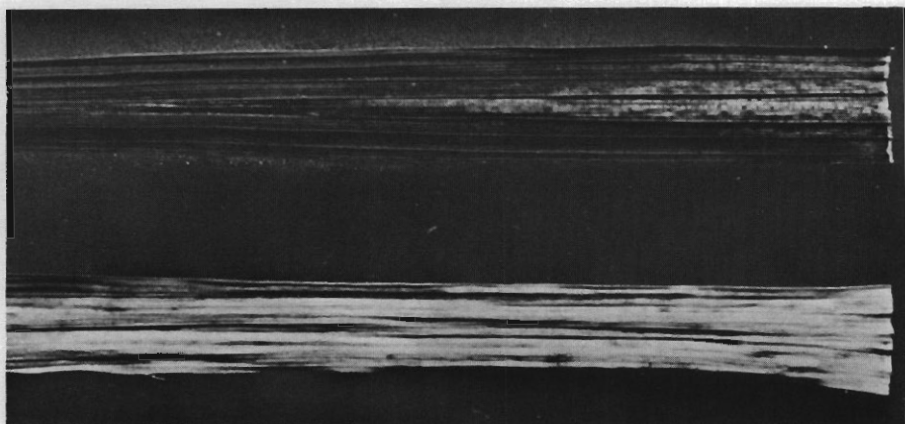
Fig. 1. Symptoms of rice Blue-bonnet-50 leaf clipping inoculated (1 x 10⁹ cells/ml) with the brown blotch bacterium (upper) and Xco (lower) 30 days after incubation in greenhouse at 24°C (28°C maximum; 16°C minimum) average and 2500 f.c. for 12 h daily photoperiod.

showed vascular necrosis and brown vessels. Generally, the development of symptoms was much slower than the Xco infection.

Colonies on Wakimoto and Yoshii medium (9) are smaller, less mucoid and more yellow than those of Xco on the same medium. The pathogen is a rod with a single polar flagellum. Some physiological and biochemical characteristics were different from those reported for Xco, especially the absorption spectrum of the yellow pigment (Fig. 2) (18), in oxidase production (6, 19), tolerance to tetrazolium chloride (TZC) (7), H_2S production from cyteine (1), esculin hydrolysis (16) and acid production from glucose, galactose, trehalose and cellobiose (Table 1, 2, and 3) (1, 12).

Varietal resistance was apparently not correlated with resistance to the strains of Xco used (Table 4).

From the cultural, physiological, and biochemical tests it is evident that this pathogen belongs to the *Xanthomonas* genus (1, 2, 18). However, the causal organism is different from Xco with respect to symptoms on rice and also from the other reported rice bacterial pathogens (9, 10, 11, 12). In addition, it differs in some cultural, physiological, and biochemical characteristics from Xco (Table 1, 2, 3). The host to the bacterial brown blotch pathogen does not appear to correlate with that of Xco (Table 4), although various pathotypes of Xco have been reported. It appears that this pathogen differs in a number of characteristics, to the extent that it could be considered as a different pathovar of Xco on rice. Its geographic distribution in America indicates that the pathogen could be a native of this continent that evolved on native grass weeds closely related to rice. It has been isolated from liendre de puerco (*Echinocloa colonum*), and samples taken from both infected plants in rice fields and weeds following crops of rice.

The taxonomic status of this bacterium as well as its ecological behavior can only be determined after further investigations in which a number of different strains of Xco are compared with those of this pathogen from different geographical areas of Latin American rice growing regions.
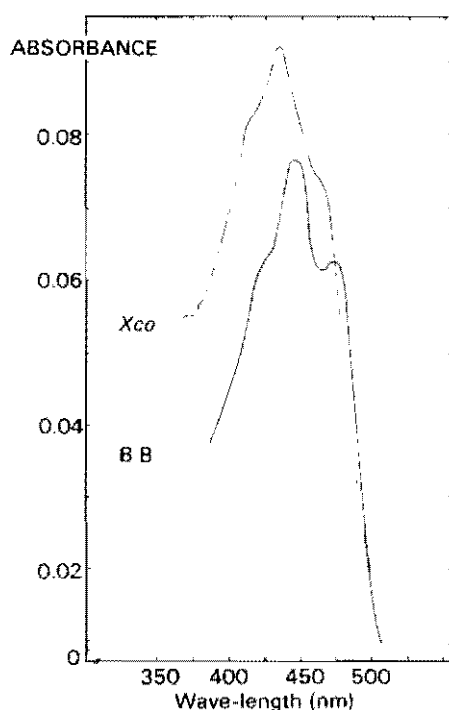
Fig. 2. Absorption spectrum *Xantho-monas campestris* pv *oryzae* (Xco) and the causal agent of brown blotch (BB) in uv Spectronic 20

Table 1. Morphological and biochemical characters of *Xanthomonas campestris* pv. *oryzae* and a yellow group of isolates from tropical America.

Character	Reference source	*X.c. oryzae*[a] (CIAT isolates 1185 & 1186)	Yellow group (CIAT isolates 1171, 1173, 1187, 1191 & 1192)
Gram stain		Gram-negative	Gram-negative
Flagellation		single polar	single polar
Bacterial pigments	18	465 - 438 nm[b]	473 - 455 nm
Oxidase	6,19	−	+
Catalase activity	1	+	+
Glucose utilization	4	Oxidative	Oxidative
Nitrate reduction	17	−	−
Tolerance to o/o TZC	7	0o/o	0.02 - 0.05o/o
Indole	5	−	−
H₂S production from cysteine	1	+	+++

[a] − = negative reaction; + = positive reaction; ± = weakly positive reaction; d = 21 to 79o/o of isolates were positive.

[b]Peak of Spectrometric absorption.

Table 2. Biochemical and physiological characters of *Xanthomonas campestris* pv. *oryzae* and a yellow group of isolates from tropical America.

Character	Reference source	*X.c. oryzae*[a] (CIAT isolates 1185 & 1186)	Yellow group (CIAT isolates 1171, 1173, 1187, 1191 & 1192)
Growth at 35°C	1	d	+
Esculin hydrolysis	16	+	−
Casein Hydrolysis	1,15	−	−
Gelatin liquefaction	12,17	±	−
Urease production	1	−	−
Growth in 5º/o NaCL	1	−	−
Acid production on:	1,12		
— Arabinose		+	−
— Glucose		+	+
— Mannose		+	d
— Galactose		+	−
— Trehalose		+	−
— Cellobiose		+	−

[a]_ = negative reaction; + = positive reaction; ± = weakly positive reaction; d =21 to 79º/o of isolates were positive.

Table 3. Biochemical and physiological characters of *Xanthomonas campestris* pv. *oryzae* and a yellow group of isolates from tropical America.

Character	Reference source	*X.c. oryzae*[a] (CIAT isolates 1185 & 1186)	Yellow group (CIAT isolates 1171, 1173, 1187, 1191, & 1192)
Tyrosinase	1	—	—
β-Glucosidase	3	Acid	—
Lipolytic activity	14	+	±
Phenylalanine deaminase	13	—	—
Ammonia production	1	±	+
Acid production on:	1,12		
— Xylose		+	—
— Fructuose		+	++
— Sucrose		+	—
— Starch		—	—
Gas production on:	1,12		
— Glucose		—	—
— Sucrose		—	—

[a] — = negative reaction; + = positive reaction; ± = weakly positive reaction; d = 21 to 79º/o of isolates were positive.

Table 4. Reaction of some rice genotypes to *Xanthomonas campestris* pv. *oryzae* and to a yellow isolate causing brown blotch from tropical America; 15 days after clipping inoculation ($\cong 1 \times 10^9$ Cells/M1).

Genotype	*X.c. oryzae* (CIAT No. 1185)	Yellow isolate (CIAT No. 1192)
Native capuringa 1	10[a]	9
CICA 4	7	9
Bbt - 50	8	10
6028 (Bahagia x IR 262)	8	9
6041 (Bahagia x IR 262)	10	10
6065 (Bahagia x Bg 90 - 2)	9	9
6067 (Bahagia x Bg 90 - 2)	7	9
Pelita 1/1	4	1
Tadukan	5	2
OS-6	4	2
5955 (Bahagia x 73 - 805)	4	3
6008 (Bahagia x 73 - 805)	4	1
6045 (Bahagia x IR 262)	6	2
6071 (Bahagia x Bg 90 - 2)	6	1
IR 2070 - 423 - 2 - 5 - 6	3	9
T 442 - 57	2	8
5979 (Bahagia x 73 - 805)	2	9
6009 (Bahagia x 73 - 805)	3	7
DV - 2	8	2
5976 (Bahagia x 73 - 805)	7	2
6025 (Bahagia x IR 262)	8	3
6068 (Bahagia x Bg 90 - 2)	9	2
6072 (Bahagia x Bg 90 - 2)	10	2
Remadja	3	1
Bg 66 - 1	3	1
5959 (Bahagia x 73 - 805)	3	2
5984 (Bahagia x 73 - 805)	2	1
5990 (Bahagia x 73 - 805)	3	2
5999 (Bahagia x 73 - 805)	3	1

[a]Disease rating following Ou's scale of evaluation (Plant Disc. Reptr. 55:17-21).

Literature Cited

1. Dye, D. W., 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* species. N. Z. J. Sci. 5:393-416.

2. Hayward, A. C., 1966. Methods of identification in the genus *Xanthomonas In:* Gibbs, B.M., and F. A. Skinner. Identification methods for microbiologists. The Society for applied Bacteriology. Technical Series No. 1. New York. Academic Press.

3. Hildebrand, D. A., and M. N. Schroth. 1964. β-glucosidase activity in phytopathogenic bacteria. Appl. Microbiol. 12:487-491.

4. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by varios Gram-negative bacteria. J. Bacteriol. 566:24-26.

5. Kiraly, X., F. Klement, and J. Solymosy. 1974. Methods in Plant Pathology. New York. Voros Elsevier Scientific Publishing Co.

6. Kovacs, N., 1956. Identification of *Pseudomonas pyocyanea* by the oxidase reaction. Nature 178:703.

7. Lovrekovich, L., and Z. Klement. 1960. Triphenyltetrazolium chloride tolerance of phytopathogenic bacteria. Phytopath. Z. 39:129-133.

8. Lozano, J. C., 1977. Identification of Bacterial Leaf Blight in Rice caused by *Xanthomonas oryzae*, in America. Plant Dis. Reptr. 61:644-648.

9. Ou, S. H. 1972. Rice diseases England. Commonwealth Mycological Institute.

10. Ou, S. H. 1973. A handbook of rice diseases in the tropics. Philippines. The International Rice Research Institute.

11. Ou, S. H., F. L. Nuque, and J. P. Silva. 1971. Varietal Resistance to bacterial blight of rice. Plant Dis. Reptr. 55:17-21.

12. Reddy, C. R., and S. H. Ou. 1976. Characterization of *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson, the bacterial blight pathogen of rice. Ann. Phytopath. Soc. Japan 42:124-130.

13. Report of the enterobacteriaceae subcommittee of the nomenclature committee of the international association of microbiological societies. 1958. (Test No. 18). Internal. Bull. Bactiol. Nomen. and Tax. 8(1):25-70.

14. Sierra, C. 1957. A simple method for the detection of lipolytic activity of micro-organism and some observations on the influence of the contact between cells and fatty substrates. Antonie Van Leeuwenhoek 23:15-22.

15. Smith, N. R., R. E. Gordon and F. E. Clarls. 1952. Aerobies sporeforming bacteria. U. S. Dept. Agric. Monograph. No. 16, 148 pp.

16. Sneath, P. H. A. 1956. Cultural and biochemical characteristics of the genus *Chromobacterium*. J. Gen. Microbiol. 15:70-98.

17. Society of American Bacteriologists. 1957. Manual of microbiological methods. McGraw-Hill Book Co. 315 pp.

18. Starr, M. P. and W. L. Stephens. 1965. Pigmentation and taxonomy of the genus *Xanthomonas*. J. Bacteriol. 87:293-302.

19. Steel, K. J. 1961. The oxidase reaction as taxonomic tool. J. Gen. Microbiol. 25:297-306.

20. Young, J. M., D. W. Dye, J. F. Bradbury, C. G. Panagopoulos and C. F. Robbs. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N. Z. J. Agric. Res. 21:153-177.

Bacterial Wilt of *Zornia* spp. Caused by *Corynebacterium flaccumfaciens*

C. Torres G.
M. Lenné
CIAT
Cali, Colombia

J. I. Victoria
Instituto Colombiano Agropecuario
Palmira, Colombia

Abstract

During 1980 and 1981, young plants and, after cutting, mature plants of the promising tropical forage legumes *Zornia brasiliensis* and *Zornia* sp. (CIAT 7847) became chlorotic, stunted, and wilted and often died at forage evaluation sites in Colombia. The causal bacterium was identified as *Corynebacterium flaccumfaciens*. This is the first report of bacterial wilt in *Zornia* spp. and the first report of the occurrence of the pathogen in tropical Latin America. The seedlings of *Phaseolus vulgaris* were susceptible to this pathogen in inoculation tests. Since the bacterium is seed-borne, care is being taken to prevent its spread from Colombia to other countries.

Introduction

Zornia is a prostrate to erect, herbaceous, perennial, bi- or quadrifoliate legume, native to the tropical savanna regions of South America (6). Over the past 5 years evaluations in Colombia have provided evidence of the high quality and productivity of this species and its potential as a forage legume for the acid infertile soils of the tropics (1, 2).

During 1980 and 1981, young plants and, after cutting, mature plants of promising accessions of *Zornia brasiliensis* Vog. and *Zornia* sp. wilted and often died at forage evaluation sites in Colombia. Cross-sections of lower stems and taproots showed brown coloration of the outer vascular tissue. A bacterium was consistently isolated from affected tissue.

As no previous reports of this disease on *Zornia* spp. could be found in the literature, the following study was made to identify the causal organism and confirm its pathogenicity to *Zornia* spp.

Materials and Methods

Isolates

Sixteen isolates were collected from diseased plants of *Zornia* sp. CIAT 7847 at Santander de Quilichao, Cauca, Colombia. Isolates were grown on

yeast extract-dextrose-CaCO$_2$ medium (YDC) at 27°C and maintained on YDC at 15°C (4).

Morphology and Cultural Characters

Cell morphology was observed by using the Hucker modificiation of Gram stain (9) and the Fisher and Conn modification of Bayley's method was used to observe flagella (9). Cultural characters and pigment production were determined on nutrient agar (NA), tetrazolium chloride medium (TZC), and YDC (9).

Biochemical and Physiological Properties

The methods used to determine biochemical and physiological properties of the isolates have been described previously (5).

Pathogenicity

Inocula for pathogenicity tests were grown in Petri plates of YDC for 48 h at 27°C. Cells were suspended in sterile distilled water at a concentration of 10^6 cell/ml. Young plants at the three and four leaf stage of *Zornia* sp. CIAT 7847 and 3-week old plants of *Phaseolus vulgaris* P 635, *P. lunatus*, and *Glycine max* were inoculated by leaf cutting and by needle puncture. The controls were inoculated with sterile distilled water using the same methods. Plants were rated for disease reaction 12 days after inoculation. Reisolations were made from inoculated plants and Koch's postulates were completed.

Seed Tests

The presence of bacteria in seed was tested on nutrient agar with seed of *Zornia* sp. CIAT 7847. Seeds were surface sterilized in 1% sodium hypochlorite solution, washed in sterile distilled water, placed on nutrient agar and incubated at 27°C for 48 h. All bacteria that grew from seeds were compared with isolates from affected plants of *Zornia* sp. CIAT 7847.

Results

Bacteria Characteristics

All isolates were Gram-positive, non-spore forming short rods with rounded ends, with a size range of 0.6 to 3.0 x 0.3 to 0.5 nm. Cells were motile with various flagella. Colonies on nutrient agar after 24 h at 27°C were circular, convex, entire, and butyrous. On YDC, the colonies were colored creamy-yellow (Table 1).

All isolates were catalase-positive, hydrolyzed starch, grew at 37°C, grew on tetrazolium chloride medium, produced yellow colonies on NBY (5), and produced acid from cellobiose, rhamnose, mannose, ribose, mannitol, sorbitol, and inuline (Table 2). In addition, all isolates were oxidase-negative, did not reduce nitrates to nitrites, did not form levan, did not produce a blue pigment in YDC, and did not reduce sucrose (Table 2). Isolates had a variable reaction in the production of acid from melezitose (Table 2).

Table 1. Characters used to differentiate common genera.[a]

Characters	Isolates from Zornia sp. CIAT 7847	Corynebacterium	Agrobacterium	Erwinia	Pseudomonas	Xanthomonas
Growth in common media	+	+	+	+	+	+
Gram stain	+	+	—	—	—	—
Colonies yellow or orange on media YDC, NBY	+	+	—	V⁻	—	+
Fluorescent pigment on KB	—	—	—	—	V+	—
Anaerobic growth	—	—	—	+	—	—

a   From the Laboratory Guide for Identification of Plant Pathogenic Bacteria. Ed. N. W. Schaad.
+ = Result positive; — = result negative; and V = result variable.

Table 2. Determination of four pathogenic species of *Corynebacterium*[a,b] and comparison with an isolate from *Zornia* sp. CIAT 7847.

Characters	C. michiganense	C. ilicis	C. fasciens	C. flaccumfaciens	Isolate from Zornia sp. CIAT 7847
Motility	—	+	—	+	+
Maximum temperature of growth	29—35	37	34—36	35—37	37
Catalase	+	+	+	+	+
Oxidase	—	—(c)	—	—	—
Nitrates to Nitrites	—	—	—	—	—
Production of acids:					
Rhamnose	—	+	—	+	+
Mannose	V	+	+	+	+
Ribose	—	+	+	+	+
Cellobiose	V	+	—	+	+
Melezitose	—	+	—	+	V
Starch	—	—	—	—	—
Inuline	—	—	—	—	—
Mannitol	V	+	+	+	+
Sorbitol	—	+	+	V	+
Reduction of substances (Sucrose)	V	—	—	—	—
Levan	V	—	—	—	—
Hydrolysis of potato starch	V	—	+	+	+

[a]From Dye and Kemp (1977). [b]All are Gram-positive; strict aerobes. [c]Mandel *et al.* (1961) reported Oxidase positive.
+ = Result positive. — = Result negative; V = Result variable.

Pathogenicity

All isolates caused chlorosis, wilting, dieback and, in some cases, death of young plants of *Zornia* sp. CIAT 7847. The bacterium was readily reisolated from inoculated plants and Koch's postulates were successfully completed with 16 isolates.

In addition, the bacterium caused chlorosis and severe wilting of 3-week old plants of *Phaseolus vulgaris* P 635. *Phaseolus lunatus* and *Glycine max* were not affected when inoculated with isolates of the bacterium.

Seed Tests

The bacterium was readily isolated from seed of *Zornia* sp. CIAT 7847. Levels ranged from 75 to 100% of seed infected.

Discussion

The severe wilting and death of young and mature plants of *Z. brasiliensis* and *Zornia* sp. CIAT 7847 observed at forage evaluation sites in Colombia during 1980 and 1981 were found to be caused by a bacterium. On the basis of its morphological, cultural, biochemical, and physiological properties (Tables 1 and 2), this bacterium was identified as *Corynebacterium flaccumfaciens* Hedges. This is the first report of bacterial wilt of *Zornia* spp. caused by this pathogen.

Corynebacterium flaccumfaciens caused wilting of *Zornia* spp. with similar symptoms to those described in other leguminous hosts (3, 8). In addition, brown coloration of the vascular system was similar to that previously reported in alfalfa (3).

Although *P. lunatus* and *G. max* were not affected by the bacterium, *P. vulgaris* P 635 was most susceptible. In the United States, *C. flaccumfaciens* can cause severe losses in beans (8). As the presence and importance of the pathogen in Latin America is unknown (8), this first report on the bacterium and its pathogenicity in beans should be well noted.

As has been shown in beans (8), the bacterium is seedborne in *Zornia* spp. Importing of seed of infected *Zornia* spp. is being avoided to prevent the spread of this disease.

Further studies are in progress to determine the host range of the pathogen among tropical forage legumes, the survival of the bacterium in soil, and methods of producing clear seed.

Literature Cited

1. Annual Review, 1976. Centro Internacional de Agricultura Tropical, CIAT, Cali, Colombia. p. C11.
2. Annual Review, 1978. Centro Internacional de Agricultura Tropical, CIAT, Cali, Colombia. p. B19.
3. A compendium of alfalfa diseases. 1979. American Phytopathological Society, St. Paul, Minnesota, U.S.A. p. 13-14.
4. Baigent, N.E. J. E. de Vay, and M.P. Starr. 1963. Bacteriophages of *Pseudomonas syringae*. N. Z. J. Sci. 6:75-100.

5. Dye, D. W. and W. J. Kemp. 1977. A taxonomic study of plant pathogenic *Corynebacterium* species. N.Z.J. Agric. Res. 20:563-582.

6. Mohlenbrock, R. H. 1961. A monograph of the leguminous genus *Zornia* Webbia XVI:1-142.

7. Schuster. M. L., D. P. Coyne, and K. Singh. 1964. Population trends and movement of *Corynebacterium flaccumfaciens* var. *aurantiacum* in tolerant and susceptible beans. Plant Dis. Reptr. 48:823-827.

8. Schwartz, H. F. and G. E. Galvez. 1980. Bean production problems. Centro Internacional de Agricultura Tropical, CIAT, Cali, Colombia. 424 pp.

9. Society of American bacteriologists. 1957. Manual of Microbiological Methods. McGraw-Hill Book Co., Inc., New York.

Abstracts

OCCURRENCE OF *Pseudomonas solanacearum* **ON** *Phaseolus* **BEANS IN BRAZIL.** Akiba, F., P. S. T. Brioso, R. de L. D. Ribeiro, O. Kimura, J. P. Pimentel, and C. F. Robbs. Universidade Federal Rural do Rio de Janeiro, Seropédica, Rio de Janeiro, Brazil.

In 1980, a severe outbreak of bacterial wilt occurred on pole beans (*Phaseolus vulgaris* L.) at the locality of Magé, State of Rio de Janeiro, Brazil. The pathogen was characterized as race 1, Hayward's biovar I of *Pseudomonas solanacearum*. Pathogenicity of the bacterium was checked in the greenhouse by means of inoculations using the tooth-pick method and inoculum levels of ca. 10^8 viable cells per ml. The bean isolates were pathogenic to tomato (cv. Kada), eggplant (cv. Florida Market) and soybean (cv. Santa Rosa), but not to tobacco (cv. Amarelinho). Inoculations were made on 203 cultivars of dry beans; of these, 49 were rated as resistant and 154 as susceptible. Among the susceptible ones were the cultivars Rico-23 and Venezuela-350, two of the most important black beans grown commercially in Brazil. Bacterial wilt is a potential danger for Brazil, although it has been reported only in one locality in the State of Rio de Janeiro.

(Paper presented at the Conference; manuscript not received in time to publish.)

EVIDENCE OF A BACTERIUM ASSOCIATED WITH THE PLANTAIN PSEUDOSTEM ROT DISEASE IN THE DOMINICAN REPUBLIC. Angeles Ramos, R. Departamento de Sanidad Vegetal, CESDA, San Cristóbal, R. D.

This disease is producing great losses in the main plantain regions of the Dominican Republic. Its most conspicuous symtoms are: (1) Pseudostem watersoaked rot, that advances from the outside to the inside and from the upper part of the tree toward the rhizome and (2) necrosis beginning on the oldest leaves and later advancing to the youngest ones. Internal leaf sheaths show initially water-soaked spots that later become rotted and show a brown color. Eventually, the plant bends over and dies. For many years this disease was suspected to be caused by the fungus that causes "The Panama Disease" (*Fusarium oxysporum* f. sp. *cubense*). In this study we have found that the probable causal agent, is a bacterium belonging to the genus *Erwinia*. This is a gram-negative, rod-shaped, motile and facultative anaerobe bacterium. Colonies on nutrient agar and yeast extract dextrose calcium carbonate are creamy-white, smooth, and slightly mucoid. Gelatin liquefaction variable, starch not hydrolized, oxidase negative. Sufficient acid to change the color of Bromothymol blue was produced from rhamnose, mannose, and methyl D-glucoside, but not from sorbitol. In pathogenicity tests we were able to reisolate the bacterium from rotted spots produced in the inoculated leaf-sheath tissues.

(Paper presented at the Conference; manuscript not received in time to publish.)

Session II

New Developments in Microbial Identification and Taxonomy

Edwin L. Civerolo, Chairman

New Developments in Microbial Identification and Taxonomy

Introductory Remarks
by E.L. Civerolo, Chairman, Session II

Recently, many previously recognized species of phytopathogenic bacteria were combined, based on their overall similarities. Those phytopathogenic bacteria which cannot be adequately differentiated on the basis of phenotypic characteristics have been given the infrasubspecific epithet pathovar, based on their distinctive pathogenicity to one or more host plants.

Based on work with several strains of *Xanthomonas campestris* pathovars, various forms of enzyme-linked immunosorbent assay (ELISA) appear to be useful for detecting and identifying, as well as evaluating, the serological relationships among pathovars and strains of phytopathogenic bacteria (including fastidious prokaryotes). In indirect ELISA, analyses of competitive antigen or antibody inhibition curves, rather than binding curves, may be particularly useful for quantitatively determining antigenic similarities among these phytopathogens. However, as with other serological techniques, the applicability of ELISA for identification and taxonomy in phytobacteriology depends upon adequate determination and evaluation of the parameters of specific techniques. Although the taxonomic significance of specific antigenic determinants is not completely understood, improved taxonomic resolution at the species and subspecies levels might be achieved by further adaptation or modifications of ELISA (or other serological techniques).

The serological cross-reactions between *Corynebacterium sepedonicium, C. michiganense, C. insidiosum,* and an unidentified coryneform bacterium observed by indirect immunofluorescence using immunoglobulin prepared from gluteraldehyde-fixed cells of *C. sepodonicum* differed quantitatively between antisera from different rabbits and was lowest in antisera soon after beginning immunization.

Using immunoglobulin prepared from antisera against gluteraldehyde-fixed whole cells of *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae,* no serological cross reactions between these pathovars were detected in immunodiffusion or direct immunofluorescence (IF) tests. Thus, serological relationships based on cross reactions may depend upon the immunization schedule for antiserum production, antigen preparation, and the specific serological test. Using indirect IF strains, *C. sepodonicum* was detected in symptomatic tissue; however, results obtained by this technique need to be interpreted cautiously as cross-reacting gram-positive, gram-negative, and gram-variable bacteria were ostensibly associated with diseased and

healthy potato plants. In addition, low populations of phytopathogenic bacteria may limit the ability to detect "latent" infections by indirect IF.

The use of electrophoretic analyses of proteins in polyacrylamide gels to indicate relationships at the species level was illustrated with *Pseudomonas andropogonis* strains. *P. andropogonis* strains were distinguishable from other pseudomonads based on comparative one-dimension SDS-polyacrylamide electrophoretic protein analyses. In addition, the synonymy of the nomen-species *P. andropogonis* and *P. stizolobii* was supported by these protein analyses.

A simple method based on detection of characteristic xanthomonadin pigments was useful for identifying and distinguishing *X. campestris* pathovars from other yellow saprophytic bacteria. This was generally more reliable than colony appearance, induction of a hypersensitive response in incompatible hosts, or physiological tests.

Future work might focus on other chemotaxonomic markers, protein analyses, development of new, improved, or modified serological techniques as related to phytopathogenic specialization or ecological characteristcs. The specificity, sensitivity, and reliability of these techniques to resolve the taxonomic significance of complex antigenic determinants in phytobacteriology may be clarified by international standardization of serological procedures and techniques. It seems that the potential of molecular methods, such as computer-assisted protein analyses, to analyze subgeneric hetergeneity among phytopathogenic bacteria (and other fastidious prokaryotes) that might have taxonomic significance has not been fully developed. This does not preclude other approaches, such as numerical analyses of biochemical and physiological characteristics, genetics, gene-product analyses, bacteriophage reactions, selective isolation media, or analyses of characteristics that are ecologically relevant to resolving taxonomic problems.

E.L. Civerolo

Value of Xanthomonadins for Identification of Pigmented *Xanthomonas campestris* Pathovars

M. S. Irey
R. E. Stall

University of Florida
Gainesville, FL

Abstract

Colonies of pigmented *Xanthomonas campestris* pathovars often can not be distinguished visually from colonies of other yellow-pigmented bacteria. Physiological tests that characterize the genus *Xanthomonas* were not useful for distinguishing *Xanthomonas* isolates from other yellow bacteria. Necrosis in tobacco also was not a consistent characteristic for many xanthomonads. However, thin-layer chromatography of yellow pigments was a simple and accurate method for identification of the pigmented *X. campestris* pathovars. The *Xanthomonas* pigment (xanthomonadins) had an Rf value of 0.42-0.49 when extracted with methanol and developed with methanol on precoated plastic sheets of silica gel 60 that was 0.2 mm thick. A pigment of that Rf was absent from other yellow bacteria.

Introduction

The genus *Xanthomonas* Dowson, as it is now recognized, is a group of rod-shaped, aerobic, Gram-negative bacteria which cause diseases in plants (22). This group was separated from other plant pathogenic bacteria by Dowson (8), who urged that the yellow bacteria, motile with one polar flagellum and mostly producing acid in lactose, were deserving of generic rank and should be elevated to a new genus, *Xanthomonas*. In naming the genus Dowson emphasized the yellow color of the bacteria, saying that the color is one of the most significant characteristics of its species. Since then, the yellow color has been an invaluable aid in the identification of *Xanthomonas*. No other characteristic has been used consistently to separate the genus *Xanthomonas* from ecologically and taxonomically related genera (5, 9, 10, 11, 16).

Chromogenic bacteria are also found in other currently recognized genera of phytopathogenic bacteria (4). In addition, chromogenic saprophytic bacteria are commonly encountered in ecologically adjacent locations. The difficulty in quick and routine identification of *Xanthomonas*

spp. from other pigmented bacteria prompted studies on the nature of the pigmentation in the genus *Xanthomonas*. Stephens and Starr (26) reported the presence of membrane-bound carotenoid pigments in *X. juglandis*. Starr and Stephens (24) examined the pigments of a number of xanthomonads as well as a number of yellow non-xanthomonad isolates and concluded that members of the genus *Xanthomonas* possessed a unique carotenoid pigment complex that could not be found in any non-*xanthomonad*. The *Xanthomonas* carotenoids had many characteristics of a carotenoid alcohol. Starr and Stephens (24) proposed that a Gram-negative, polarly flagellated, oxidative, rod-shaped bacterium with a carotenoid alcohol having absorption maxima at 418,437, and 463 nm (in petroleum ether) be placed in the genus *Xanthomonas*. This proposal was criticized by Lelliot (16), who suggested that Starr and Stephens were misled by assuming that 19 isolates of the *X. campestris* group were representative of the four other taxospecies in the genus.

Andrewes *et al.* (2, 3) and Andrewes (1) reinvestigated the nature of the pigmentation of *X. juglandis*. They found the pigments consisted of mixtures of unusual, previously unknown, brominated aryl-polyene esters, rather than of carotenoids as reported by Starr and Stephens (24). Determinations of the structure of the *X. juglandis* pigments resulted in the discovery of xanthomonadin I [17-(4-bromo-3-methoxyphenyl)- bromo-heptadeca-2, 4, 6, 8, 10, 12, 14, 16-octaenoic acid] (3). Starr *et al.* (23) then reported that xanthomonadin pigments occurred in all recognized taxo-species of the genus *Xanthomonas*, except *X. ampelina*. On the other hand, the pigments of many other yellow bacteria are known to be carotenoid in nature (17, 18, 19, 21), thus the *Xanthomonas* pigments appear unique.

The main thrust of the present research was to determine whether the xanthomonadin pigments could be used as a chemotaxonomic marker in routine laboratory identification of pathovars of *Xanthomonas campestris*. There were two objectives: (1) to develop a quick method to compare isolates for the presence of xanthomonadin pigments, and (2) to compare a number of *Xanthomonas* and non-xanthomonad isolates for pigmentation, physiological characteristics, and pathogenicity.

Materials and Methods

Cultures

Cultures of 36 isolates of *Xanthomonas*, 20 unknown bacteria which produced yellow colonies on plates of 0.8% nutrient broth solidified with 1.5% Difco agar (NA), 2 species of *Corynebacterium*, and 3 isolates of *Erwinia herbicola* were included in this study. All were isolated from plant material, except two of the unknown bacteria which were laboratory contaminants. The designations, sources, origins, and hosts of isolation for the cultures are listed elsewhere (13). All cultures were stored in sterile deionized water in screw cap tubes at room temperature, except for *X. albilineans* and *X. axonopodis* isolates, which were stored on Wilbrink's medium containing Noble agar (WA) and kept at 4°C (6).

Physiological Tests

Three to 4 days after streaking a stock culture onto NA a single colony was suspended in water. This suspension was used to inoculate the various media. Stocks of *X. albilineans* and *X. axonopodis* were streaked onto WA. All tests were performed in duplicate and incubated at room temperature (24 to 27°C) unless otherwise noted. Bacteria that were known to give positive and negative reactions for each test were always included.

The Gram stain was applied to air dried films of bacteria by standard techniques (20), except that two drops of a 4% aqueous solution (w/v) of sodium bicarbonate was added to the crystal violet solution.

The oxidase test was performed by placing 1 drop of a 1% aqueous solution (w/v) of N, N, N, N, tetramethylpara phenylene-diamine-dihydrochloride solution on 3-day-old cultures (7). A reaction was considered to be positive if a purple color developed on treated colonies within 10 seconds. Negative reactions were those in which no color developed within the specified 10 seconds.

Oxygen requirements were determined with the glucose medium of Hugh-Liefson (12). Immediately after inoculation, one of two tubes was sealed with 2 ml of sterile mineral oil. Color change of the medium was recorded after 7 days at 30°C.

The medium of Dye (9) was used to determine the utilization of L-asparagine as the sole source of carbon and nitrogen. The medium was prepared by adding 0.5 ml of a 2% solution (w/v) of filter sterilized L-asparagine to 4.5 ml of autoclaved basal medium. All glassware was soaked overnight in 0.01M HCL and rinsed three times in deionized water prior to use. Tubes were inoculated with 0.05 ml of a bacterial suspension and were examined for turbidity after 7 days of incubation.

Bacteria were streaked onto NA containing 0.02%, or 0.1% triphenyl-tetrazolium chloride (TTC). Enough of a sterile, autoclaved solution of TTC was added to autoclaved NA to make the final concentrations. Plates were checked for growth of bacteria after 4 days at 30°C.

The procedures of Dye (9) were used to determine action in purple milk and reduction of nitrate. Tubes were observed for reactions after 7 days in the purple milk test and 4 days in the nitrate test.

Pathogenicity Tests

Cultures in nutrient broth (NB) or Wilbrink's broth (WB) were allowed to grow for 24 hr, then were pelleted, and resuspended in sterile deionized water. Suspensions were diluted to an $OD_{600\ nm}$ of 0.3 as measured with a spectrophotometer. Leaves of 6- to 8-week-old plants of *Lycopersicon esculentum* Mill, 'Bonny Best'', *Capsicum annuum* L. '10 R', *Solanum nigrum* L., and *Nicotiana tabacum* L. 'F$_2$C^1' were injected with the suspensions as described by Klement (15). Six cultures were tested per plant. Three replicates were included, each on a different plant. Inoculated plants were incubated at 30°C, 90 to 100% relative humidity, and under a 12-hr light/dark photoperiod with a light intensity of 1500 lux measured at the level of the top leaves. Plants were scored for necrosis of the inoculated area 4 days after inoculation.

Thin Layer Chromatography of Pigments

Xanthomonas albilineans and *X. axonopodis* were cultured in WB and the other bacteria were cultured in NB. Cultures were streaked on NA or WA to check for purity before use. After each culture was centrifuged at 5520 X g for 15 min at 20°C, the supernatent fluid and loose bacterial slime were discarded. Each bacterial pellet was resuspended in 40 ml of anhydrous, spectrophotometry grade methanol. The methanolic suspension was quickly brought to a boil by immersing it in a 90°C water bath. After each suspension cooled, it was centrifuged at 1300 X g for 15 min to remove cell debris. The methanolic extract was evaporated to near dryness *in vacuo* at 75°C, after which just enough methanol was added to redissolve any yellow crystal that had for.ned on the sides of the flasks. Each extract was again centrifuged at 1300 X g for 15 min after which a clear, yellow, concentrated extract was obtained.

Extracts that had an OD_{443nm} of 0.4 or higher were spotted with capillary pipets, in 5-nl amounts on precoated, thin-layer chromathography plastic sheets of silica gel 60 of 0.2 mm thickness, and without fluorescent indicators (E. Merck, Darmstadt, Germany). Each 5-nl sample was allowed to dry before the application of the next 5-nl. A total of 25 nl was applied per spot. The extracts were placed 24 mm apart on a plate. Plates were placed in a developing apparatus (Eastman Kodak, Rochester, New York) with anhydous spectrophotometry grade methanol used as the solvent. Care was taken to saturate the chromatograph's chamber with solvent as completely as possible.

Absorption Spectrum of Pigments

Pigments of selected isolates were extracted by scraping the growth from 4-day-old NA or 10-day-old Wa plates and suspending the cells in 4 ml of methanol in screw-cap tubes. The tubes were immersed in a water bath at 90°C for 5 min and then centrifuged at 1400 x g for 15 min. The absorption spectrum of the pigment extracts was determined with an ACTA II double-beam scanning spectrophotometer.

Selected pigment extracts were concentrated by evaporation of the methanol *in vacuo* as before. The concentrates were applied in a thin band 2 cm from the bottom of a silica gel thin-layer plate and chromatographed. Approximately 400 nl of extract were applied per plate. After development with methanol, the pigment band corresponding to the the R value of 0.40 to 0.50 was scraped from the plate and eluted with methanol. The eluates were then filtered and analyzed for their absorption spectra.

Results

Physiological Tests

All isolates of *Xanthomonas* form a fairly homogeneous group based on physiological tests (Table 1). They were strictly aerobic and Gram-negative bacteria. No xanthomonad could utilize L-asparagine as the sole source of carbon and nitrogen, or reduce nitrate to nitrite. None of the xanthomonads acidified purple milk. All were inhibited by 0.1% TTC. Their oxidase reaction was variable, but most of them were weakly positive. Five of 36 isolates did not cause proteolysis of milk.

Table 1. Percentages of cultures demonstrating various physiological characteristics[a].

	Xanthomonas albilineans	Xanthomonas axonopodis	Xanthomonas fragariae	Xanthomonas campestris	Yellow bacteria	Corynebacterium	Erwinia herbicola
Number of cultures	2	1	2	31	20	2	3
Gram negative	100	100	100	100	90	0	100
Fermentative	0	0	0	0	15	0	100
Oxidase positive	0	0	100	94[c]	85	0	100
Growth in L-asparagine	0	0	0	0	25	50	100
Nitrate reduction	0	0	0	0	40	50	100
Acid in milk	0	0	0	0	5	0	100
Proteolysis of milk	0	0	100	94	10	0	0
Growth in 0.02% TTC[b]	0	0	50	32	50	0	100
Growth in 0.1% TTC	0	0	0	0	20	0	100

[a] All isolates were tested in duplicate.
[b] TTC refers to triphenyl-tetrazolium chloride.
[c] Weakly positive.

The isolates of yellow bacteria were heterogeneous in the characters examined. Based on the nine physiological characters, 14 different bacteria were in the group. Eight of the yellow bacteria could not be distinguished from *Xanthomonas*. The yellow bacteria were most often distinguished from *Xanthomonas* by no proteolysis of milk, but this test was not always positive for xanthomonads.

Pathogenicity Tests

Necrosis of inoculated test plants was evidence of the isolate's pathogenicity. All cultures of *Xanthomonas*, except *X. albilineans* induced necrosis in one or more of the four test plants used in this study (Table 2). None of the yellow bacteria caused necrosis in any plant.

Tomato was the superior plant for detection of pathogenicity by *Xanthomonas*. Tobacco was the least reliable plant for selection of the pathogenic organisms.

Chromatographic Analysis of Pigments

The R_f values of pigments of some of the bacteria are given in Table 3. All isolates of *Xanthomonas*, except *X. albilineans* and *X. axonopodis* produced characteristic pigment spots with an average R_f value of about 0.45 (range of 0.42 to 0.49). Occasionally, when more than 25 nl of pigment extract was applied to the plates, additional spots of higher R_f values also were found. When that happened, the spot corresponding to the R_f value of 0.45 was usually distorted (tailing) probably due to excessive sample size. Chromatography of methanol extracts of *X. albilineans* and *X. axonopodis* was unsucceessful.

Pigments extracted from the yellow bacteria gave spots with R_f values considerably different from those of *Xanthomonas*. The R_f values of pigment spots of the yellow bacteria ranged from 0.07 to 0.85, with some extracts having up to four distinct spots. In general, the pigments of the yellow bacteria were more distinct on the thin layer plates, and did not fade as quickly after development as did the pigments of the xanthomonads.

Absorption Spectra of Pigments

The absorption maxima of the crude pigment extracts of 11 selected isolates were determined. Isolates of *X. albilineans, X. axonopodis*, and of the pathovars of *X. campestris* had absorption maxima at 443 nm, with ill-defined peaks or broad shoulders around 420 and 467 nm. The absorption maxima of the non-xanthomonads varied, but were always different from those of xanthomonads.

The pigment extracts of four *Xanthomonas* isolates were chromatographed and eluted and absorption spectra were determined on them. The absorption maxima of the pigments with an R_f of 0.40 to 0.50 were identical with those of the crude pigment extracts of the same isolates.

Table 2. Percentages of cultures that caused necrosis in leaves of four plants[a].

Bacteria	Numbers of cultures	*Lycopersicon esculentum*	*Solanum nigrum*	*Capsicum annuum*	*Nicotiana tabacum*
X. albilineans	2	0	0	0	0
X. axonopodis	1	100	100	0	0
X. fragariae	2	100	100	50	0
X. campestris	31	100	77	71	48
Yellow bacteria	20	0	0	0	0
Corynebacterium	2	50	0	100	0
Erwinia herbicola	3	33	33	33	33

[a] Leaves of three plants were inoculated by infiltration of about 10^8 cells ml^{-1} into a spot. Necrosis in two of the three was scored as a positive reaction.

Table 3. R_f values of yellow pigment.

Isolates	R_f value[a]
X. fragariae	.48
X. camp. pv. *campestris* 528	.45
X. camp. pv. *begoniae* 066-1778	.46
X. camp. pv. *cannae* 068-1097	.44
X. camp. pv. *cucurbitae* JPJ	.45
X. camp. pv. *dieffenbachiae* 067-1260	.44
X. camp. pv. *hederae* 017-988	.43
X. camp. pv. *maculifoliigardinae* 069-2413	.43
X. camp. pv. *malvacearum* $ATCR_2$	.46
X. camp. pv. *nigromaculans* 069-1200	.45
X. camp. pv. *phaseoli* 080-1339	.44
X. camp. pv. *physalidis* 1926	.46
X. camp. pv. *poinsettiaecola* 071-424	.47
X. camp. pv. *vesicatoria* 69-13	.44
YB-1[b]	.76
YB-2	.73, .79, .83
YB-3	.66
YB-4	.53
YB-5	.73
YB-6	.63, .70, .79
YB-7	.71
YB-8	.57, .66, .77
YB-9	.57, .64, .74
YB-10	.63, .69, .80
YB-11	.78
YB-12	.75
YB-14	.80, .84
YB-19	.87
Corynebacterium michiganense	.75
Erwinia herbicola GW-27	.85

[a] R_f values calculated from the average of two silica gel plates.

[b] YB refers to yellow bacteria.

Discussion

The physiological tests used in this report were the ones listed in Bergey's Manual (4) for characterizing *Xanthomonas*. The cultures used in this study were uniform for five of the tests. However, some non-xanthomonads had similar characteristics. A single test is needed that consistently identifies an isolate of *Xanthomonas,* such as the production of fluorescin, which is used to identify certain pseudomonads (14). Utilization of asparagine has been cited as a useful character for the separation of *Xantomonas* spp. from non-xanthomonad yellow bacteria and pseudomonads (25). However, many non-xanthomonads are similar to *Xanthomonas* in this characteristic. The same criticism applies to the tests of inhibition by TTC, oxidative respiration, acid in milk, and nitrate reduction. Proteolysis of milk had the best possibility of being a single test to distinguish *Xanthomonas* from saprophytic yellow bacteria. However, some exceptions occurred with this test, also.

As the genus *Xanthomonas* is currently defined, yellow color and pathogenicity are the most reliable characteristics that can be used for identification. Pathogenicity is often determined by the methods of Klement (15) and tobacco is commonly used as the test plant. Tobacco is an excellent indicator plant for pathogenicity of members of the *Pseudomonas syringe* group of bacteria (15) but it was less than satisfactory for the determination of the pathogenicity of the *Xanthomonas* isolates.

The use of pathogenicity to distinguish xanthomonads is probably responsible for the absence of saprophytic members in the genus. A better system for distinguishing xanthomonads may be pigment analysis. Saprophytic xanthomonads then might be discovered.

Attempts to chromatograph the yellow pigments of isolates of *X. albilineans* and *X. axonopodis* were unsuccessful. Possibly, this was not because of the nature of the pigments, but because of the slimy growth of these isolates. Neither species grew well on NA, so a complex medium containing sucrose (WA) was used. It has long been known that on sugar-containing media, members of the genus *Xanthomonas* produce large amounts of slime (2, 5, 9). Andrewes *et al.* (2) reported that the production of slime by xanthomonads inhibited their studies of the pigmentation of the genus. In the present study, however when pigments were examined spectrophotometrically, the absorption spectra of the pigments of *X. albilineans* and *X. axonopodis* were indistinguishable from those of members of the *X. campestris* group. This was considered to be evidence that at least three species of the genus have pigments in common. The same results and conclusions were also reached by Starr *et al.* (23).

Young *et al.* (27) listed 119 pathovars of *X. campestris* and among them are listed 5 non-pigmented pathovars. Little has been published on these white xanthomonads, and their taxonomic position has not been positively determined. It would be easy to assign these non-pigmented plant pathogenic bacteria to the genus *Xanthomonas,* because as found here, the physiological characteristics of *Xanthomonas* are not exacting. If these non-pigmented bacteria are truly xanthomonads they could not be identified by pigment extraction, of course.

The identification of pigmented *X. campestris* pathovars can be relatively

quick and easy by pigment analysis. A simple technique would be to grow them on a standard peptone medium that contains no sugar. The bacteria should be scraped from the medium and suspended in a small volume of methanol (2 to 3 ml) in a screw-cap tube. The tube should be placed in a 90°C water bath for 5 min and then centrifuged to remove cell debris. The open test tube then should be placed in the 90°C water bath until approximately 0.5 ml of pigment extract remains (2-3 min). The concentrated extract should then be chromatographed on thin layer silica gel plates with methanol as a solvent. The *Xanthomonas* pigments have distinct Rf values.

Literature Cited

1. Andrewes, A. G. 1973. Synthesis of methyl 17-(3,5-dibromo-4-methoxyphenyl)-heptadeca-2,4,6,8,10,12,14,16-octaenoate. Acta Chem Scand. 27: 2574-2580.
2. Andrewes, A. G., S. Hertzberg, S. Liaaen-Hensen, and M. P. Starr. 1973. The *Xanthomonas* "cartenoids" — non-cartenoid brominated aryl-polyene esters. Acta Chem. Scand. 27:2383-2395.
3. Andrewes, A.G., C. L. Jenkins, M.P. Starr, J. Shepherd and H. Hope. 1976. Structure of xanthomonadin I, a novel di-brominated aryl-polyene pigment produced by the bacterium *Xanthomonas juglandis*. Tetrahedron Lett. 45:4023-4024.
4. Buchanan, R.E., and N. E. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology. (8th ed.) Williams and Wilkins. Baltimore, MD. 1268 p.
5. Burkholder, W. H. and M.P. Starr. 1948. The generic and specific characters of phytopathogenic species of *Pseudomonas* and *Xanthomonas*. Phytopathology 38.494-502.
6. Dean. J. L. 1974. A method for isolating *Xanthomonas albilineans* from sugarcane leaves. Plant Dis. Rep. 58:439-441.
7. Department of the Air Force and the Army. 1977. Clinical laboratory procedures: bacteriology. Air Force Manual 160-52. 232 p.
8. Dowson, W. J. 1939. On the systematic position and generic names of the Gram negative bacterial plant pathogens. Zentralbl. Bakt., II. Agt. 100:177-193.
9. Dye, D.W. 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N. Z. J. Sci. 5:393-416.
10. Dye, D. W. 1963. Comparative study of the biochemical reactions of additional *Xanthomonas* spp., N. Z. J. Sci. 6:483-486.
11. Dye, D. W. 1966. Cultural and biochemical reactions of additional *Xanthomonas* spp. N. Z. J. Sci. 9:918-919.
12. Hugh, R., and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various Gram negative bacteria. J. Bacteriol 66:24-25.
13. Irey, M. S. 1980. The taxonomic value of the yellow pigment of the genus, *Xanthomonas*. M. S. Thesis. Univ. Fla., Gainesville, Florida. 38 p.
14. King, G.O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
15. Klement, Z., G. L. Farkas, and L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.

16. Lelliot, R. A. 1971. The genus *Xanthomonas*. Pages 269-272. H. P. Mass Geesteranus, ed. *In* Proc. 3rd Int. Conf. Plant Pathogenic Bact. Centre Agr. Pub. and Doc. Wageningen, the Netherlands 365 pp.

17. Norgard, S., A. Jorben, and S. Liaanen. 1970. Carotenoids from *Corynebacterium poinsettiae* including four new C_{50}-diols. Acta Chem. Scand. 24:2183-2197.

18. Palleroni, N. J., and M. Doudorff. 1972. Some properties and taxonomic subdivisions of the genus *Pseudomonas*. Annu. Rev. Phytopathol. 10:73-100.

19. Prebble, J. 1968. The carotenoids of *Corynebacterium fasciens* strain 2Y. J. Gen. Microbiol. 52:15-24.

20. Schaad, N. W. 1980. Initial identification of common genera. Pages 2-3. N. W. Schaad, ed. *In* Laboratory Guide for Identification of Plant Pathogenic Bacteria. Amer. Phytopathol. Soc., St. Paul, MN. 72 pp.

21. Sobin, B. and G. L. Stahly. 1942. The isolation and absorption spectrim maxima of bacterial carotenoid pigments. J. Bacteriol. 44:265-276.

22. Starr, M. P. 1959. Bacteria as plant pathogens. Annu. Rev. Microbiol. 13:211-238.

23. Starr, M. P., C. L. Jenkins, L. B. Bussey, and A. G. Andrewes. 1977. Chemotaxonomic significance of the xanthomonadins, novel brominated arylpolyene pigments produced by bacteria of the genus *Xanthomonas*. Arch. Microbiol. 113.1-9.

24. Starr, M. P., and W. L. Stephens. 1964. Pigmentation and taxonomy of the genus *Xanthomonas*. J. Bacteriol. 87:293-302.

25. Starr, M.P., and J. E. Weiss. 1943. Growth of phytopathogenic bacteria in a synthetic asparagin medium. Phytopathology 33:314-318.

26. Stephens, W. L., and M. P. Starr. 1963. Localization of carotenoid pigment in the cytoplasmic membrane of *Xanthomonas juglandis*. J. Bacteriol. 86:1070-1074.

27. Young. J. M., D. W. Dye, J. F. Bradbury, C. G. Panagopoulos, and C. F. Robbs. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N.Z.J. Agr. Res. 21:153-177.

Relationships Between *Xanthomonas* c. pv. *manihotis, X. c.* pv. *cassavae* and Colombian Yellowish Isolates

As Reflected in Physiological, Biochemical, and Serological Tests.

F. N. Elango, Centro Internacional de la Papa, Lima, Peru
J. C. Lozano, CIAT, Cali, Colombia
J. F. Peterson, McGill University, Quebec, Canada

Abstract

Physiological/biochemical tests were inadequate in separating *Xanthomonas campestris* pv. *manihotis, X. c.* pv. *cassavae,* and some Colombian yellowish isolates. However, serological techniques such as the Ouchterlony double diffusion and direct immunofluorescence indicated antigenic differences between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae.* The Colombian yellow isolates were serologically similar to *X. c.* pv. *cassavae* but not to *X. c. manihotis.* Therefore, retaining the distinction between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* is probably justified.

Introduction

Xanthomonas campestris pv. *manihotis* (Berthet and Bondar 1915) Dye 1978 and *X. c.* pv. *cassavae* (Wiehe and Dowson 1953) Maraite and Weyns 1979 are two of four bacterial pathogens which attack cassava (*Manihot esculenta* Crantz) (24,37). While *X. c.* pv. *manihotis* occurs in most cassava growing areas of the world (25), *X. c.* pv. *cassavae* is limited to the African highlands of Rwanda and Kenya at 1,600 m (26) and, possibly, sea-level in the eastern plains of Colombia (4). Because the early leaf spot symptoms are so similar, workers frequently have difficulty distinguishing the two pathogens. It has been reported that the pathogens are synonymous (29), but other evidence (16, 26) suggests the contrary.

Taxonomically, *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* are grouped in Bergey's Manual of Determinative Bacteriology (2) under *X. campestris* group, but as two distinct pathovars. However, the newly proposed nomenclature which came into use January 1, 1980 (39) considers *X. cassavae* as a *nomen dubium,* probably on the basis of controversial evidence (29). Therefore, the relationship between these two pathogens needs a clear distinction to avoid confusion for quarantine purposes (26). Moreover, reports that *X. c.* pv. *cassavae* may have evolved from *X. c.* pv.

manihotis need to be confirmed. "Deeper" taxonomic studies have been suggested to resolve this problem (26) and such studies could include genetics, bacteriophage, and serology (36).

This study was undertaken in a continued effort to clarify the relationship between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae.*

Materials and Methods

Physiological and biochemical tests were used, methods and references are indicated in Table 1. The replica plating technique (22) was used to study the utilization of carbohydrates, nitrates, fatty acids, amino acids, amines, organic acids, and sodium and tetrazolium chloride salts.

Antisera were produced against *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae.* Cultures used for antisera production were prepared according to the method of Allan and Kelman (1), i.e., using glutaraldehyde fixed whole cells to immunize New Zealand white rabbits. Part of the crude antiserum was preserved for the agglutination test (35) and the Ouchterlony double diffusion assay (19), while another portion was fractionated following the methods of Allan and Kelman (1) and Cherry (5). The precipitated globulin was conjugated with Fluorescein isothiocyanate (FITC) (5). Samples were stored in vials at -20°C until needed.

To determine the serological relationship between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* and some Colombian xanthomonads, isolates were grown for 48 hours on Pseudomonas isolation agar (7). Dilutions of samples were smeared onto alcohol cleaned slides, air-dried, heat fixed and later stained with fluorescent antibody conjugate. The stained preparations were examined under an X 100 objective fitted to an Orthomat microscope with an oil immersion condenser.

The relationship between *X. c.* pv. *manihotis, X. c.* pv. *cassavae,* and the Colombian yellowish isolates was studied by the method of Klement (19). All gel-diffusion tests were made at room temperature (25°C) Photographs of double diffusion plates were taken in an immunodiffusion Camera (Cordis) using Polaroid 107 black and white film.

Results

Results shown in Table 2 indicate that the three xanthomonads could not be separated adequately by physiological and biochemical tests. However, all tests marked with two asterisks showed some differential value between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* on the one hand and the Colombian isolates on the other hand. The differences cited were mainly in the rate of utilization of several carbohydrate and other nutritional sources. Other tests showing differences included the hypersensitivity tests, tolerance to tetrazolium chloride salt, growth rate, pigmentation, and sodium polypectate. Tests marked with one asterisk showed an inconsistent differential value. Fatty acids, amino acids, amines, and organic acids were not useful in separating the species.

Glutaraldehyde-fixed whole cells were used as antigens, and they induced the production of highly specific antisera. The agglutination titre of

Table 1. Tests and methods used for physiological and biochemical tests.

Test	Method (Reference)
Oxidades	(7, 21)
Catalase	(8, 35)
Mode of glucose utilization	(15)
Gelation liquefaction	(17, 28)
Hydrolysis of starch	(17)
Indole production	(17)
Nitrate reduction	(17)
Tyrosinase	(8, 38)
Hydrolysis of Lipase	(31)
NaCl tolerance	(30)
Tetrazolium chloride tolerance	(23)
Urease	(6, 13)
H_2S production	(17)
Carbohydrate utilization	
Nitrate utilization	
Fatty acids	(27)
Organic acids	
Amino acids	
Amines	
Soft rot of potato and cassava roots	(25)
Tobacco hypersensitivity reaction	(20)
Pectate liquefaction	(13)
Hydrolysis of casein	(12)
Litmus milk reaction	(7, 17)
Ammonia production	(17)
Phenylalanine deaminase	(32)
Methyl red and Voges proskeur	(17)
Arginine dihydrolase	(27)
Hydrolysis of Esculin	(34)
Sodium polypectate	(3)
B-glucosidase	(14)
Levan production	(27)
Fluorescence on King's B Medium	(18)
Growth rate	
Pigmentation	
Sensitivity to antibiotics	(7)

Table 2. Physiological/biochemical characterization of *Xanthomonas campestris manihotis*, *X. c.* pv. *cassavae*, and some Colombian yellowish isolates.

Test	Reaction		
	X. manihotis	*X. cassavae*	Colombian Yellowish isolates
Carbohydrate utilization[a]	+ —[c]	+	++
Nitrate utilization	—	—	—
Amines	+	+	+
Amino acids	+	+	+
Organic acids	—	—	—
Antibiotic sensitivity[b]	+	+	++
Hydrolysis of casein	—	—	—
Hydrolysis of gelatin	+	+	+
Hydrolysis of starch	+	+	+
Litmus milk reaction	+	+	+
Production of ammonia	+	+	+
Production of H_2S	—	—	—
Production of indole	—	—	—
Nitrate reduction	—	—	—
Methyl red and V.P. test	—	—	—
Mode of glucose utilization	Oxidative	Oxidative	Oxidative
Lipase	+	+	+
Catalase	+	+	+
Oxidase	—	—	—
Arginine dihydrolase	—	—	—
Phenylalanine deaminase	+	+	+
Tyrosinase	—	—	—
Urease	—	—	—
Aesculin hydrolysis[b]	+	+	++
B-glucosidase	+	+	+
Soft rot of potatoes & cassava roots	—	—	—
Levan production	+	+	+
Hypersensitivity in tobacco leaves[a]	—	—	+
Salt tolerance (Max.)	2.5%	2.5%	2.5%
Tetrazolium chloride (tolerance [a]Max.)	0.02%	0.02%	0.1%
Sodium polypectate[a] utilization	—	—	+
Fluorescence on King's B. medium	—	—	—
Growth rate[a]	moderate	slow	fast
Yellow pigment[a]	—	+	+

[a] Tests with distinct differential value.

[b] Tests with non-consistent differential value.

[c] + + = strongly positive; + = positive; — = negative.

Table 3. Serological identity between *Xanthomonas campestris* pv. *manihotis* (Berthet and Bondar 1915) Dye 1978, *X. c.* pv. *cassavae* Wiehe and Dowson 1953 (Maraite and Weyns 1953) and some Colombian yellowish isolates.

Antigen (Whole cells) 1×10^9/ml	Antibody Reaction[a,b]			
	Double Diffusion		Immunofluorescence	
	Xcm[a]	Xcc	XcmA/FITC	Xcca/FITC
X. manihotis (60 isolates)	+	–	+	–
X. cassavae (6 isolates)	–	+	–	+
Colombian yellowish isolates (3 isolates)	–	+	–	+

[a] Xcm = *X. c.* pv. *manihotis*; Xcc = *X. c.* pv. *cassavae*; XcmA/FITC = *X. manihotis* antibody conjugated with Fluorescein isothiocyanate; XccA/FITC = *X. cassavae* antibody labelled with Fluorescein isothiocyanate.

[b] += positive reaction; – = negative reaction.

antisera produced against *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* was between 2560 and 5120. Some cross-agglutination occurred between *X. c.* pv. *manihotis* and the Colombian yellowish isolates but this was not confirmed by the Ouchterlony double diffusion or by the immunofluorescence tests. No cross-reaction occurred between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* by any of the serodiagnostic methods used (Table 3). However, Immunodiffusion plates showed the presence of antigens common to all the isolates studied (Fig. 1 and 2). In both the double diffusion (Fig. 1 and 2) and immunofluorescence tests, all isolates of *X. c.* pv. *manihotis* were serologically identical and through these tests *X. c.* pv. *manihotis* could be differentiated from *X. c.* pv. *cassavae* and the Colombian yellowish isolates. The Colombian isolates were serologically identical to *X. c.* pv. *cassavae* (Fig. 1) and related to *X. c.* pv. *manihotis*. All *X. c.* pv. *cassavae* isolates were also serologically identical.
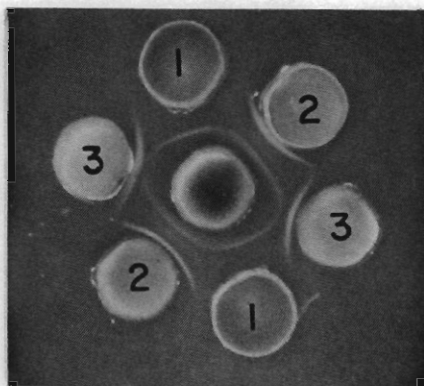
Fig. 1 Central well holds antiserum to *X. c.* pv. *cassavae* (CIAT 1148). Peripheral wells contain: (1) *X. c.* pv. *manihotis* antigen; (2) *X. c.* pv. *cassavae;* (3) Colombian yellowish isolate.
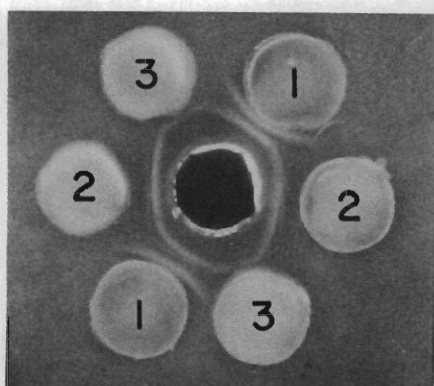
Fig. 2. Central well contains crude antiserum to *X. c.* pv. *manihotis* (CIAT 1105). Peripheral wells: (1) *X. c.* pv. *manihotis* antigen (washed whole cells); (2) *X. c.* pv. *cassavae* antigen (washed whole cells); (3) Colombian yellowish isolate (washed whole cells).

Discussion

Results show that *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* are biochemically similar and serologically related but distinct. This agrees with previous findings (11, 16). While *X. c.* pv. *manihotis* was serologically distinct from *X. c.* pv. *cassavae,* *X.* pv. *cassavae* and the Colombian yellowish isolates had common antigenic properties, even though differences were found in their pathogenicity. The synonymy between *X. c.* pv. *manihotis* and *X. c.* pv. *cassavae* reported by Robbs *et al.* (29) was based on biochemical and a few pathogenicity characteristics, but not on serology.

Therefore, the decision to consider *X. cassavae* as a *nomen dubium* (39) is probably unjustified. Based on our results on the serological and pathogenicity relationships between these two pathogens, and their geographical distribution, it is probably unlikely that *X. c.* pv. *cassavae* evolved from *X. c.* pv. *manihotis*. The presence of *X. c.* pv. *cassavae* in Colombia or elsewhere in Latin America therefore remains to be established.

The specificity of our XMA/FITC and XCA/FITC conjugates indicates the high specificity of antiserum produced using glutaraldehyde-fixed whole cells as reported by Allan and Kelman (1) in studies on *Erwinia carotovora* var. *atroseptica*. Similar results have recently been reported (33) in studies with *Corynebacterium sepedonicum*.

Literature Cited

1. Allan, E. and A. Kelman, 1977. Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. Phytopathology 67.1305-1312.
2. Buchanan, R.E. and N.E. Gibbons. 1974. (Eds.) Bergey's Manual of Determinative Bacteriology (8th. Edition). Williams and Wilkins company, Baltimore. 1268 pp.
3. Burkholder, W.H. and M.P. Starr. 1948. The generic and specific characters of phytopathogenic species of *Pseudomonas* and *Xanthomonas*. Phytopathology 38:494-502.
4. Centro Internacional de Agricultura Tropical. Annual Report, 1976.
5. Cherry, W.B. 1974. Immunofluorescence techniques. *In* Manual of Clinical Microbiology 2nd. Ed. pp 29-44. E.H. Lennette, E. H. Spaudling, and J.P. Truant (Eds.). American Society for Microbiology.
6. Christensen, W.B. 1946. Urea decomposition as a means of differentiating proteus and paracolon cultures from Salmonella and Shigella. J. Bacteriol. 52:461-464.
7. Difco Manual. 9th. Ed. 1977. Difco Laboratories. Inc., Detroit Michigan, 350 pp.
8. Dye, D.W. 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas spp*. N.Z.J. Agric. Sci 5:393-416.
9. Dye, D.W. 1968. A taxonomic study of the genus *Erwinia* N.Z.J. Agric. Sci. 11:590-607.
10. Elango, F.N. 1980. Relationship between *Xanthomonas manihotis* (Arthaud-Berthet and Bondar) Starr and *Xanthomonas cassavae* (Wiehe and Dowson), and some epidemiological studies on cassava bacterial blight. Ph.D. thesis, McGill University, Montreal, Canada, 301 pp.
11. Elango, F.N., J.C. Lozano, and J.F. Peterson. 1979. Physiological and Serological relationship between *Xanthomonas manihotis and Xanthomonas cassavae*. (Abstr.). Proc. 5th Symp. Int. Soc. Tropical Root Crops, Manila, Philippines (*In* Press).
12. Hayward, A.C. 1966. Methods of identification in the genus *Xanthomonas* pp. 9-14. In Identification methods for microbiologists. B.M. Gibbs and F.A. Skinner (Eds.). Academic Press, New York.
13. Hildebrand, D.C. 1971. Pectate and pectin gels for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens Phytopathology 61:1430-1436.
14. Hildebrand, D.C. and M.N. Schroth. 1964. B-glucosidase in phythopathogenic bacteria. Appl. Microbiol. 12:487-491.

15. Hugh, R. and E. Leifson. 1953. The taxonomic significance of fermentative versus oxidative metabolism of Carbohydrates by various gram-negative bacteria. J. Bacteriol. 66:24-26.

16. Ikotun, B. 1975. Cassava bacterial blight disease caused by *X. manihotis* (Arthaud-Berthet et Bondar) Starr. Ph.D. thesis, Imperial College of Sci. & Tech., London, 242 pp.

17. Kiraly, Z., Z. Klement, F. Solymosy, and J. Voros. 1974. Methods in Plant Pathology. Elsevier Scientific Publishing Co., New York, 509 pp.

18. King, E.O., M.K. Ward, and D.E. Raney. 1954. Two Simple Media for the demonstration of Pyocyanin and fluorescence. J Lab. Clin. Med. 44:301-307.

19. Klement, Z. 1975. Methods for the separation of closely related bacteria by gel diffusion test. pp 8-10. *In* R.N. Goodman (Ed.). Proceeding of the 1st Workshop on Phytobacteriology (3rd. Ed.) Univ. of Missouri, Columbia. 73pp.

20. Klement, Z., G.L. Farkas and L. Lovrekovich. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.

21. Kovacs, N. 1956. Identification on *Pseudomonas pyocyanea* by the Oxidase reaction. Nature (London) 178:703.

22. Lederberg, J. and E.M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol 63:399-406.

23. Lovrekovich, L. and Z. Klement. 1960. Triphenyl tetrazolium chloride tolerance of phytopathogenic bacteria. Phytopath. Z. 39:129-133.

24. Lozano, J.C. 1975. Bacterial Blight of Cassava. PANS 21:38-43.

25. Lozano, J.C. and L. Sequeira, 1974. Bacterial Blight of Cassava in Colombia: etiology. Phytopathology 64:74-82.

26. Maraite, H. and J. Weyns. 1979. Distinctive physiological, biochemical and pathogenic characteristics of *Xanthomonas manihotis* and *Xanthomonas cassavae*. pp. 103-117 *In* H. Maraite and J.A. Meyer (Eds.). Diseases of Tropical food crops, U.C.L. Belgium. 316 pp.

27. Misaghi, I. and R.G. Grogan. 1969. Nutritional and Biochemical comparisons of plant pathogenic and saprophytic fluorescent *Pseudomonads*. Phytopathology 59:1436-1450.

28. Reddy, C.R. and S.H. Ou, 1976. Characterization of *Xanthomonas oryzae* (Uyeda et Ishiyama). Dowson, the bacterial blight pathogen of rice. Ann. Phytopath. Soc. Jpn. 42:124-130.

29. Robbs, C.F., R. de L.D. Ribeiro, O. Kimura, and F. Akiba. 1972. (Variacoes em *Xanthomonas manihotis* (Arthaud-Berthet) Starr. Revista da Sociedade Brasileira de Fitopatologia 5:67-75.

30. Sands, D.C., M.N. Schroth, and D.C. Hildebrand. 1970. Taxonomy of phytopathogenic Pseudomonads. J. Bacteriol. 101:9-23.

31. Sierra, G. 1957. A simple method for the detection of lipolytic activity of microorganisms and some observation of the influence of the contact between cells and fatty substrates. Antonie Van leewenhoek J. Microbial Serology 23:15-22.

32. Skerman, V.B.D. 1967. A guide to the identification of the genera of bacteria (2nd. Ed.) The Williams and Wilkins Co., Baltimore, Maryland. 303 pp.

33. Slack, S.A., A. Kelman and J.B. Perry. 1979. Comparison of three serodiagnostic assays for the detection of *corynebacterium*. Phytopathology 69:186-189.

34. Sneath. P.H.A. 1956. Cultural and biochemical characteristics of the genus *Chromobacterium*. J. Gen. Microbiol. 15:70-98.

35. Society of American Bacteriologists. 1957. Manual of Microbiological Methods. McGraw-Hill Book Co., New York, 315 pp.

36. Starr, M.P. 1979. A concept of the genus *Xanthomonas* and its spp. pp. 393 (Abstr.), *In* Proceedings of the IVth Inter. Conf. on Plant Path. Bact., Angers, France.
37. Terry, E.R. 1978. Cassava Bacterial diseases. pp. 75-84. *In* T. Brekelbaum, A. Bellotti, and J.C. Lozano (eds.). Proceedings Cassava Protection Workshop. CIAT, Cali, Colombia, 7-12 November, 1977.
38. Waksman, S.A. 1967. The Actinomycetes. The Ronald Press Co., New York, 270 pp.
39. Young, J.M., D.W. Dye, J.F. Bradbury, C.G. Panagopoulos, and C.F. Robbs. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N.Z.J. Agric. Res. 21:153-177.

Acknowledgment

Indirect Enzyme-Linked Immunosorbent Assay of *Xanthomonas campestris* pv. *citri.*

E. L. Civerolo and C. Helkie

Agricultural Research Center
U.S. Dept. of Agriculture
Beltsville, MD, U.S.A.

Abstract

Citrus bacterial canker disease (CBCD) is caused by *Xanthomonas campestris* pv. *citri.* At least three distinct forms of CBCD are currently recognized. These are the Asiatic form (CBCD-A), the cancrosis B form (CBCD-B), affecting primarily lemon, and the 'Galego' lime form (CBCD-C). The serological relationships of *X. c.* pv. *citri* strains associated with these forms of CBCD were evaluated by indirect immunosorbent assay.

Antisera were prepared in White New Zealand female rabbits using immunogen preparations containing intact or heat-killed cells of *X. c.* pv. *citri* strains XC62 (CBCD-A), XC64 and XC69 (CBCD-B), and XC70 (CBCD-C). Immunoglobulin (Ig) was precipitated from pre-immune serum and antiserum with ammonium sulfate. Goat antirabbit Ig conjugated with alkaline phosphate was obtained commercially. Antigen preparations were saline suspensions of washed cells from pure culture, heated at 100°C for 30 min, and diluted in carbonate coating buffer (pH 9.6).

In indirect ELISA, CBCD-A strains were serologically related to but distinct from CBCD-B and CBCD-C strains. CBCD-B and CBCD-C strains were more closely related serologically to each other than to the CBCD-A strains. However the serological distinction between these strains was variable and not conclusive. No positive reactions in indirect ELISA occurred with antigen preparations from several strains of *Agrobacterium tumefaciens, Corynebacterium fascians, C. flaccumfaciens* pv. *flaccumfaciens, C. michiganense* pv. *michiganense,* Erwinia herbicola, E. stewartii, and *Pseudomonas pseudoalcaligenes* subsp. *citrulli.*

Introduction

Citrus bacterial canker disease (CBCD) is caused by *Xanthomonas campestris* pv. *citri.* The extent of pathogenic variation among strains of this bacterium is not completely known. However at least three distinct forms of CBCD are currently recognized (4). The most widespread form, the Asiatic form of CBCD (CBCD-A) occurs on grapefruit, sweet oranges, sour orange and other varieties. The cancrosis B (CBCD-B) form of the disease occurs primarily on lemon in South America but strains of *X. c.* pv.

citri associated with this form of the disease may infect other citrus varieties. Under natural conditions, the third form of CBCD occurs only on 'Galego' lime (CBCD-C). The causal agent of CBCD has been designated as *X. citri* n.f. sp. *aurantifolia* (13).

The serological relationships among different *X. c.* pv. *citri* strains (1, 3, 9, 10, 11), and of *X. c.* pv. *citri* strains to several other *X. campestris* pathovars and species of phytopathogenic bacteria have also been studied (1, 3, 15). Based on the results of these studies, collectively, the CBCD-A, CBCD-B, and CBCD-C strains of *X. c.* pv. *citri* are seroligally related, but distinct (1, 3, 9, 10, 11). Although CBCD-A strains are clearly distinct from the CBCD-B and CBCD-C strains the degree of serological relatedness among CBCD-B and CBCD-C strains is not clear (3, 11).

Because of its sensitivity and specificity, indirect ELISA (14) is potentially useful for qualitatively comparing and quantitatively comparing the serological relationships among different bacterial strains (2). The results of indirect ELISA using immunoglobulin (Ig) prepared from antisera against four strains of *X. c.* pv. *citri* are presented here.

Materials and Methods

Bacteria

The bacterial strains used are presented in Tables 1 and 2. All strains were maintained and cell suspensions prepared as previously described (3).

Table 1. *Xanthomonas campestris* pv. *citri* strains used in enzyme-linked immunosorbent assays.

Lab strain designation	Source strain no. or designation[a] (origin)	CBCD form[b]	Source[c]
XC59	IBBF-164 (Brazil)	A	1
XC62	6501 Japan	A	2
XC63	7801 Japan	A	2
XC64	B-4 Argentina	B	3
XC69	Xc-11 ("B" TYPE-4) Argentina	B	4
XC70	IBBF-512 (Brazil)	C	4

[a] Strain designations assigned in the Fruit Laboratory, HSI, Beltsville, Maryland 20705.

[b] CBCD = Citrus bacterial canker disease. Form A is the type A canker, Asian canker, cancancrosis A or true canker form of CBCD. Form B is the type B canker, cancrosis B, canker B or false canker form of CBCD. Form C is the 'Galego' lime cancrosis in Brazil.

[c] 1 = V. Rossetti, Divisao de Patologia Vegetale, Instituto Biológico, Sao Paulo, Brazil.
2 = M. Koizumi, Fruit Tree Research Station, Kuchninotsu, Nagasaki, Japan.
3 = M. Goto, Shizuoka University, Shizuoka, Japan.
4 = J. W. Miller, I.N.T.A., Bella Vista, Argentina.

Table 2. Non-xanthomonad strains used in enzyme-linked immunosorbent assays.

Lab strain designation[a]	Species	Source[b]
AT1	*Agrobacterium tumefaciens* (15955)	1
AT2	" " (C58)	2
CF1	*Corynebacterium fascians* (ICPBCF6)	3
CF1c1	*C. flaccumfaciens* pv. *flaccumfaciens* (ICPBCF6)	3
CM1	*C. michiganense* pv. *michiganense* (ICPBCM9)	3
EH1	*Erwinia herbicola*	4
ES1	*E. stewartii*	5
PPscl	*Pseudomonas pseudoalcaligenes* subsp. *citrulli*	5

[a] Strain designations assigned in the Fruit Laboratory, HSI, Beltsville, MD 20705.

[b] 1 = American Type Culture Collection, Rockville, Maryland 20852.
2 = R. H. Hamilton, The Pennsylvania State University, University Park, Pennsylvania 16802.
3 = R. Harris, Applied Plant Pathology Laboratory, Plant Protection Institute, Beltsville, Maryland 20705.
4 = Isolated from lemon leaf naturally infected with *Xanthomonas campestris* pv. *citri*. This leaf was provided by R. Stall and designated as being affected by the cancrosis B form of citrus bacterial canker disease.
5 = R. Goth, Vegetable Laboratory, Horticultural Science Laboratory, Beltsville, Maryland 20705.

Antisera Production

The production of antisera against intact (I), live cells is described elsewhere (3). In addition, antisera were produced commercially in white New Zealand female rabbits against heat-killed (HK) cells of CBCD-B strains XC64. Immunogen preparations were cell suspensions obtained as before (3) and containing approximately 10^9 colony-forming units (CFU)/ml sterile 0.85% NaCl. Rabbits were immunized by interveinal injection of 0.2, 0.4, 0.8, 1.6, 3.2 and 5.0 ml on day 1, 4, 7, 10, 13, and 17, respectively. On day 24, the rabbits were exsanguinated.

Antigen Preparations

Antigens were prepared from suspensions of pure culture cells as previously described (3). Saline cell suspensions (50% T at 620 mm, 1 cm light path) were heated in boiling water bath for 30 min and diluted 10-fold in PBS-Tween.

ELISA

The Ig preparation and reagents used were the same as previously described (3). Indirect ELISA tests were done essentially as described by Voller, *et al.* (14). Generally, microtiter plates (Dynatech) were coated with 200 nl of heated antigen preparation containing the equivalent of about 10^6

CFU/ml carbonate coating buffer (pH 9.6) for 1 hr at 37°C. After washing the plates successively three times with PBS-Tween 200 nl of Ig preparation containing 10 ng protein/ml was incubated in each well for 1 hr at 37°C. There were at least three replicate wells for each antigen-antibody combination. After washing as before, 200 nl goat anti-rabbit Ig (Miles-Yeda Ltd., No. 61-275; 100-250 enzyme units/ml), conjugated with alkaline phosphatase diluted 1:3000 in PBS-Tween, was incubated in each well for 1 hr at 37°C. The washing was repeated. Then 200nl of 1 mg para-nitrophenyl phosphate/ml substrate buffer was incubated in each well for 30 to 60 min at room temperature. After adding 50 nl 3M NaOH to each well, the reactions were quantitated with a Titertek Multiskan photometer (Flow Laboratories, McLean, VA 22102) equipped with a 405 nm filter. In addition, the ratio of the absorbance at 405 nm of the heterologous to that of the homologus antigen-antibody (A_{405} Ht/Hm) combination was calculated for comparison.

Results

Coating Ig

Indirect ELISA using Ig (0-25 ng protein/ml) obtained from pre-immune serum and anti-XC64 (HK) serum is shown in Fig. 1. Generally, the amount of homologous antibody specifically attached to antigen passively immobilized on the solid phase was directly proportional to the Ig concentration up to about 10 ng of Ig protein/ml. There was no significant non-specific attachment of enzyme-conjugated antiglobulin using pre-immune serum Ig up to 25 ng Ig protein/ml.

Comparative Indirect ELISA of *X. c.* pv. *citri* Strains

Generally, A_{405} values in indirect ELISA were higher for homologous than heterologous antigen-antibody combinations (Table 3). Using anti-strain XC62 (I) serum Ig, the A_{405} Ht/Hm ratios obtained with the heated antigen preparations from CBCD-B and CBCD-C strains were about 0.58 (avg. value for XC64 and XC69) and 0.54, respectively (Table 3). Generally, the A_{405} values using anti-XC64 (I) and anti-XC64 (HK) sera Ig were higher for heated antigen preparations from CBCD-B strains (A_{405} Ht/Hm = 0.65-0.98) than from CBCD-A strains (A_{405} Ht/Hm = 0.31-0.51). Similarly, A_{405} values using anti-XC70(I) serum Ig were higher for heated preparations from CBCD-B strains (A_{405} Ht/Hm = 0.46-0.88) than from CBCD-A strains (A_{405} Ht/Hm = 0.17-0.41).

Thus, there was significant cross-reactivity between heated antigen preparations from CBCD-B strains (XC64, XC69) and the CBCD-C strain (XC 70) in indirect ELISA using Ig from antiserum to a CBCD-A strain (XC62). Similarly, there was significant cross reactivity between heated antigen preparations from CBCD-A strains (XC59, XC62, and XC63) in indirect ELISA using an anti-CBCD-B strain (XC64) or anti-CBCD-C strain (XC 70) serum Ig. Clearly, however, the CBCD-A strains were serologically distinct from the CBCD-B and CBCD-C strains.

The CBCD-B and CBCD-C strains used here were not as readily distinguishable from one another in these indirect ELISA tests (Table 3). With anti-XC69(I) serum Ig, the ELISA reactions with antigen preparations
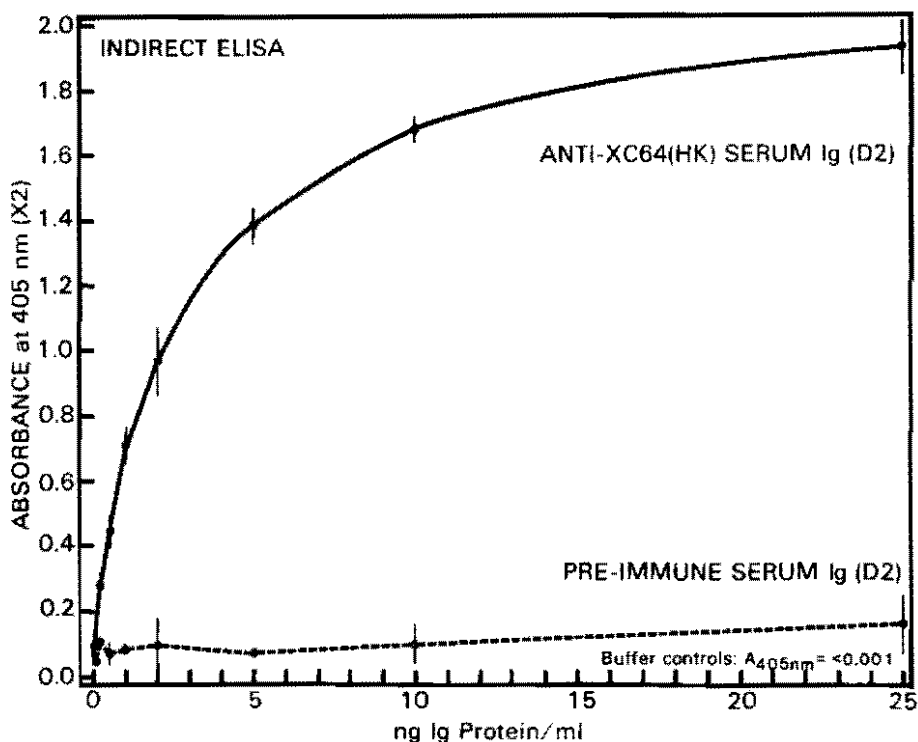
Fig. 1. Indirect ELISA of *Xanthomonas campestris* pv. *citri* (strain XC64) using immunoglobulin from pre-immune serum and anti-XC 64 (HK) serum.

from CBCD-B strains (XC64 and XC69) were about 1.3 times higher than with a similar preparation from the CBCD-C strain (XC70). However, this difference was not observed consistently. The A_{405} values with heated antigen preparations from CBCD-B strains using the anti-CBCD-C strain (XC70) serum Ig were 0.88 and 0.46 times those of the homologous antibody-antigen combination for strain XC64 and strain XC69, respectively. The A_{405} values with heated antigen preparations from the CBCD-C strain (XC70) using anti-XC64(I) and anti-XC64(HK) sera Ig were about 0.84 times those of the homologus antibody-antigen combinations.

Comparative Indirect ELISA with Antigen Preparations from Non-xanthomonad Strains

There were no significant reactions in indirect ELISA using anti-XC62 (I), anti-XC64(I), anti-XC64 (HK), or anti-XC70(I) serum Ig with heated antigen preparations from two strains of *Agrobacterium tumefaciens*, and one strain each of *Corynebacterium michiganense*, *C. fascians*, *C. flaccumfaciens*, *E. herbicola*, *E. stewartii*, and *Pseudomonas pseudoalcaligenes* subsp. *citrulli*.

109

Table 3. Indirect enzyme-linked immunosorbent assays of six *Xanthomonas campestris* pv. *citri* strains and one *Erwinia herbicola* strain using immunoglobulin from antisera against four *X. c.* pv. *citri* strains.

Antigen source strain	A_{405} [a]				
	Ig from antiserum against				
	XC62(I)	XC64(I)	XC64(HK)	XC69(I)	XC70(I)
XC59	1.54 + 0.05	0.49 ± 0.06	0.56 ± 0.08	ND	0.16 ± 0.08
XC62	1.49 + 0.07	0.43 ± 0.03	0.49 ± 0.01	0.21 ± 0.03	0.36 ± 0.06
XC63	1.14 + 0.05	0.60 ± 0.01	0.60 ± 0.01	ND	0.38 ± 0.02
XC64	0.98 ± 0.04	1.18 + 0.02	1.08 + 0.01	0.66 + 0.07	0.82 + 0.04
XC69	0.76 ± 0.03	0.70 + 0.04	0.97 + 0.04	0.67 + 0.07	0.43 + 0.02
XC70	0.80 ± 0.09	0.99 + 0.06	0.91 + 0.01	0.49 + 0.07	0.93 + 0.03
EH1	0.14 ± 0.03	0.06 ± 0.01	0.09 ± 0.06	0.01	0.08 ± 0.02
None	0.01 ± 0.01	0.05 ± 0.02	0.08 ± 0.01	0.01	0.04 ± 0.01
Control: Pre-immune serum Ig	ND	0.11 ± 0.02	0.06 ± 0.03	ND	0.01 ± 0.01
No Ig	0.10 ± 0.02	0.02 ± 0.01	0.05 ± 0.01	0.01	0.01 ± 0.01

[a] Each figure is the mean ± standard deviation of the A_{405} values of three replicate wells.

Discussion

Based on the cross-reactivity in indirect ELISA tests of six *X. c.* pv. *citri* strains of the bacteria associated with three forms of CBCD, these strains are serologically related. However, the CBCD-A strains (XC59, XC62 and XC63) are serologically distinct from the CBCD-B strains (XC64, XC69) and CBCD-C strains (XC70). The serological distinction between the CBCD-B strains (XC64, XC69) and CBCD-C strains (XC70) in indirect ELISA was variable and not conclusive. Additional strains of the bacteria associated with CBCD-B and CBCD-C are needed to evaluate further the serological relationships between these strains.

Additional information about the serological relationships between these strains might also be obtained by using Ig prepared from antisera against different specific immunogens and/or antisera cross-adsorbed with heterologous antigen preparations, or by adaptation of modified ELISA techniques (5, 6, 7, 8, 12).

Literature Cited

1. Bach, E. E., A. P. C. Alba, A. Lima, G. Pereira, A. G. Zagetto, and V. Rossetti. 1978. Serological studies of *Xanthomonas citri* (Hasse) Dowson. Arch. Inst. Biol., Sao Paulo 45:229-236.
2. Cambra, M., and M. M. Lopez. 1979. Titration of *Agrobacterium radiobacter* var. *tumefaciens* antibodies by using enzyme-labelled anti-rabbit - globulins [(ELISA) indirect method]. Proc. 4th Int. Conf. Plant Path. Bact., Angers, 1978, pp. 327-331.
3. Civerolo, E. L., and F. Fan. 1981. *Xanthomonas campestris* pv. *citri* detection and identification by enzyme-linked immunosorbent assay. Plant Disease in press.
4. Garnsey, S. M., E. P. DuCharme, J. W. Lightfield, C. P. Seymour, and J. T. Griffiths. 1979. Citrus canker. The Citrus Industry 60:5-6, 8, 10, 13.
5. Goding, J. W. 1978. Use of staphylococcal protein A as an immunological reagent. J. Immunol. Methods 20:241-253.
6. Hsu, S.-M., L. Raine, and H. Fanger. 1981. The use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: A comparison between ABC and unlabelled antibody (PAP) procedures. J. Histochem. Cytochem. 29:577-579.
7. Kohler, G. and C. Milstein. 1975. Continuous cultures of fused cells secreting antibody of predefined specificity. Nature 256:495-497.
8. Lutz, H., J. Higgins, N. C. Pedersen, and G. H. Theilen. 1979. The demonstration of antibody specificity by a new technique: The gel electrophoresis derived enzyme-linked immunosorbent assay (GEDELISA) and its application to antibodies for feline leukemia virus. J. Histochem. Cytochem. 27:1216-1218.
9. Messina, M. A. 1976. Los métodos serológicos en el estudio de la bacteria que produce la "cancrosis cítrica" en la Argentina. I. Hallazgo de un nuevo serotipe. Metodología empleada en el estudio completo. INTA, Concordia, Estación. Experimental Agropecuaria, Serie Técnica No. 46, pp. 1-9.
10. Messina, M. A. 1980a. Los métodos serológicos en el estudio de la bacteria que produce la "cancrosis cítrica" en la Argentina. III. Diferenciación entre las bacterios de la "cancrosis A" o "asiática" y las de la "cancrosis

B" or "sudanamericana" en la país. INTA, Concordia, Estación Experimental Agropecuaria, Serie Técnica No. 50, pp. 3-8.

11. Messina, M.A. 1980b. Los métodos serológicos en el estudio de la bacteria que produce la cancrosis cítrica en la Argentina. IV. La técnica de immunofluorescencia. Su applicacion como elemento de diagnóstico de laboratorio. Resumenes II Congreso Nacional de Citricultura, 27-31 October 1980, Concordia (E.R.), Argentina, p. 52.

12. Morley, S. J., and D. G. Jones. 1980. A note on a highly sensitive modified ELISA technique for *Rhizobium* strain identification. J. Appl. Bacteriol. 49:103-109.

13. Namekata, T. 1971. Estudor comparativos entre *Xanthomonas citri* (Hasse) Dow., agent causal do "Cancro Citrico" e *Xanthomonas citri* (Hasse) Dow., n.f. sp. *aurantifolia*, agente causal de "cancrose do Limoero Galego". Ph.D. Thesis. Escola Superior de Agricultura "Luís de Queiroz", Piracaiba, Brazil.

14. Voller, A., A. Bartlett, and D. E. Bidwell. 1978. Enzyme immunoassays with special reference to ELISA techniques. J. Clinical Pathol. 31:507-520.

15. Yano, T., A. F. Pestana de Castro, J. A. Lauritius, and T. Namekata. 1979. Serological differentiation of bacteria belonging to the *Xanthomonas campestris* group by indirect hemagglutination test. Ann. Phytopathol. Soc. Jpn 45 1-8.

Specificity of Elisa to
Xanthomonas campestris
pv. *phaseoli* Identification

L. Afanador
CIAT
Cali. Colombia

J. I. Victoria
Instituto Colombiano Agropecuario
Palmira, Colombia

Abstract

Rabbit antisera were produced against glycoprotein bacterial extracts and whole cell of *Xanthomonas campestris* pv. *phaseoli* isolate xp-5-51 and *Xanthomonas* spp, *Pseudomonas* spp., and *X. campestris* pv. *phaseoli* isolates from different sites were determined by Ouchterlony double diffusion (ODD) test and indirect ELISA Method (IEM). Better antiserum specificity was obtained with IEM and glycoprotein as antigens than with ODD and whole cells as antigens. Sensitivity for the former combination was estimated in 5 x 10^5 cells/ml of antigen and 20 ng/ml of antiserum as minimum for good reaction.

Introduction

Beans (*Phaseolus vulgaris* L.) are important in Colombia as in the rest of the Latin American countries as a source of protein for a majority of the population.

But bean production is diminishing due to damages caused by bacteria, fungi and viruses. Among the diseases caused by bacteria is common bacterial blight caused by *Xanthomonas campestris* pv. *phaseoli*. Its occurrence and severity varies with variation in climatic conditions. It is probably the most widely distributed disease of beans. Yield losses due to common bacterial blight run 20 to 80%.

More than 50% of the pathogens that cause diseases in beans are seed transmitted. For that reason, a program aimed at producing clean seed is needed.

X. campestris pv. *phaseoli* in seeds occurs in a mixture with other types of bacteria, making it necessary to develop a method that permits its differentiation from them. Serological methods are considered to be some of the most reliable in identification of bacteria and other plant pathogens.

However, there are differences in reliability, sensitivity and specificity of these methods, making it necessary to develop a method that has all these characteristics.

Materials and Methods

Pathogenicity Tests

Isolate xp-5-51 of *X. campestris* pv. *phaseoli* grown on YDC (yeast extract, 10 gr; dextrose, 20 gr; $CaCO_3$, 20 gr; agar, 18 gr; distilled water, 1000 ml) for 48 hr was used to make the bacterial suspension.

The turbidity of the latter was adjusted to an optical density of 0.5 (600 nm) corresponding to a concentration of 5×10^8 cells/ml, using a Spectronic 20 (Bausch and Lomb Co., Rochester, N.Y.) colorimeter. A dilution was made to obtain a final concentration of 5×10^7 cells/ml which was used for inoculation, by the scissors method (5). Inoculation was made on plants 15 to 20 days old.

Production of Antigen

Two types of the antigens were produced, the whole cells and the extracelluler glycoproteins. Forty-eight hr old cultures grown on nutrient agar (peptone, 5 gr; beef extract, 3 gr; agar, 20 gr; and distilled water, 100 ml) for whole cells antigen production and on YDC for glycoprotein antigen production were used.

To obtain whole cells antigen, bacterial cells (obtained by centrifugation at 10,000 for 15 minutes and suspended in 0.85% saline solution) were killed by submerging them in a hot (100°C) water bath (3) for 2 hr. Extracellular glycoprotein antigen was obtained by precipitation of glycoproteins with a saturated ammonium sulphate solution (6). The precipitate was removed from the solution using a thin glass rod. Both types of antigens were used in immunization of the rabbits.

Immunization Proccess

Immunization using the antigens was done with New Zealand white rabbits. The whole cell antigens were injected intravenously. For extracellular glycoprotein antigens one part was mixed with 2 parts of phosphate buffer solution (PBS), 1/2 (NaCl, 8 gr; KH_2PO_4, 02 gr; $Na_2HPO_4.12H_2O$ 2.9 gr; KCl, 0.2 gr; NaN_3. 0.2 g and distilled water, 1000 ml); the mixture was combined with an equal volume of Freuds incomplete adjuvant and then injected intramuscularly.

For both antigens, 0.5 ml was used as the initial whereas 2 ml were used as the final dose.

Antisera Collection

A total of five bleedings were made to evaluate the antisera titers. In the first bleeding, 4 ml of blood were collected whereas 50 ml were collected in each of the last 4 bleedings. The antisera obtained by blood centrifugation at 5000 rpm for 15 minutes, were used in the microagglutination tests (1) to evaluate the titers obtained. When the desired titers were obtained, the antisera were purified and γ -globulin was obtained by precipitation, using a saturated solution of ammonium sulphate (4) and the concentration of

the former was calculated by measuring its absorbence in a Beckman DB Spectrophotometer at a wavelength of 280 nm.

Evaluation of Antisera

The antisera were tested with the homologus isolate xp-5-51, *X. c.* pv. *phaseoli* isolates from and out of Colombia, various pathovars of *X. campestris* and isolates of other genera (Table 2.)

The methods used in antisera and antigen testing were, the Ouchterlony Double Diffusion (ODD) test (4) and the Indirect ELISA (2).

For homologous reactions with the ODD method, antigen dilutions of 5 x 10^8 to 5 x 10^6 cells/ml and antisera dilutions of 1 mg/ml to 50 ng/ml were used, whereas with the Indirect ELISA, antigen dilutions of 5 x 10^8 to 5 x 10^4 cells/ml and antiserum dilutions of 1 mg/ml to 10 ng/ml were used (Table 1).

The corresponding concentrations used for the heterologous reactions with ODD method were 5 x 10^8 cells/ml of the antigen and 1 mg/ml of the antisera. With the Indirect ELISA method, the concentrations used were 5 x 10^6 cells/ml of the antigen and 50 and 20 ng/ml of the antisera.

Identification of *X. campestris* pv. *phaseoli* Isolated from Seeds

Seeds apparently healthy, were surface sterilized twice in 1% sodium hypochlorite and then rinsed twice with sterile distilled water. They were then plated in YDC medium with the hilum touching the medium. Nineteen isolates were obtained, which could be grouped into 2 groups based on the color of their colonies. Eleven isolates had yellow colonies, whereas 8 had cream colonies. The yellow group were tested with the Indirect ELISA method and later for their pathogenicity on 15-20 day old plants using the

Table 1. Different antigen and antisera dilutions used in the ODD and Indirect ELISA methods.

Method	Dilutions utilized																			
	Antigen concentrations (cells/ml)																			
ODD	5 x 10^8			5 x 10^7			5 x 10^6													
	Antiserum concentrations (mg-ng/ml)																			
	1	75	50	1	75	50	1	75	50											
	Antigen concentrations (cells/ml)																			
Indirect ELISA	5 x 10^8			5 x 10^7			5 x 10^6			5 x 10^5			5 x 10^4							
	Antiserum concentrations (mg-ng/ml)																			
	1	75	50	20	10	1	75	50	20	10	1	75	50	20	10	1	75	50	20	10

Table 2. Isolates tested with the ODD and Indirect ELISA methods

Isolates tested			Identification
Xanthomonas campestris pv. *phaseoli*			xp-5-51
"	"	"	CBP-057
"	"	"	CBP-004
"	"	"	CBP-047
"	"	"	CBP-048
"	"	"	CBP-045
"	"	"	CBP-001
"	"	"	CBP-003
"	"	"	CBP-107
Xanthomonas cassavae			CIAT-1148
"	*vesicatoria*		CIAT-1123
"	*oryzae*		CIAT-1185
"	*malvacearum*		
"	*manihotis*		
Agrobacterium tumefasciens			CIAT-1182
Pseudomonas solanacearum			CIAT-1001
"	*syringae*		CIAT-1021
"	*toloasi*		
"	*syringae* pv. *glicinea*		
"	*phaseolicola*		Pp-20
Corynebacterium flacumfasciens			
Erwinia carotovora			

scissors inoculation method (5). Isolates of the cream group were plated on Kings medium B (bacto peptone, 20 g; glycerol, 15 g; $MgSO_4$, 3 g; K_2HPO_4, 2 g; agar, 18 g and distilled water, 1000 ml) and evaluated for fluorescence under UV-light, due to pigment production (8).

Results

Pathogenicity Test

Isolate xp-5-51 was pathogenic and plants inoculated showed high disease severity.

Production of Antigen

To produce somatic antigens, use of levan peptone glucose agar (LPGA) (levan extract, 5 g; pancreatic peptone, 5 g, glucose, 10 g; agar, 20 g;

distilled water, 1000 ml) as a medium for bacterial growth, resulted in exuberant growth of the organism, due to high glucose content. The heavy mucous production makes centrifugation of the bacterial suspension difficult and impedes the separation of free cells from the bacterial exudates. This problem was avoided by using nutrient agar, which permitted good bacterial growth without producing much exudate.

The extraction method for somatic antigen (5) production used, resulted in consistent and considerable amounts, appropriate for the process of immunization. The titers of the antiserum obtained with this antigen were, however, low and the quantity γ -globulin purified from it was not sufficient to give an optimum concentration for serological tests (1 mg/ml). To produce extracellular glycoprotein antigens, using the saturated solution of ammonium sulphate (6), LPGA medium was replaced with YDC for bacterial growth. The YDC medium permits abundant bacterial growth with production of extracellular polysaccharides. This method permitted production of appreciable quantities of the antigen and of γ globulin needed for serological tests.

Immunization Process

Extracellular glycoprotein antigens induced more antibody production, resulting in higher antisera titers than those given with whole cell antigens. Immunization by intramuscular injection gave high production of antibodies, although in a slower but more prolonged manner, whereas, low production of antibodies resulted from intravenous injection. This had a direct relationship with the quantity of the γ globulins that could be produced from the antisera. More γ globulin were produced when the quantities of the antibodies were optimum (high antisera titers), hence, in the antisera of extracellular glycoproteins the quantity of γ globulins was high (levels more than 1 mg/ml) but less in the antisera whole cells (levels much below 1 mg/ml).

Antisera Collection

Higher antisera titers were obtained with the extracellular glycoprotein antigens than with the whole cell antigens, while injecting the rabbits with the same dose and frequency. Starting from the third bleeding, it was observed that the titers stabilized and did not increase despite continued antigen application (Table 3). The highest titer obtained with whole cells was 256 whereas with extracellular glycoprotein titers of 2560 to 5120 were attained which establishes a clear comparison between the two forms of antigens used and their practical application as antisera in serological tests.

Evaluation of the Antisera

With the ODD method, it was possible to detect bacterial concentration of 5×10^8 cells/ml when the antiserum concentration was 1 mg/ml, but no detection of bacterial concentration of 5×10^7 nor 5×10^6 cells/ml was possible. Bacterial concentration of 5×10^8 cells/ml was only detected when the γ globulin concentration was 1 mg/ml and not at lesser concentration (Table 4).

Table 3. Titers obtained with the different antigens used.

Antigen	Titers obtained in their order of bleeding				
	I	II	III	IV	V
Whole cells	1:64	1:128	1:128	1:256	1:256
Extracellular glycoproteins	1:128	1:128	1:256	1:2560	1:2560-5120

Table 4. Comparison of ODD-ELISA at different concentrations of the antigen and the antisera.

Method	Antigen concentrations (cells/ml)														
	5×10^8			5×10^7			5×10^6			5×1^{-5}			5×10^4		
	Antiserum concentrations (mg/ng/ml)														
	1	75	50	1	75	50	1	75	50	1	75	50	1	75	50
ODD	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ELISA	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

All isolates of *X. c.* pv. *phaseoli* from and out of Colombia gave positive reactions, forming bands with the antiserum except for isolate CBP-048 (Table 5). There was no band formation with either different pathovars of *X. campestris* or isolates of other genera used, at any antigen concentration level tested.

Table 5. ODD Test-Isolates tested at different concentrations of the antigen and the antiserum.

Identification of the isolate	Antigen concentrations		
	5×10^8 cells/ml		
	Antiserum concentrations (mg/ng/ml)		
	1	75	50
Control xp-5-51	+	—	—
Control PBS	—	—	—
CBP-057	+	—	—
CBP-004	+	—	—
CBP-047	+	—	—
CBP-048	—	—	—
CBP-045	+	—	—
CBP-001	+	—	—
CBP-003	+	—	—
CBP-107	+	—	—

Indirect ELISA

It was possible with this method, to detect bacteria at concentrations of 5 to 10^5 cells/ml with antiserum concentration from 1 mg/ml to 10 ng/ml (Table 4). Using an antigen concentration of 5×10^5 cells/ml and antisera concentrations of 50 and 20 ng/ml, all isolates of *X. campestris* pv. *phaseoli* tested gave a positive reaction except for isolates CBP-045 and CBP-048 (Table 6). However, all isolates of pathovars of *X. campestris* other than *phaseoli* and isolates of other genera used, gave negative reactions.

To qualitatively measure the reaction that occurred by using the Indirect ELISA, the change and intensity of the pigment produced by the enzyme-substrate reaction were quantitatively measured in a Beckman DB Spectrophotomer at 400 nm. The quantity of the product liberated corresponded to color intensities produced at different antigen and antiserum concentrations.

It was noted that the absorbence values of the antigen samples decreased with the decrease in its concentration, whereas, there was no significant change in absorbence with the decrease in the antiserum concentration.

Identification of *Xanthomonas campestris* pv. phaseoli Isolated from Seeds

The yellow group of isolates, that had been isolated from seeds were tested using the indirect ELISA method. Positive reaction was obtained

Table 6. Indirect ELISA test; isolates tested at different antiserum concentrations and 5×10^6 cells/ml of the antigen.

Isolates 5×10^6 cells/ml.	Antiserum concentrations	
	50 ng /ml	20 ng/ml
Control xp-5-51	+	+
Control PBS	−	−
CBP-057	+	+
CBP-107	+	+
CBP-048	−	−
CBP-045	−	−
CBP-047	+	+
CBP-001	+	+
CBP-003	+	+
CBP-004	+	+

with isolates 8, 9, 10, and 11; the rest showed negative results. The 4 isolates that gave positive reaction were later tested for pathogenicity by inoculation of bean plants 15 to 20 days old using the scissors method (5). They were all found pathogenic and proved that they were isolates of $X. c.$ pv. *phaseoli.*

Discussion

The use of the two antigen types in rabbit immunization established which one of them could produce more antibodies. The amount of the latter has a direct relationship to the concentration of γ globulin in the antisera obtained. Extracellular glycoprotein gave antiserum of high titers and therefore higher concentration of γ globulin could be purified from it. This made us choose and use this antiserum as more appropriate in all serological tests.

The results obtained with the ODD method indicated that the method can be considered specific and reliable but not sensitive, as bacterial concentrations of 5×10^8 cells/ml or more are required for their detection and also it requires high antiserum concentration (1 mg/ml). This means that, a large quantity of the antisera would be required to evaluate bean materials, making the method less economical. Furthermore, it does not permit evaluation of a large number of samples and results are only obtained after 48 hr.

The indirect ELISA method proved to be more reliable and sensitive than the ODD method. It is possible to detect bacterial concentration of up to 5×10^5 cells/ml with minimum antiserum concentration (1 mg/ml), although, in reducing it more, quantitative estimation of the method showed that absorbence does not decrease much between dilutions of the antiserum, indicating that, there is sufficient antiserum and it is possible to have it in lower concentrations and still detect bacterial populations.

The extracellular glycoprotein antiserum was specific for *Xanthomonas campestris* pv. *phaseoli,* hence, permitting differentiation of the latter from a group of bacterial isolates belonging to a genus other than *Xanthomonas* when using both methods (ODD and ELISA). The ELISA method, however, demonstrated more sensitivity and reliability.

The indirect ELISA method is reliable in that *X. campestris* pv. *phaseoli* reacts specifically with an homologous antiserum; and in this form, it can be used in testing bacterial cultures whose identity is doubtful or in separation of *Xanthomonas* from a group of unknown bacteria. Due to its sensitivity, low concentrations of the antiserum (10 ng/ml and possibly less) can be used in detecting bacterial concentration as low a 5×10^6 or 5×10^5 cells/ml. Knowing that high bacterial concentrations in seeds are uncommon, the use of enriched liquid media, followed by centrifugation can increase their concentration and aid identification when using Indirect ELISA. For this reason the method serves as a useful tool in detection of *X. campestris* pv. *phaseoli* and its distinction from other bacteria population in the seed.

The method saves time in diagnosis and permits evaluation of a large number of materials more economically.

Literature Cited

1. Ball, E.M., 1974. Serological tests for the identification of plant viruses. The American Phytopathological Society. INC. United States of America. Pp. 4-6.
2. Berger, *et al.* 1979. Calorimetric enzyme-linked immunosorbent assay for the identification of strains of rhizobium in culture and in the nodules of lentils. Applied and Enviromental Microbiology. 37. 2:642-646.
3. Benquist, K.R. and R. P. Elrod. 1948. The somatie antigens of the genus Agrobacterium. Proc. SD Acad. Sel. 27:104-111.
4. Clark, M. F. and A. N. Adams. 1977. Characteristic of the microplate methods of enzyme-linked inmunosorbent assay for the detection of plant virus. J. Gen. Viral. 34:475-483.
5. Correa, V. F. and J. I. Victoria. Desarrollo de un sistema de evaluación y pruebas de resistencia del añublo común del fríjol (*Xanthomonas phaseoli* Dowson). Degree Thesis. Faculty of Agronomy. Palmira, Colombia.
6. Digat, B. and M. Cambra. 1976. Specificity of antigens on *Pseudomonas solanacearum* E. F. Sm. and application of serology for studying bacterial wilt. Planning conference and workshop on the ecology and control of bacteria wilt caused by *Pseudomonas solanacearum*. North Carolina State University, Raleigh, North Carolina, July 18-23. Pp 38-55.
7. Digat, B. Selection sanitaire des boutres de Pelargonium et de Begonia X. Elatior "Reiger" vis-a-vis des bacterioses por utilisation de I'mmunofluorescence. Centre de Recherches agronomiques d'Angers. I. N. R. M. 66-70.
8. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocianin and fluorescein. J. Lab. Clin. Med. 44:301-307.

Antigenic Relationships Between *Xanthomonas* Strains Causing Disease of Cabbage, Lettuce, and Onion in Hawaii

A. M. Alvarez, S. R. Kuniyuki, and K. Lou.

University of Hawaii
Honolulu, Hawaii, U.S.A.

Abstract

Rabbit antiserum made to *Xanthomonas campestris* pv. *campestris* strain A249 isolated from cabbage grown in Maui, Hawaii was used as a standard in the enzyme-linked immunosorbent assay (ELISA) to determine its antigenic similarities with *Xanthomonas* strains isolated from cabbage and other crucifers, lettuce, and onion from different locations. Inhibition curves were used to compare antigenic similarities between homologus strain A249, 23 other heterologus *Xanthomonas* strains, and one *Erwinia herbicola* strain. The quantitative data provided by ELISA could be used to distinguish among closely related *Xanthomonas* pathovars and strains causing diseases of cabbage, other crucifers, lettuce, and onion.

Introduction

Conventional determinative tests have been largely inadequate for distinguishing among *Xanthomonas campestris* pathovars (9). Colony morphology, pigmentation, and bacteriological tests were inadequate for distinguishing among strains isolated from cabbage, lettuce, and onion in Hawaii and other locations. Serological methods based on precipitation or agglutination reactions have been inadequate for differentiating among *Xanthomonas* species because antigenic determinants are shared within the genus (10). The enzyme-liked immunosorbant assay (ELISA) has been widely used as a diagnostic tool in recent years because of its sensitivity and practicality (5, 13, 21, 23, 24). The availability of this technique has prompted a renewed effort to differentiate plant pathogens using surface antigens on intact cells because it may be those antigens which play the greatest role in recognition mechanisms between host and pathogen. Since ELISA has the potential of providing a quantitative measure of antigenic determinants on cell surfaces (14, 15), studies were undertaken to determine whether this assay could be used to characterize the

relationships among *Xanthomonas campestris* pathovars causing diseases of cabbage, lettuce, and onion in adjacent fields in Hawaii.

Materials and Methods

Strains

X. campestris pv. *campestris* strains A249, KC-3-12, A673-2A, A673-2B, and A675-1 were isolated from diseased cabbage plants on farms in Maui, Hawaii. Strain OK_2 was obtained from M. Goto, Japan, and strain EE XC 118 was obtained from E. Echandi, Raleigh, N.C. Strain A342 was isolated from diseased broccoli seedlings in Kula, Maui, and strain PHW-RR 68 was isolated from radish seed and obtained from P. H. William, Madison, Wisconsin. The six lettuce strains 10-TB7, 10-TB-10, 10S6, 10S7-2, A674-4A, and A676-2A were all isolated from diseased lettuce showing necrotic leaf spots in Kula, Maui. Onion strains were isolated from onion variety Yellow Grannex showing symptoms of leaf blight; six strains, A206-2a, A226-3, A-227-1, A551-3, A554-3, and A54-7 came from Kula, Maui; and 3 strains, A30-2a, A88-3, and A255-4, came from Molokai.

Antiserum

Whole cells of *X. campestris* pv. *campestris* strain A249 were prepared as immunizing antigen and injected into New Zealand white rabbits as previously described (1). The specificity of the antiserum was determined by double diffusion and immunofluorescence using *X. campestris* pathovars and 36 unrelated pathogens and saprophytes (8).

Coating Antigen

Whole cells of strain A249 were prepared by the same procedure as for the immunizing antigen. After heat treatment, cells were washed and resuspended in saline, and the optical density (OD) was read at 600 nm. The suspension was adjusted to the desired cell count by using OD readings calibrated to total cell counts and resuspending the cells in the appropriate volume of carbonate-bicarbonate buffer (pH 9.6) made by mixing equal volumes of 15 nM Na_2CO_3 (1.59 g/1) with 35 nM $NaHCO_3$ (2.94 g/1). The best coating procedure was determined by varying the coating time, temperature, and method (air-dry vs. wet coat).

Conjugate

Goat-anti-rabbit IgG, conjugated to horseradish peroxidase (GARP) was purchased from Cappel Laboratory, Cochranville, PA 19330, lot #13466. The optimum concentrations of GARP, coating antigen, and antibody were determined from a series of titrations and antibody-antigen binding curves at different GARP concentrations (2).

Enzyme Substrate

A stock solution of 1% w/v *ortho-phenylenediamine* (OPD) (Sigma Lot #10F-0076) was prepared in citrate buffer (pH 7) made by mixing 98.6 parts of 0.1 M citric acid and 101.4 parts of 0.2 M Na_2HPO_4. One ml of the stock solution was mixed with 0.1 ml of 3% H_2O_2 in 99 ml of citrate buffer.

Microplates

Polystyrene microplates with 8 rows of 12 wells, flat-bottomed for photometric reading, were obtained from Linbro Division, Hamdon, CT 06517. Test liquids were dispensed with a Repetman (Rainin Instruments, Emeryville, CA 94608) or an 8-tipped multichannel pipetter (Titertek, Flow Laboratories, Inglewood, CA 90301).

ELISA Procedure

Coat plates by adding 200 nl of homologus antigen A249 to each well of the microplate. Cover with parafilm and incubate in a water bath (70°C) for 4 hr. Refrigerate overnight. Drain antigen. Flood with phosphate-buffered saline plus Tween-20 (pH 7.4) made by mixing 8.0 g NaCl, 0.2 g KH_2PO_4 and 1.15 g Na_2HPO_4 an 0.5 ml Tween-20 per liter (PBST); allow to sit 3 minutes. Drain and repeat for a total of three washes. Add 200 nl antiserum (rabbit-anti-A249) prepared in PBST. Incubate 0.5 hr at 37°C. Drain antibody. Wash three times as previously. Add 200 nl/well of GARP. Incubate 0.5 hr at 37°C. Wash as previously. Add fresh preparation of enzyme substate (200 nl/well) and incubate in dark for 0.5 hr at 37°C. Add 25 nl of 8N H_2SO_4 to stop the reaction. Read optical density (492 nm) of individual wells using a multiple-channel photometer (Titertek Multiscan) (16).

Inhibition Curves

Whole cells of homologous and heterologous strains were prepared as previously, formalin-heat killed, and adjusted to a predetermined cell count using OD readings calibrated to the total cell count for each strain. A 2-fold dilution series in PBST was made for each strain and an equal volume of rabbit IgG-anti A249 in PBST was added to each tube and shaken immediately. Two hundred nl of each inhibition mixture were placed in each well of the precoated microplates. Tests were run in quadruplicate, placing paired samples on separate plates. Plates were incubated for 0.5 h at 37°C, and the protocol for ELISA was continued as previously described. Control wells contained (i) buffer alone, (ii) non-reacting serum (NRS).

Statistical Analysis

Absorbance values were converted to percent inhibitions of the binding reaction. Percent inhibition was plotted against the $\log_{10}$ of the number of cells added as competitive antigen. Regression coefficients were calculated, and the number of cells to achieve 50% inhibition of the homologous standard was extrapolated for each heterologous antigen. Statistical parameters, means, standard deviations, standard errors of the mean, slopes, confidence limits, and t-tests to compare slopes were calculated from ELISA data by the methods of Crow, et al. (7). Linear regression coefficients were calculated using all points in the linear portion of a plot of percent inhibition vs. $\log_{10}$ of the number of inhibitor cells added. Confidence limits were calculated by using a two-tailed t-test at the 0.05 probability level.

Results

Standardization of ELISA Procedure

Coating antigen, varying in concentration from 100 to 10^9 cells per ml, gave readable reactions only after the concentration reached 10^4 cells/ml or higher. At all antibody concentrations OD readings were greatest when the antigen was coated at 10^9 cells/ml. Coating efficiency reached a maximum when plates were left in a water bath (70°C) for 4 h. Whole live cells could also be air-dried on to plates overnight, at 24°C or 37°C. Antisera having agglutination titers of 640, 1280, and 2560 (expressed as reciprocal of dilution factor) showed optimum reactions in ELISA when diluted 300, 600, and 3600-fold, respectively. A standard binding curve was made for each serum. The concentration of GARP was selected at a dilution of 1:6000, because this value gave OD readings near 1.0 at optimum antigen-antibody concentration. Incubation times between additions of reactants (antibody, GARP, substrate) were established at 0.5 h at 37°C or 1 h at 24°C. Measurable differences between these methods did not occur, and the shorter incubation period was selected for convenience. Whole cells to be used as antigen for inhibition mixtures could be prepared in advance and stored in PBST for at least three weeks without loss of activity. Once the antibody is added, the inhibition mixtures must be dispensed into wells of precoated microplates within 2 to 4 h. If mixtures were refrigerated overnight a reversal of the reaction was observed as an upward shift in OD readings at high antigen concentrations.

Standardization of Inhibition Curves

A standard inhibition curve was drawn by plotting OD_{492} against the number of inhibitor cells in the reaction mixture. At low cell numbers, OD readings were near maximum for the binding reaction between antibody and its homologous coating antigen. As the amount of the competitive antigen increased, OD readings were reduced, reaching a minimum when inhibitor cell numbers were greater than 5×10^8 cells/ml. As an average of 14 inhibition curves made on different plates and assay dates, the binding reaction was inhibited 50% at an OD reading of 0.490 absorbance units, which was extrapolated to $5 \pm 2 \times 10^7$ cells/ml. Heterologous cabbage strain KC-3-12 and lettuce strain 10-TB-10 gave curves very similar to inhibition curves formed by the homologous inhibitor A249. White onion strain A255-4 showed little inhibition. Optical density values were converted to percent inhibition by the following equation:

$$\% \text{ inhibition} = \left[1 - \frac{OD_i}{OD_o} \right] \times 100$$

where OD_i is the optical density given by a mixture of antibody with a known number of inhibitor cells and OD_o is the point at which no inhibition of the binding reaction occurred (eg. the tube to which no competitive antigen was added). Regression coefficients for the 92 inhibition curves plotted for these samples average 0.968 (range 0.994-0.857). Slopes and confidence limits were calculated. These data showed that cabbage strains

A249 and KC-3-12 did not produce significantly different inhibition curves, while lettuce strain 10-TB-10 showed slightly flatter slopes and required each 5-fold more inhibitor cells for 50% inhibition of the binding reaction. Onion strain A255-4 showed a shallow slope and required 28-fold more inhibitor cells for 50% inhibition. Using the quantitative analysis of inhibition curves and the numbers of cells required for 50% inhibition, the relationships between the 23 strains of X. campestris pathovars could be determined.

Two cabbage strains KC-3-12 and OK_2 had values for slope (b) closely approximating the slope produced by the homologous inhibitor A249, and the numbers of cells required for 50% inhibition were not significantly different, indicating antigenic similarity between these strains. Other heterologous crucifer strains and lettuce strains produced inhibition curves showing less similarity with A249. Little inhibition was produced by onion strains, showing that these organisms shared few antigenic determinants with A249. Erwinia herbicola caused no inhibition, showing that this organism was serologically unrelated.

Discussion

Quantification of ELISA depends on the consistency with which a number of parameters in the ELISA protocol can be controlled (2, 4, 5, 14-24). Variability in the adsorption of antigen onto tubes and microplates and antigen density are important considerations (12, 13). A supra-optimal concentration of antigen may impose steric hindrance in the antigen-antibody reaction (17). Although coating with large numbers (1 x 10^8 cells/ml) of bacteria gave consistent results in our ELISA protocol, a reduction in density of coating antigen may reduce non-specific reactions and thus provide better data for determining relationships between heretologous strains. Optimal quantities of all reactants must be determined for each serological system (3, 6, 11) as well as the pH of coating buffer and substrate. When the prescribed ELISA protocol was strictly followed, inhibition curves were found to be highly reproducible and could be used as quantitative measures of antigenic similarity between closely related pathovars.

It was interesting to note that two cabbage strains, A249 and KC-3-12, were isolated from neighboring cabbage farms while isolates obtained from more distant locations were less closely related. These results are encouraging for the prospects of using ELISA as a tool in determining inoculum source.

Although lettuce and onion strains shared some antigenic determinants with crucifer strains, the ELISA data showed that they were not serologically identical. Onion strains in particular produced inhibition curves with significantly different slopes and required several-fold more inhibitor cells to produce 50% inhibition of the binding reaction. Since both the slope and the number of inhibitor cells must coincide for serological identity, the onion and cabbage strains examined here should be considered serologically different. This use and interpretation of inhibition curves may provide useful criteria on which to compare serological relatedness among strains of plant pathogens.

Literature Cited

1. Alvarez, A. M., I. W. Buddenhagen, E. S. Buddenhagen, and H. Y. Domen. 1978. Bacterial blight of onion, a new disease caused by *Xanthomonas* sp. Phytopathology 68:1132.
2. Avrameas, S. and B. Guilbert. 1972. Enzyme-immunoassay for the measurement of antigens using peroxidase conjugates. Biochimie 54:837.
3. Bartlett, A., D. E. Bidwell, and A. Voller. 1976. Practical methods for the detection of antigens by ELISA. Protides Biol. Fluids Colloq. 24:767.
4. Bidwell, D. E., A. Bartlett, and A. Voller. 1977. Enzyme immunoassay for viral diseases. J. Infect. Dis. 136 Suppl.:S274.
5. Bullock, S. L. and K. W. Walls. 1977. Evaluation of some of the parameters of the enzyme-linked immunospecific assay. J. Infect. Dis. 136 Suppl.:S279.
6. Carlsson, H. E. and A. A. Lindberg. 1978. Application of enzyme immunoassay for diagnosis of bacterial and mycotic infections. Scand. J. Immunol. 8, Suppl. 7:97.
7. Crow, E. L., F. A. Davis, and M. W. Maxfield. 1960. *Statistics Manual.* Dover Publications, New York. 288 pp.
8. Domen, H. Y.* and A. M. Alvarez. 1978. Detection of *Xanthomonas campestris* in soil using a direct immunofluorescent technique. Proc. 4th Int. Conf. Plant Bact. Angers, France. 301.
9. Dye, D. W. 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N. Z. J. Sci. 5:393.
10. Elrod, R. P. and A. C. Braun. 1947. Serological studies of the genus *Xanthomonas*. Cross-agglutination relationships. J. Bacteriol. 53:509.
11. Engvall, E. and P. Perlmann. 1972. Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labeled antiimmunoglobulin in antigen-coated tubes. J. Immunol. 109:129.
12. Kricka, L. J., T. J. N. Carter, S. M. Burt, J. H. Kennedy, R. L. Holder, M. L. Halliday, M. E. Telford, and G. B. Wisom. 1980. Variability on the adsorption properties of microtitre plates used as solid supports in enzyme immunoassay. Clin. Chem. 26:741.
13. Lehtonen, O. P. and M. K. Viljanen. 1980. Antigen density in ELISA: effect on avidity dependency. J. Immunol. Meth. 36:63.
14. Pesce, A. J., D. J. Ford, M. Gaizutis, and V. E. Pollak. 1977. Binding of protein to polystyrene in solid-phase immunoassay. Biochim. Biophys. Acta 492:399.
15. Pesce, A. J., D. J. Ford, and M. A. Gaizutis. 1978. Qualitative and quantitative aspects of immunoassay. Scand. J. Immunol. 8, Suppl. 7:1.
16. Ruitenberg, E. J., V. M. Sekhuis, and B. J. M. Brosi. 1980. Some characteristics of a new multiple-channel photometer for through-the-plate reading of microplates to be used in enzyme-linked immunosorbent assay. J. Clin. Micro. 11:132.
17. Salonen, E. M. and A. Vaheri. 1979. Immobilization of viral and mycoplasma antigens and of immunoglobulins on polystyrene surface for immunoassay. J. Immunol. Meth. 30:209.
18. Schaad, N. W. 1979. Serological identification of plant pathogenic bacteria. Ann. Rev. Phytopathol. 17:123.
19. Voller, A., A. Bartlett, D. E. Bidwell, M. F. Clark, and A. N. Adams. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33:165.
20. Voller, A., D. E. Bidwell, and A. Bartlett. 1976. Enzyme immunoassays in diagnostic medicine. Theory and practice. Bull. W.H.O. 53:55.

21. Voller, A., A. Bartlett, and D. E. Bidwell. 1978. Enzyme immunoassays with special reference to ELISA techniques. J. Clin. Path. 31:507.
22. Voller, A. 1980. Heterogeneous enzyme-immunoassays and their applications. In *Enzyme Immunoassay.* E. T. Maggio, Ed. CRC Press.
23. Wisdom, G. B. 1976. Enzyme-immunoassay, a review. Clin. Chem. 22:1243.
24. Yolken, R. H. and P. J. Stopa. 1980. Comparison of seven enzyme immunoassay systems for measurement of cytomegalovirus. J. Clin. Micro. 11:546.

Proteins and Plasmids of
Pseudomonas andropogonis

A. K. Vidaver, P. A. Lambrecht,
D. Muenchau, R. R. Carson,

University of Nebraska
Lincoln, NB, U.S.A.

Abstract

Plasmid, and protein profiles of ten *Pseudomonas andropogonis* strains, isolated from four hosts, were compared in electrophoretic gels. All strains contained two to four plasmids, ranging in mass from 30 to 145 megadaltons. The plasmid profiles could not be correlated with the host of origin. The protein patterns of all *P. andropogonis* strains were essentially indistinguishable. As a group, however, their patterns were clearly distinguishable from other non-fluorescent pseudomonads.

Introduction

The status of the nomenspecies *Pseudomonas andropogonis* (Smith 1911) Stapp 1928 and *P. stizolobii* (Wolf 1920) Stapp 1935 is ambiguous at present. The 1980 Approved Lists of Bacterial Names includes *P. andropogonis* but not *P. stizolobii*. The latter nomenspecies was considered synonymous with *P. andropogonis* by Goto and Starr (5). The nomenspecies *P. stizolobii*, however, is still being used (3). Both species were relegated to Addendum I in the latest edition of Bergey's Manual (2); this addendum lists nomenspecies which have been "incompletely described, but which appear to conform to the generic definition."

In an effort to resolve the question of taxonomic status, we examined strains of both nomenspecies in two different ways. This paper describes the analyses of plasmids and proteins in the nomenspecies *P. andropogonis* and *P. stizolobii*.

Materials and Methods

Bacterial Strains

The bacterial strains used in this study are shown in Table 1 and Figure 3.

Media and Cultural Conditions

The bacteria were maintained and grown as previously described (8). Bacteria were grown at 30°C for plasmid and protein extractions.

Table 1. *Pseudomonas andropogonis* strains analyzed.

Nomenspecies	Strain	Host	Source
P. andropogonis	NCPPB 934[a]	sorghum	Indiana, U.S.A.
P. andropogonis	GB209	sorghum	Nebraska, U.S.A.
P. andropogonis	NCPPB 933	corn	Indiana, U.S.A.
P. andropogonis	PI3195	corn	Iowa, U.S.A.
P. andropogonis	MCP	corn	Nebraska, U.S.A.
P. andropogonis	YP1	corn	Nebraska, U.S.A.
P. andropogonis	X1131A	corn	Iowa, U.S.A.;
P. stizolobii	NCPPB 1024	*Stizolobium deeringianum*	Rhodesia, Africa
P. stizolobii	NCPPB 1127	*Bougainvillea* sp.	Rhodesia, Africa

[a] NCPPB = National Collection of Plant Pathogenic Bacteria, England. The remaining strains were isolated by the authors.

Preparation of DNA for Agarose Gel Electrophoresis

The procedure of Gonzalez & Vidaver (4) was used with minor modifications to improve lysis of the cells. Cells (1×10^9 CFU/ml) were resuspended after low speed centrifugation in an equal volume of lysozyme (1 mg/ml), 25% sucrose (w/v) solution, made up in Tris-EDTA buffer (4). The suspension was shaken at 250 rpm on a rotary shaker for 1 hr at 37°C, centrifuged at low speed for 15 min at 4°C, and resuspended in TE buffer with predigested Pronase as before (4). This mixture was then shaken for 15 min at 37°C, Sarkosyl (Ciba-Geigy) was added as before (4), and the sample was incubated on a shaker for an additional 40 to 45 min at 37°C. The resulting solution generally was clear and viscous. After overnight storage at 4°C, 4 ml aliquots were sheared, denatured, and renatured as described previously (4).

Agarose Gel Electrophoresis

Ethanol-precipitated DNA was subjected to electrophoresis as previously described (4).

Polyacrylamide Gel Electrophoresis

Cell proteins were extracted and visualized as previously described (1), except that the more sensitive method of silver staining was used (7).

Results

Plasmid Analysis of Strains

All strains of both nomenspecies examined contained detectable plasmid (Fig. 1). The number of plasmids per strain was 2 to 4 and the mass ranged from 30 to 145 megadaltons (Table 2). Several strains appeared to have plasmid of common mass. The strains from *Stizolobium* and
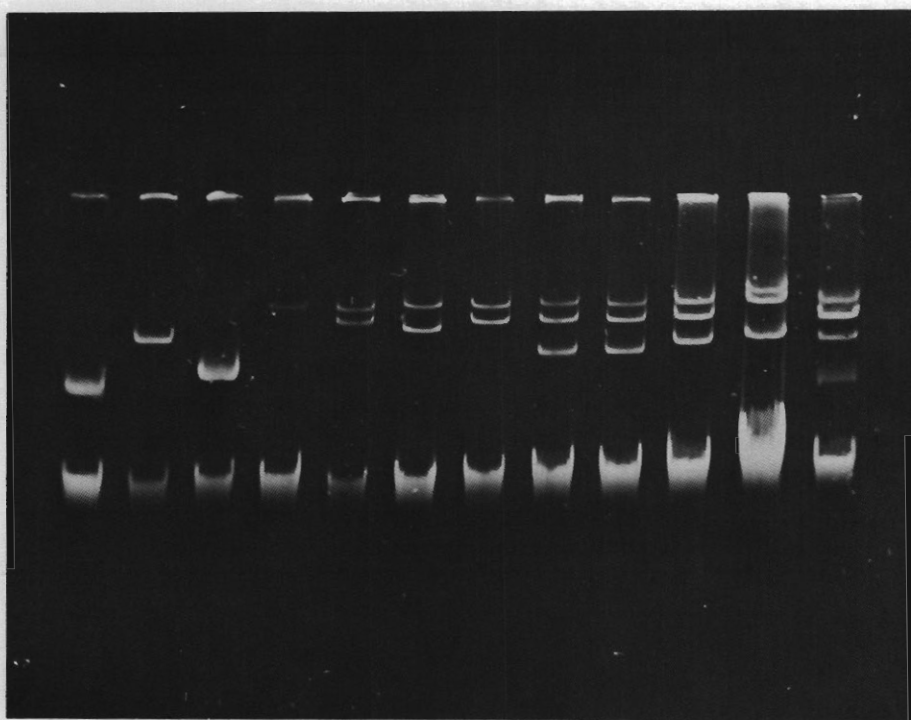
Fig. 1. Agarose gel electrophoresis of ethanol-precipitated plasmid DNA from cleared lysates of *Pseudomonas andropogonis* and reference strains. Lanes show (A) *P. syringae* HS 191 (pCG131); (B) *Escherichia coli* J53 (Rldrd 19); (C) *E. coli* J5 (RP1); (D) NCPPB 933; (E) YPI; (F) MCP; (G) X1131A; (H) NCPPB 934; (I) PI3195; (J) pI 101; (K) NCPPB 1024, and (L) NCPPB 1127. Chromosomal DNA migrated to the same position for all strains. Marker plasmids were 33 (pCG 131) 39 (RPI) and 62 (Rldrd 19) megadaltons, respectively.

Bougainvillea, however, contained the largest and smallest plasmids, respectively.

Polyacrylamide Gel Electrophoresis of Cellular Proteins

The patterns of the protein bands of the nomenspecies *P. andropogonis* and *P. stizolobii* were very similar (Fig. 2). The strains isolated from corn differed as much from each other as from the remaining strains of *P. andropogonis* and *P. stizolobii*. These variations were minor, particularly when *P. andropogonis* proteins were compared with the proteins of strains of other non-fluorescent pseudomonads (Fig. 3). *P. andropogonis* clearly differed from all other nomenspecies, including representatives of non-fluorescent phytopathogenic pseudomonads.

Discussion

The minor modification made in the lysis procedure enabled consistent detection of plasmids in the nomenspecies *P. andropogonis* and *P.*

Table 2. Plasmids of *Pseudomonas andropogonis*.

	Strain	Number of plasmids	Mass (megadaltons)[a]			
1.	NCPPB 933	2	76 ±3;	118 ±1		
2.	YP1	2	82 ±5;	107 ±3		
3.	MCP	2	74 ±3;	113 ±3		
4.	X1131A	2	90 ±5;	118 ±7		
5.	GB209	2	90 ±2;	125		
6.	NCPPB 934	3	51 ±1;	85 ±4;	113 ±4	
7.	PI3195	3	51 ±2;	85 ±4;	122 ±5	
8.	PI101	3	57 ±3;	85 ±4;	118 ±8	
9.	NCPPB 1024	3	64 ±8;	122 ±20;	145 ±10	
10.	NCPPB 1127	4	30 ±2;	61 ±5;	90 ±10;	118 ±8

[a] Results are from two to four independent experiments.
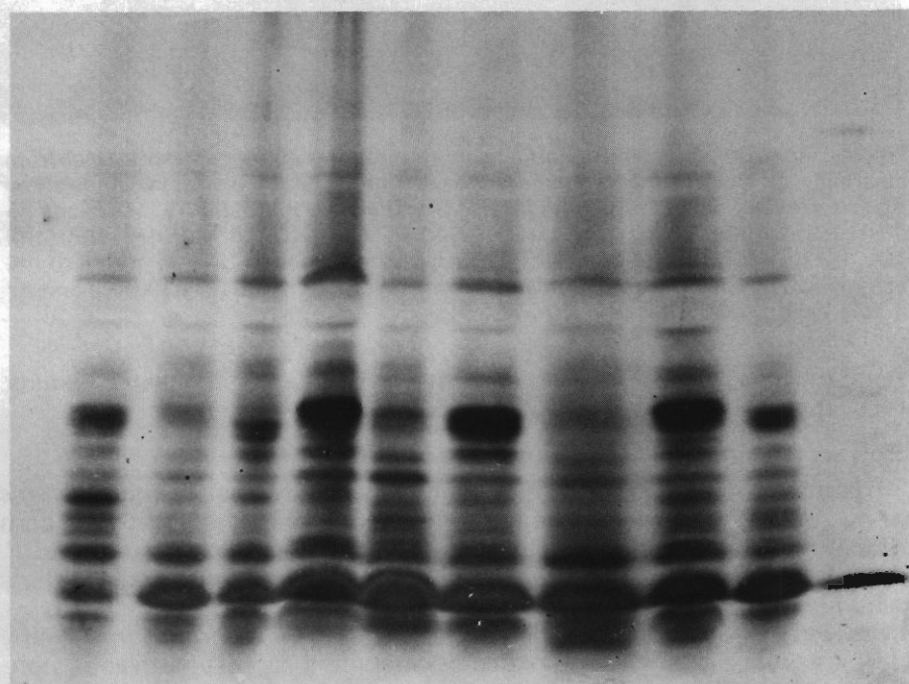
Fig. 2. Protein patterns of *Pseudomonas andropogonis* in a polyacrylamide slab gel. Samples were electrophoresed from top to bottom. Strains are: (A) PI3195; (B) PI101; (C) YPI; (D) MCP; (E) X1131A; (F) NCPPB 933; (G) NCPPB 934; (H) NCPPB 1127; (I) NCPPB 1024. Lane (J) contains standards, from top to bottom, bovine serum, albumin (MW 66,300), carbonic anhydrase B (MW 28,000), and lysozyme (MW 14,300).

132
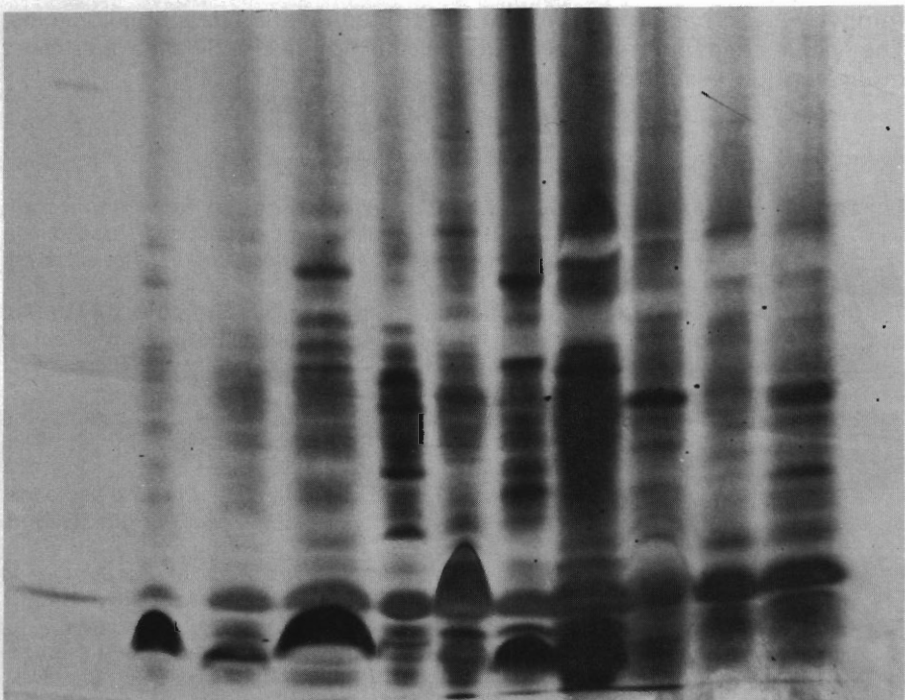
Fig. 3. Protein patterns of *Pseudomonas andropogonis* and other non-fluorescent pseudomonads. Samples were electrophoresed as in Fig. 2 with standards in lane (A). Strains are (B) *P. mendocina* ATCC17588; (C) *P. alcaligenes* ATCC14909; (D) *P. pseudoalcaligenes* ATCC17440; (E) *P. cepacia* ATCC25416; (F) *P. testeroni* ATCC11996; (G) *P. acidovorans* ATCC15668; (H) *P. delafieldii* ATCC17505; (I) *P. solanacearum* K60; (J) *P. andropogonis* NCPPB 934; and (K) *P. caryphylli* ATCC25418.

stizolobii. Other methods, for example, that of Gonzalez and Vidaver (4), either did not lyse the cells or gave irreproducible results.

All strains of the nomenspecies *P. andropogonis* and *P. stizolobii* contained 2, 3, or 4 plasmids, some of which appeared to be of common mass. However, no single plasmid of common mass was found in all corn and sorghum strains. Thus, it would appear that no particular plasmid determines host specificity for these strains; the two remaining strains from dicotyledonous hosts, may be different. Nevertheless, plasmids may determine host range, as in *Agrobacterium* (6).

All strains of both nomenspecies showed a sheathed flagellum (Gonzalez and Vidaver, unpublished results). Such flagella are absent from the other nonfluorescent pseudomonads.

The powerful tool of protein analysis by polyacrylamide gel electrophoresis (1) independently supports the conclusion of Goto and Starr (5) that *P. andropogonis* and *P. stizolobii* are a single species. *P. andropogonis* is recognized as having priority (5). Based on the work of Goto and Starr (5) and this study, we recommend that the next edition of Bergey's Manual

133

remove *P. andropogonis* from the list of incompletely described species. The species appears relatively homogenous and is clearly separable from other nonfluorescent pseudomonads.

Literature Cited

1.   Carlson, R.R., and A. K. Vidaver. 1981. *Taxonomy of Corynebacterium* plant pathogens, including a new pathogen of wheat, based on polyacrylamide gel electrophoresis of cellular proteins. In. J. Syst. Bacteriol. (In press).
2.   Doudoroff, M., and N. J. Palleroni. 1974. Genus 1. *Pseudomonas*, p 217-243. *In* R. E. Buchanan and N. E. Gibbons (eds.), Bergey's Manual of Determinative Bacteriology, 8th ed., Williams and Wilkins Co., Baltimore, Md.
3.   Fuerst, J. A., and A. C. Hayward. 1980. The effect of temperature on the formation of sheathed flagella by *Pseudomonas stizolobii*. J. Gen. Microbiol. 117:111-117.
4.   Gonzalez, C.F., and A. K. Vidaver. 1979. Bacteriocin, plasmid and pectolytic diversity in *Pseudomonas cepacia* of clinical and plant origin. J. Gen. Microbiol. 110:161-170.
5.   Goto, M., and N. P. Starr. 1971. A comparative study of *Pseudomonas andropogonis*, *P. stizolobii* and *P. albo-precipitans*. Ann. Phytopathol. Soc. Jpn. 37:233-241.
6.   Loper, J. E., and C. I. Kado. 1979. Host range conferred by the virulence-specifying plasmid of *Agrobacterium tumefaciens*. J. Bacteriol. 139:591-596.
7.   Merril, C. R., D. Goldman, S. A. Sedman, and M.H. Ebert. 1981. Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins. Science 211:1437-1438.
8.   Vidaver, A.K. and R.R. Carlson, 1978. Leaf spot of field corn caused by *Pseudomonas andropogonis*. Pl. Dis. Reptr. 62:213-216.

Abstracts

SEROLOGICAL CROSS-REACTION WITH *Corynebacterium sepedonicum* **ANTISERA.** De Boer, S.H. Agriculture Canada, Research Station, Vancouver, B.C., Canada.

Antisera were produced in New Zealand white rabbits against glutaraldehyde-fixed cells of *Corynebacterium sepedonicum,* the incitant of bacterial ring rot of potato. The IgG fractions of antisera were purified by column chromatography of Sephacryl S300 and used for indirect immunofluorescence staining. Whole antiserum was used for immunodiffusion tests. *Corynebacterium sepedonicum* cells could be detected by immunofluorescence in potato stems and tubers infected with the bacterial ring rot disease. However, cross-reacting bacteria were also found to be associated with diseased and healthy potato plants. Gram-positive, gramnegative, and gram-variable cross-reacting bacteria were isolated from ostensibly healthy potato stems. These strains were morphologically different from *C. sepedonicum* and were not pathogenic on eggplant. *Corynebacterium insidiosum* and *C. michiganense* also cross-reacted with *C. sepedonicum* antiserum but other plant pathogenic *Corynebacterium* spp., except *C. fasciens* which gave a very weak reaction, did not cross-react in immunofluorescence tests. *Corynebacterium insidiosum, C. michiganense* and some of the cross-reacting strains from potato also cross-reacted with *C. sepedonicum* antisera in agar double diffusion. Specific activity (immunofluorescence titer/mg protein) of IgG antisera fractions was determined with *C. sepedonicum, C. insidiosum, C. michiganense,* and an undetermined coryneform bacteria isolated from a potato stem. Specific activity with *C. sepedonicum* was greater than with cross-reacting bacteria in antisera obtained up to 12 weeks after beginning immunization, but was equal to specific activity of cross-reacting bacteria in antisera obtained 20 weeks after beginning immunization. An antigenic fraction was extrated from acetone-dried *C. sepedonicum* cells with hot phenol and antigenic activity was detected in culture filtrates. The culture filtrate, but not the phenol extract, sensitized shepp erythrocytes in indirect hemagglutination. Both the extract and culture filtrate produced precipitin bands in agar double diffusion. Hot phenol extracts and culture filtrates of *C. insidioeum* and *C. michiganense* also produced precipitin bands with *C. sepedonicum* antiserum in agar double diffusion.

(Abstract provided but paper was not presented at the Conference.)

SEMI-SELECTIVE MEDIA FOR DETECTION AND MONITORING OF SOME *Xanthomonas campestris* **PATHOVARS.** B. N. Dhanvantari, Agriculture Canada, Research Station, Harrow, Ontario, Canada NOR 1GO.

A soluble starch medium (DSX) of the following composition developed opaqueness when stored at 5°C for a minimum of 9 days, thus aiding the visualization of starch hydrolysis by *Xanthomonas campestris* pvs.

campestris and *phaseoli:* soluble starch, 10.0 g; yeast extract, 5.0 g; $NH_4 H_2 PO_4$, 0.5 g; $MgSO_4$. $7H_2 O$, 0.2 g; NaCl, 5.0 g; agar, 15 g; distilled water to make up to a litre; pH adjusted to 6.8; and pimaricin, 50 mg added after autoclaving. The bacterial colonies developed yellow pigmentation characteristic of the xanthomonads, and starch hydrolysis was coincident with the appearance of the colonies in 3 to 5 days. The medium permitted the production of diffusible fuscous pigment by the *fuscans* strains of the pv. *phaseoli*. The antibiotics nalidixic acid (up to 64 ng/ml), penicillin (up to 101 international units), and vancomycin (up to 64 ng/ml) were tolerated by the strains of pvs. *campestris* and *phaseoli* tested. The DSX medium alone, or supplemented by one or more of these antibiotics was selective enough for detection and monitoring of these bacteria, obviating the necessity for addition of dyes or iodine used in other media for this purpose.

The DSX medium is not applicable to *X. pruni* whose capacity to hydrolyze starch is poor. Because of its ability to utilize cellobiose, the following medium (XP) was found suitable for its isolation and autecological studies: cellobiose, 3.0 g; $NH_4 H_2 PO_4$, 0.5 g; $K_2 HPO_4$, 0.5 g; $MgSO_4$. $7H_2 O$, 0.2 g; NaCl, 5.0 g; sodium taurocholate, 2.5 g; Tergitol-7 (anionic), 0.1 ml; distilled water to make up to a litre; pH adjusted to 7.2; nicotinic acid (10 mg) and pimaricin (50 mg) added after autoclaving. The colonies of *X. pruni* appeared in 4 to 5 days on this on this medium and were off-white to cream-colored.

(Paper presented at the Conference; manuscript not received in time to publish.)

A NUMERICAL TAXONOMIC STUDY OF THE GENUS *Erwinia.* Dye, D. W. Plant Diseases Division, D.S.I.R., Auckland, New Zealand.

Using biochemical and physiological characters of 206 *Erwinia* cultures representing 25 nomenspecies, four numerical analyses have been made and dendograms prepared. These dendograms are presented as the basis for proposals concerning the nomenclature of the genus *Erwinia*. None of the analyses support a clear break into the groups previously known as the "amylovora," "herbicola," and "atypical *Erwinia*" groups. However, the synonymy of a number of nomenspecies is confirmed and other relationships are indicated. On the information available it is proposed that the 25 nomenspecies are best classified as 13 species with a number of pathovars.

(Abstract provided but paper was not presented at the Conference.)

RICKETTSIA-LIKE ORGANISM (RLO) FROM PLANTS. Misra, A. Botany Dept., Mithila University, Darbhanga, India.

It is now well known that several "virus" diseases of plants with antibiotic sensitivity and yellow types of symptoms belong to the mycoplasmal etiology. In addition to those RLOs, we also know that Rickettsia are also involved in certain important diseases of plants. The relationship between bacteria, mycoplasma, Rickettsia, and the related organisms will be discussed. The identification of RLOs and the problems of their taxonomy and identification as well as nomenclature will be commented upon. Better understanding of the taxonomy of mollicutes,

especially Rickettsia should help us to plan a better strategy of control of these diseases. Proposals for the creation of an International Committee for the Taxonomy of Rickettsia and Mycoplasma have been made, and cooperation from animal workers has been sought.

(Abstract provided but paper was not presented at the Conference.)

Session III

Ecology of Bacterial Pathogens
Introductory Remarks

Leopold Fucikovsky, Chairman

Ecology of Bacterial Pathogens

Introductory remarks by

Leopold Fucikovsky
Chairman, Session III

This session deals with epidemiology, dispersal, and survival of plant pathogenic bacteria. Emphasis is on survival of these bacteria, not only on the aerial surfaces or internal parts of vegetatively propagative material or botanical seeds, but also in the rhizosphere of cultivated plants or weeds or in soil.

In the past, various selective media or other sensitive methods have been used to detect or monitor the bacterial population, especially on the surfaces of plants. These methods are being refined every day and we shall hear of some new advances in this field. Other aspects, such as symptomless plants with a potential of maintaining low populations of bacteria for prolonged periods may be of great importance to the future of a healthy agriculture.

It is well known that various ambient factors such as rain, humidity, temperature, and microflora as well as age, nutrition of the plant tissues, and other factors, may have a profound influence on the survival of bacteria on or near susceptible host plants. We will hear a great deal about these factors as they influence populations of different pathogenic bacteria.

Some of the most recent advances in ecology of plant pathogenic bacteria have been with *Pseudomonas tomato* (5) where a field temperature between 13 and 28°C and a high relative humidity and free water on the leaves have been directly correlated with the bacterial speck of tomato. Yield losses were as high as 75% in plants infected at an early stage.

Another recent and important development has been the demonstration by sensitive methods used by Elango and Lozano (1) that *Xanthomonas manihotis* can be detected in the embryo of the botanical seed of cassava, also in pollen, and later transmitted to new plants. The seed transmission could be as high as 40%.

Other interesting advances on ecology of bacterial pathogenesis were made by Surico *et al.* (4). They found that *Pseudomonas syringae* pv. *glycinea* could be areosolized and could add significantly at relatively low temperatures to the epidemic potential of the pathogen on soybeans by establishing itself in the field during the early part of the growing season.

An aspect of bacterial motility of *Erwinia amylovora* has been studied recently by Raymundo and Ries (2), showing that environmental factors such as temperature, chelating agents, pH, energy sources, and oxygen, affect motility and flagellation of these bacteria. Low temperature favors flagella growth; addition of chelating agents enhances motility. Cells were actively motile between pH 6 and 8. Also, certain energy sources like mannitol and glucose stimulate motility. Oxygen stimulates motility of this bacterium, too. Inside the host tissue, *E. amylovora* was not motile.

It is interesting that *Corynebacterium fascians* could be transmitted by tobacco seeds, as we shall hear in this session. Our own work on *Corynebacterium fascians* and Shasta daisy showed that the bacteria could be transmitted by mollusks feeding on these plants (3).

Reflecting now on research for the future, I would say from some of the cited examples that more work will be needed on population studies of other plant pathogenic bacteria, and on environmental factors that affect their motility inside and outside of the plants. Factors and characteristics such as the ones mentioned could be of great importance for the survival of the bacteria in nature and, consequently, the adaptation of the best control measures in the future.

Literature Cited

1. Elango, F., and J. C. Lozano. 1980. Transmission of *Xanthomonas manihotis* in seed of cassava (*Manihot esculenta*). Plant Disease 64:784-786.
2. Raymondo, A. K., and S. M. Ries. 1980. Motility of *Erwinia amylovora*. Phytopathology 70:1062-1065.
3. Rodriguez, M. E. and L. Fucikovsky. 1979. *Corynebacterium fascians* (Tilf.) Dowson, agente causal de la fasciación del margaritón (*Chrysanthemum maximum* Ram.) Agrociencia 43:73-78.
4. Surico, G., B. S. Kennedy, and G. L. Ercolani. 1981. Multiplication of *Pseudomonas syringae* pv. *glycinea* on soybean primary leaves exposed to aerosolized inoculum. Phytopathology 71:532-536.
5. Yunis, H., Y. Bashan, Y. Okon, and Y. Henis. 1980. Weather dependence, yield losses and control of bacterial speck of tomato caused by *Pseudomonas tomato*. Plant Disease 64:937-939.

Incidence, Perpetuation, and Control of Bacterial Blight of Guar

S. S. Karwasra, J. N. Chand, G. S. Saharan

Haryana Agricultural University
Hissar, India

Abstract

The incidence of *Bacterial blight* of guar increased with the increase in amount of rainfall and number of rainy days. Carryover of the pathogen on the seed plays an important role in the perpetuation of the pathogen from one season to another. The pathogen can be eliminated from the seed by a hot water treatment of 10 min at 56°C.

Introduction

Guar [*Cyamopsis tetragonoloba* (L) Taub.] is a well established crop for forage, vegetable, and green manuring in India. This crop has also assumed industrial importance recently, because of gum content in its seeds. The production of guar has been limited by bacterial blight disease caused by *Xanthomonas cyamopsidis.* This disease was first reported from India in the 1950s (1, 2). It causes blighting of leaves, resulting in premature defoliation. In severe cases, blackening and cracking of stem occurs, resulting in the death of well established plants. During the last few years the disease has been causing considerable damage to the crop. After taking into consideration the severity of the disease, and the influence of weather on its incidence and mode of perpetuation, a control by hot water treatment was worked out.

Materials and Methods

Effect of Meteorological Factors on Incidence of Bacterial Blight

Two varieties of Guar, FS 277 and Guar No. 2, were used to study effects of meteorological factors on the incidence and perpetuation of *Xanthomonas cyamopsidis.* These varieties were sown in a randomized block design on 3 m x 2 m plots on which guar had been grown the preceding year. The sowing was done on four different dates (Table 1). Observations were recorded three months after the date of sowing. Infected plants were graded as follows: (0) healthy; (1) very few lower

143

leaves showing the blight symptoms, stem healthy; (2) 20% of leaves in the lower half showing blight symptoms, stem healthy; (3) 50% of leaves blighted, stem showing some streaks near the base; and (4) more than 50% leaves blighted, stems cracking and blackening.

Disease index was calculated as:

$$\frac{\text{No. of plants affected}}{\text{Total No. of plants observed}} \times \frac{\text{Respective grade}}{\text{Maximum numerical value (4)}} \times 100$$

Role of Seed and Plant Debris in Perpetuation of Bacterium

For this study, seeds and plant debris from heavily infected plants of varieties FS 277 and Guar No. 2 were collected and incubated at room temperature. Monthly isolations were made from this material to check for presence of the pathogen on nutrient dextrose agar medium, according to standard isolation technique.

Elimination of Seed Borne Pathogen Through Hot Water Treatment

Hot water treatment was given as suggested by Verma *et al.* (7) in cotton. Seeds of variety FS 277 were divided into 20 g lots and kept in muslin cloth bags tied with a thread. The lots were exposed to desired temperatures and time in a hot water bath. Later the lots were removed in water at room temperature and sown directly in 3 m x 2 m field plots. The germination percentage was recorded after 10 days. Elimination of infection was confirmed through observations taken on cotyledonary leaves of seedlings.

Results and Discussion

Influence of Meteorological Factors

Different environmental factors influencing the average infection index of bacterial blight of crops sown on different dates are given in Table I. It is evident that there was no significant difference in temperature and humidity during the growth period of the crop. However, amount of rainfall and number of rainy days differed significantly from 1st of June to 1st, 10th and 20th of July. The maximum average disease index was recorded in cultivars FS 277 (33.5%) and Guar No. 2 (29.5%), when the crop was planted on 21st of June. The minimum disease index (11.1 and 10.3%) was recorded on the 20 th of July planting. It appears that the high disease indexes in the crops sown on June 21 and July 1 were due to higher precipitation and greater number of rainy days. The July 10th and 20th plantings encountered less rainfall and fewer rainy days. It is well known that splashing rains favor secondary spread of the disease (3, 4, 6) caused by bacterial pathogens.

Role of Seed and Plant Debris in Perpetuation of the Bacterium

It is evident from Table 2 that the seed played an important role in perpetuation of the bacterium. The bacterium could be isolated up to 12 months after harvest from the infected seeds. However, plant debris yielded the bacterium only for two months after harvest. These results agree with those of Srivastava and Rao (5).

Table 1. Meteorological factors affecting average disease index.

Date of sowing	Average disease index		Max. Temp. °C	Mini. Temp. °C	Rainfall (mm)	Humidity (o/o)	No. of rainy days
	FS 277	Guar No. 2					
21st, June	33.50	29.51	34.1	25.9	199.9	85.2	42
1st, July	29.87	28.90	33.4	23.0	160.9	87.3	36
10th, July	11.20	10.50	33.1	25.4	117.2	88.9	30
20th, July	11.10	10.30	33.1	26.5	40.2	83.4	27

Table 2. Role of seed and plant debris in perpetuation of bacterium.

Date of Isolation	Infected Guar Seed		Plant Debris	
	FS 277	Guar No. 2	FS 277	Guar No. 2
13/11/75	+	+	+	+
13/12/75	+	+	+	+
13/ 1/76	+	+	–	–
13/ 2/76	+	+	–	–
13/ 3/76	+	+	–	–
13/ 4/76	+	+	–	–
13/ 5/76	+	+	–	–
13/ 6/76	+	+	–	–
13/ 7/76	+	+	–	–
13/ 8/76	+	+	–	–
13/ 9/76	+	+	–	–
13/10/76	+	+	–	–

+ Indicates the presence of bacterium
– Indicates the absence of bacterium

Hot Water Treatment for Elimination of Seed Borne Inoculum

The inoculum was eliminated from seed by giving it a hot water treatment for 10 min at 56°C. The other treatments proved ineffective for the elimination of bacterium from the seeds. There was a 13% reduction in seed germination in comparison with the control, but the pathogen was eliminated (Table 3). Srivastava and Rao (6) also found hot water treatment effective for elimination of the pathogen.

Table 3. Hot water treatment for the elimination of seed borne inoculum.

Temperature °C	Time (Min.)	Germination (o/o)	Cotyledonary infection
50	10	70	+
52	10	68	+
54	10	64	+
56	10	62	–
Control	–	75	+

+ : Infection
– : No infection.

Literature Cited

1. Patel, A. J. and M. K. Patel. 1958. A new bacterial blight of guar, *Cyamopsis tetragonoloba* (L.) Taub. Curr. Sci. 27:258-259.
2. Patel, M. K., G. W. Dhande, and Y. S. Kulkarni. 1953. Bacterial leaf spot on *Cyamopsis tetragonoloba* (L.) Taub. Curr. Sci. 22:183.
3. Shekhawat, G. S., and D. N. Srivastava. 1972. Mode of seed infection and transmission of bacterial leaf streak in rice Ann. Phytopath. Soc. Japan 38:4-6.
4. Shekhawat, G. S. and D. N. Srivastava. 1972. Epidemiology of bacterial streak of rice. Ann. Phytopath. Soc. Japan 38:7-14.
5. Srivastava, D.N. and Y. F. Rao. Bacterial blight of guar. Indian Phytopath. 16:69-75.
6. Srivastava, D. N. and Y. P. Rao. 1970. Epidemiology and control of bacterial blight of guar (*Cyamopsis tetragonoloba* (1.) Taub. Bull. of Ind. Phytopath. Society. No. 6:1-4.
7. Verma, J. P., R. P. Singh, and M. L. Nayak. 1974. Bacterial blight in cotton and steps for its eradication. Cotton Development 4:23-27.

Pseudomonas solanacearum
Distribution in Potato Plants;
Establishment of Latent
Infections

L. Ciampi-Panno
L. Sequeira
E. R. French

Universidad Austral de Chile, Valdivia, Chile
University of Wisconsin-Madison, U.S.A. and
International Potato Center, Lima, Peru, cooperating

Abstract

When 1-mo-old plants of a wilt-resistant clone of *Solanum phureja* (1386.15) were stem-inoculated with three strains of *Pseudomonas solanacearum* (K60, S123, and S206), the bacteria multiplied rapidly at the point of inoculation and then moved in the vascular system to other parts of the stem. Resistant plants showed a remarkable ability to support relatively high populations of the bacterium in the absence of disease symptoms. Although multiplication in this resistant clone was substantially less than in susceptible Russet Burbank potato plants, large numbers of bacteria (up to 624×10^4 cells of K60 per 5 - cm stem segment) reached the base of the stem of plants maintained at high temperature ($28°C$) for 20 days after stem inoculation. From the base of the stem, the bacteria moved rapidly into the roots and tubers. Strains of *P. solanacearum* differed in their ability to cause latent tuber infection in different resistant potato clones. When *S. phureja* x *S. tuberosum* hybrids were stem-inoculated, maintained at $28°C$ for 3 weeks, and then grown to maturity at $20°C$, most of the clones yielded tubers infected by one or more strains. The race 1 strain (K60) was the most infectious; 53.8% of all tubers harvested from all plants inoculated with this isolate carried latent infections. Because one clone (BR 53.1) never yielded infected tubers, there appear to be genetic factors which may be useful in breeding programs aimed at eliminating latent tuber infection. Some infected clones did not yield diseased tubers, however. The development of symptoms above ground was not correlated with the incidence of tuber infection in any particular clone. No tuber infection occurred in tolerant or resistant clones grown in infested soil at cool ($12-22°C$) temperatures.

Tubers were inoculated directly in an attempt to evaluate the ability of bacteria to multiply in these tissues at different temperatures. Highly virulent strains of *P. solanacearum* survived in susceptible tubers in higher numbers and for longer periods than in resistant ones. Low temperature ($4°C$) had a deleterious effect on survival of the bacterium in tubers, but did not completely eliminate the pathogen even after 40 days.

Introduction

There are numerous reports that *Pseudomonas solanacearum* E. F. Smith, the agent of bacterial wilt of potatoes, persists in infected tubers in the interim between harvest and subsequent planting (see review by Kelman, 1953). Many tubers remain symptomless, particularly when susceptible potato clones are infected late in their growth cycle. The problem of latent infection of potato tubers is particularly severe in developing countries, where certified seed is not readily available.

Infected tubers are important sheltered sites for long-term survival of *P. solanacearum* in the field; approximately 10% of tubers infected with race 3 strains of the pathogen, when grown, in noninfested soil, give rise to diseased plants (Graham *et al.*, 1979). Furthermore, when seed potato tubers from an infested field were assessed for infection by *P. solanacearum*, both visually and by direct isolation, the visual method failed to detect two-thirds of the infected tubers after 6 weeks of storage at 28°C (González, 1977). As resistant cultivars become more widely used, movement of virulent strains may be a significant problem because these cultivars may remain symptomless after inoculation with virulent strains of the pathogen (Sequeira and Rowe, 1969), and thus would not be rogued out in the field or culled out after the tubers are harvested.

Latent infection is also an important problem in breeding programs in which progenies are screened at the seedling stage by root inoculation (González *et al.*, 1973). The tubers that are produced months later by the resistant plants may carry the bacterium (Ciampi *et al.*, 1980).

Bacterial cells reach potato tubers via the vascular system after initial multiplication in roots or stems. Although there is a considerable amount of information on the multiplication and movement of *P. solanacearum* in susceptible potato (Grieve, 1943), tomato (Grieve, 1943; Wallis and Truter, 1978), and tobacco (Copeman, 1969) plants; there is no information as to the rate of movement, or the populations of the bacterium that can occur in resistant potato clones. It is known that resistance originating from *S. phureja* breaks down under high ambient temperature (Sequiera *et al.*, 1969). It seemed likely, therefore, that infected plants that remained symptomless at low ambient temperatures might carry substantial numbers of bacteria and that these might reach the tubers. Thus, the objectives of these investigations were to determine: a) how movement of *P. solanacearum* within resistant potato plants might lead to latent infection of the tubers; b) how varieties of the host and strains of the pathogen influence the phenomenon of latent carryover of bacteria, and c) how readily resistant potato clones can be infected when grown in infected soils and if such plants can yield latently infected tubers.

Materials and Methods

Strains of *P. solanacearum* used in this work were obtained from the culture collection maintained in the Department of Plant Pathology, University of Wisconsin-Madison. Strain K60 (race 1, biovar I) was isolated from tomato in Wake Co., NC, strain S123 (race 1, biovar III) was isolated

from *Eupatorium odoratum* in Coto, Costa Rica, and strain S206 (race 3, biovar II) was isolated from potato in Las Palmas (Medellín), Colombia. Strains CIP 077, 108, 141, and 142 were obtained from the culture collection maintained at the International Potato Center, Lima, Perú; all isolated from potato (Yurimaguas, Perú). Stock cultures were maintained in distilled water at room temperature; to obtain fresh cultures, stock suspensions were streaked on Kelman's tetrazolium chloride agar (TZC) (Kelman, 1954). Cultures were grown for 48 h at 30°C.

The resistant clone of *S. phureja* (1386.15) had been selected previously in our laboratories from open-pollinated seed supplied by H. D. Thurston; resistant hybrids of *S. phureja* x *S. tuberosum* were obtained from the Potato Introduction Station, Sturgeon Bay, Wisconsin, U.S.A. At the prebud stage, when plants were 6 to 8 weeks old, they were stem-inoculated (19) by placing a drop of inoculum containing approximately 10^9 cells/ml on the axil of the third fully expanded leaf from the top and thrusting a needle through the drop and into the stem. After inoculation, plants were transferred to a greenhouse maintained at 28°C ± 2°C.

Another group of wilt-resistant potato clones (*S. phureja* x *S. tuberosum* hybrids) were provided by Dr. Robert Hanneman, Potato Introduction Station, Sturgeon Bay, or by Dr. Carlos Martin, Pathology Department, International Potato Center, Lima, Perú. Washed tubers were planted singly in 20-cm clay pots containing a mixture of sterilized soil, peat moss, and Jiffy Mix (Jiffy Products of America, West Chicago, IL 60185). Plants were grown in the greenhouse at 20 to 24°C with and without supplemental lighting. Lighting was provided by Sylvania "Gro-Lux" and General Electric "Cool White" fluorescent tubes. The plants were inoculated 60 days after planting by adding to the soil in each pot 100 ml of a bacterial suspension containing approximately 1.5 x 10^7 cells/ml, as determined by measuring OD600nm. After inoculation, the plants were grown under cool (range: 12-22°C) or warm (range: 24-28°C) greenhouse conditions, without supplemental lighting.

To isolate bacteria from stem-inoculated plants, the stem was divided into four segments, each 5 cm in length. The central section (b) included the site of inoculation, which was in the middle of the segment, and the other two segments were immediately above (a) and below (c) segement b. The fourth segment (d) was the basal 5 cm of the stem. Each stem piece was surface-sterilized with 70% ethanol (3 min) and 1% sodium hypochlorite (3 min), and then was rinsed twice in sterile distilled water. Each stem piece was ground with sterile mortar and pestle in 10 ml of sterile water. Samples (0.1 ml) from log_{10} dilutions were spread on plates of TZC. Two or four samples were taken from each of two or four plants at each incubation period; two or four plates per dilution were used, depending on the numbers of samples that could be handled conveniently at any particular time. All bacterial colony counts were made after 48 h incubation at 28°C.

In the soil infestation experiment the number of healthy appearing or diseased (bacterial ooze present at the eyes) tubers was recorded at harvest (approximately 110 days after planting). Isolations were attempted, bacteria were isolated from stems and/or tubers of healthy-appearing plants. For isolation from stems, a 5-cm section was surface-sterilized with

70% ethanol followed by 1% sodium hypochlorite, and then rinsed twice with sterile water. Each piece was ground with sterile mortar and pestle in 10 ml of sterile water and a loopful was spread on a plate of TZC. Two to four samples from each suspension were assayed; plates were incubated at 30°C for 48 h and samples were considered positive when one or more typical colonies of *P. solanacearum* were present on any of the assay plates.

To assay for possible latent infection, potato tubers were surface-sterilized with 1% sodium hypochlorite (3 min), rinsed in sterile distilled water (30 sec), dipped in 70% ethanol, and then flamed. Then each tuber was cut into small slices (approximately 2 x 1 x 0.5 cm); eight slices were selected at random and two groups of four were each placed in a test tube containing CPG medium (casamino acids 1.0, peptone 10 g, and glucose 5.0 g liter) (Kelman, 1954). The slices were incubated at 28°C for 24 h and then two loopfuls of the medium were each streaked on a plate of TZC. After 48 h incubation at 28°C the presence or absence of *P. solanacearum* was determined on the basis of colony morphology (Granada and Sequeira, 1975).

To determine rates of multiplication of *P. solanacearum* in potato tubers, a direct injection method, similar to that described by De Boer and Kelman (1978), was used. Tubers were surface-sterilized, as indicated above, and 10 nl of bacterial suspension, containing approximately 10^9 cells/ml, was injected into each tuber with a microliter syringe (Hamilton No. 705). The needle was inserted 2 cm deep and the suspension was delivered as the needle was withdrawn; two inoculations per tuber were made. Circles were drawn with indelible ink around the inoculation points. At various intervals, a cylinder of tissue (2.5 cm long) containing the inoculation site was removed with a sterile No. 4 cork and then ground with sterile mortar and pestle in 10 ml of distilled water. Log_{10} dilutions were prepared and 0.1 ml samples were spread on a plate of TZC and the number of typical *P. solanacearum* colonies was determined after 48 h incubation at 30°C.

Results

Influence of Temperature on Tuber Infection

To determine whether temperature influenced the amount of tuber infection, the cultivars Mariva (tolerant) and Molinera (resistant) were grown under warm (24-28°C) or cool (12-22°C) conditions in soil infested by each of four strains (two of race 1, two of race 3) of *P. solanacearum*. All tubers from 10 plants of each clone were harvested and classified (visually) as infected or apparently healthy; percentage infection data were transformed to degrees and significant differences were determined by the analysis of variance method.

Potato cultivars grown under warm conditions had different amounts of tuber infection (Table 1), but those grown under cool conditions yielded no apparently infected tubers. Under warm conditions, cultivar Mariva had more infected tubers when grown in soil infested with the race 1 strains than in soil infested with race 3 strains. The reverse was true for cultivar Molinera. However, the cultivars did not differ significantly in their response to different strains or races of the pathogen.

Table 1. Incidence of stem and tuber infection in the potato cultivars Mariva and Molinera grown at 24 to 28° C in soil infested by *Pseudomonas solanacearum*.

Strain	Race	Cultivar Mariva[a]			Cultivar Molinera[a]		
		Total tubers	Tubers Infected[b,c] (%)	Stems Infected (%)	Total Infected Tubers	Tubers Infected[b,c] (%)	Stems Infected (%)
CIP 077	1	92	53.3 a	90	68	39.7 a	90
CIP 141	1	106	60.4 a	100	67	28.3 b	90
CIP 108	3	60	36.3 b	100	95	53.7	100
CIP 142	3	96	45.8 ab	100	65	41.5 a	90

[a] Ten plants of each cultivar per strain. The soil around the roots was infested when plants were 60 days old; tubers were harvested and stem sections were assayed 110 days after planting.

[b] Tubers checked visually for rotting.

[c] Percentage values followed by the same letter are not significantly different at P = 0.05.

Table 2. Presence or absence of *Pseudomonas solanacearum* in apparently healthy tubers harvested from two potato cultivars grown in infested soil at 24 to 28°C and then stored at 4°C.

Time of storage (days)	077 Race 1				108 Race 3				141 Race 1				142 Race 3			
	Mariva		Molinera		Mariva		Molinera		Mariva		Molinera		Mariva		Molinera	
0	+	+	+	—	+	—	+	+	+	+	+	+	+	—	—	—
14	—	—	+	+	+	+	—	—	+	+	+	+	—	—	+	+
28	+	—	+	—	—	—	—	—	+	—	—	—	+	—	+	—
42	+	—	—	—	+	—	—	—	—	—	—	—	—	—	+	—
56	+	+	—	—	+	—	—	—	+	—	+	—	—	—	—	—
70	+	+	+	+	+	+	—	—	+	—	—	—	+	+	+	—

[a](+) bacteria present; (—) bacteria absent. Two tubers per cultivar were assayed at each interval by direct plating after CPG broth enrichment.

Stem infection was detected in almost all plants that yielded diseased tubers. Some plants that showed stem infection, however, yielded no apparently diseased tubers. No stem infection was detected in plants grown at 12 to 22°C.

To test for latent infections, all tubers harvested (247 of Mariva, 233 of Molinera) from plants grown under cool conditions were placed at 28°C and assayed for the presence of bacteria. Two tubers per isolate per cultivar were tested every 7 days up to 70 days; no latent infections were detected. On the other hand, healthy-appearing tubers collected from plants grown in infested soil under warm conditions and then stored at 4°C showed numerous latent infections up to 70 days (Table 2). Thus, because of latent infection, the number of diseased tubers produced by this group of plants was considerably higher than indicated in Table 1, which represents only the results of visual inspection of tubers for overt symptoms of the disease.

Ability of Different Potato Clones to Produce Latently Infected Tubers

Eight different wilt-resistant potato clones (five plants per clone) were grown in infested soil in the greenhouse at 24 to 28°C to determine their capacity to carry latent infections in the tubers. Tubers were collected 30 days after the soil was infested with each of the two strains (CIP 077 and CIP 108); all tubers were assayed individually for presence of the pathogen. At the time of harvest, plants of most clones showed no overt symptoms of the disease. Exceptions were clones MS 1F3 and 1386.9 which showed a relatively high incidence of wilting when inoculated with CIP 077 (Table 3).

Table 3. Incidence of latent tuber infection[a] and wilting in resistant potato clones grown at 24 to 28° C in soil infested with two strains of *Pseudomonas solanacearum*.

Clone	Isolate CIP 077 (Race 1)			Isolate CIP 108 (Race 3)		
	Total tubers	Tubers infected (%)	Plants wilted (%)	Total tubers	Tubers infected (%)	Plants wilted (%)
MS 42.3	19	0	0	14	0	0
MS 36.27	36	50	0	7	75	0
MS 35.4	42	35	0	32	14	20
MS 27A.15	16	0	0	17	0	20
MS 1F3	19	90	60	20	33	0
MS 1E7	25	92	0	24	0	0
BR 63.65	12	0	0	10	0	20
1386.9	3	0	40	6	0	20

[a] All tubers from five plants of each clone were assayed by direct plating after CPG broth enrichment.

Healthy-appearing tubers from several clones were shown to carry bacteria latently, but four (CIP 077) and five (CIP 108) out of the eight clones produced bacteria-free tubers (Table 3). The ability of an isolate to wilt potato plants was not correlated with its ability to infect tubers. Based on wilt induction, strain CIP 077 had a narrow range of pathogenicity, but caused more latent infections (26.7% of the total number of tubers produced) than strain CIP 108 (9.2%). Although plants of some clones had wilted by harvest time, they produced no infected tubers.

Multiplication of *P. solanacearum* in Resistant and Susceptible Potato Tubers

Ten tubers each of a resistant line (1386.15) and a susceptible (Russet Burbank) cultivar were inoculated with strain S213 by the direct injection method. When these tubers were assayed (one tuber of each clone) at 1 to 5 day intervals during a 25-day incubation period at 28°C, bacterial populations were significantly different in the two clones by 2 days after inoculation and thereafter. Initially, the bacterial populations decreased markedly in both clones. Later, they increased rapidly in the susceptible clone, but remained at a relatively low level in the resistant one.

By 15 days after inoculation, there was a 6-fold difference in bacterial populations of resistant and susceptible tubers. An analysis of variance indicated that these differences in population were significant ($P = 0.05$) at any sampling time beyond the first day after inoculation.

In a second experiment, the effect of different ambient temperatures on multiplication of *P. solanacearum* in susceptible tubers (cultivar Desiree) was determined. Tubers were inoculated with strain K60 and then placed at 4, 20 and 28°C; bacterial populations from two tuber samples at each temperature were assayed at various intervals during a 60-day incubation period. As in the previous experiment, populations increased within a few days after inoculation and remained at relatively high but variable levels throughout the incubation period at 28 and 20°C. At 4°C, however, populations were significantly lower ($P = 0.05$) than at the higher temperatures by 1 day after inoculation and thereafter. Very few or no bacteria could be detected by 23 days after inoculation.

Multiplication of *P. solanacearum* in Resistant Potato Plants

In initial experiments, plants of the resistant *S. phureja* clone, 1386.15, were inoculated with three strains of *P. solanacearum* and bacterial populations were sampled at zero time and at seven additional times during a 20-day period. Two plants were sampled at each time and two plates per dilution were used. The colony counts showed that immediately after inoculation, only the segment containing the inoculation site (b) had detectable bacteria. By 2 days after inoculation, however, large numbers of bacteria could also be detected in a sample a, which included the 5-cm segment above the inoculated one. At this time, very few bacteria were detected 5 cm below the inoculated segment. Thereafter, bacteria multiplied in this lower segment. Although the variability among samples was high, in general, bacterial populations increased steadily until, in some cases, they reached almost 10^8 cells per segment at 16 days after inoculation. Strain K60 was the most aggressive; it was detected in

relatively large numbers in all three stem segments by 3 days after inoculation. The strain from potato (S206) grew more slowly in the plant than K60, but faster than S123.

Although all plants supported high populations of bacteria, none showed visual wilt symptoms by 29 days after inoculation.

These results were confirmed in a second experiment in which only one strain (K60) was used and four rather than two plants were sampled at each interval. The inoculated segment always had the highest number of bacteria. However, by 20 days after inoculation, there were large populations of the bacterium in all stem sections. As in the previous experiment, bacteria moved into the segment above the inoculation site sooner than into the segment below this site.

For comparison, similar experiments were run with the susceptible cultivar, Russet Burbank. Plants were stem-inoculated with the three strains of the bacterium and samples were taken from each of four plants at 0, 1, 3, 7, and 12 days after inoculation. Average colony counts from four plates per dilution indicated an exponential increase in bacterial populations between the third and seventh days after inoculation. Populations at the seventh day were considerably higher than those in the resistant clone. For all segments, populations 7 days after inoculation for K60, S206, and S123 averaged 1065 ± 79, 685 ± 49, and 908 ± 53 cells ($X10^4$) per segment, respectively. Populations remained relatively stable up to 12 days after inoculation, but by this time all plants had wilted completely.

To determine whether there were differences in the ability of different strains of *P. solanacearum* to reach the base of the stem, plants of the resistant clone 1386.15 were stem-inoculated as before with K60, S123, and S206 and bacterial populations in the basal stem segment (5 cm) were determined at various times after inoculation. One sample each from two different plants inoculated with each isolate were assayed at each sampling time. Colony counts indicated that the three strains reached and multiplied in the lower part of the stem at different times. Strains K60 and S206 were detected 2 days after inoculation, but strain S123 was not detected until 6 days after inoculation. Strain K60 multiplied faster than the other two strains and reached a maximum (about 60×10^5 cells/segment) by 16 days after inoculation. Although relatively large numbers of bacteria could be recovered from the base of all stems, the plants showed no overt symptoms.

To determine whether there were differences in the ability of the three strains of *P. solanacearum* to reach and multiply in tubers, 11 wilt-resistant potato clones were stem-inoculated (five plants per clone per strain), maintained at 28°C for 3 weeks, and then grown to maturity at 20°C. Tubers were collected 120 days after stem inoculation and tested for the presence or absence of *P. solanacearum* by the enrichment and plating method described previously.

The three strains had different capabilities to infect the tubers of stem-inoculated plants. Strain K60 was the most infectious; 53.8% of the tubers harvested from all plants inoculated with this strain were infected (Table 4). Strain S206 infected 33.8% of the tubers and strain S123 only 7.5%.

Table 4. Total number of tubers and percentage of infected tubers harvested[a] from 11 potato hybrid clones stem-inoculated with three strains of *Pseudomonas solanacearum*

Clone	Strain K60			Strain S123			Strain S206		
	Tubers harvested	Tubers infected	Percent infected	Tubers harvested	Tubers infected	Percent infected	Tubers harvested	Tubers infected	Percent infected
A-1	11	11	100	9	0	0	3	1	33.3
P-7	15	15	100	4	0	0	2	0	0.0
P-13	15	2	13.3	12	0	0	12	2	16.6
T-2	15	5	33.3	12	1	8.3	8	6	75.0
2-1	8	6	75	13	1	1.6	1	0	0.0
6.5	10	5	50	12	0	10	10	6	60.0
7-6	3	1	33.3	6	0	0	1	1	100.0
15-4	13	11	84.6	13	1	7.6	12	7	58.3
BR49.6	8	5	62.5	6	3	50	4	0	0.0
BR53.1	8	0	0	14	0	0	14	0	0.0
BR63.65	11	2	18.1	5	2	40	4	1	25.0

[a] Tubers harvested 120 days after plants were stem inoculated.

None of the strains infected the tubers produced by clone BR53.1; only strain K60 infected tubers of clone P-7. All other clones yielded tubers infected by two or more strains, but the number of tubers harvested varied considerably; thus, comparisons between clones or strains were difficult.

Discussion

The results indicate that, at relatively high temperatures, resistant or tolerant potato cultivars grown in infested soil produce tubers that may carry overt or latent infections by *P. solanacearum*. Although only a relatively small proportion of the resistant plants have wilt symptoms at harvest, most carry the bacterium systemically. These results confirm previous reports (Ciampi *et al.*, 1980) that in stem-inoculated, resistant plants, the bacterium may be present in the tubers at harvest. The results also indicate that resistant plants can reach maturity in spite of relatively high populations of the bacterium.

The incidence of tuber infection in plants grown in infested soil at high temperature varied according to the cultivar and strain of the pathogen. Mariva, a tolerant *Solanum andigena* cultivar, produced more diseased tubers in soil infested with a race 1 strain than Molinera, a resistant cultivar. On the other hand. Molinera produced more diseased tubers in soil infested with a race 3 strain. The different genetic backgrounds of both cultivars may explain these differences in tuber infection. This was indicated also in experiments in which the incidence of latent tuber infection in seven hybrid (*S. phureja* x *S. tuberosum*) clones grown in infested soil was determined.

In general, the hybrid MS clones were more susceptible to tuber infection by the race 1 strain than by the race 3 strain. This confirms reports (Sequeira and Rowe, 1969) that resistance usually is limited to a few strains of the pathogen. Resistance is controlled by multiple, dominant genes (Rowe and Sequeira, 1970; Rowe *et al.*, 1972) and because of the high degree of heterozygosity in the parents, relatively few hybrids carry many of these genes.

It is important to note that several hybrid clones, and the *S. phureja* clone 1386.9, did not produce latently infected tubers, even when the plants were grown at high temperatures. This suggests that there are genetic factors that control tuber infection; although some of the plants of clones such as MS 27A.15 and BR 63.65 had wilted by harvest time, the tubers they produced were free of the bacterium. These results have important implications for breeding programs aimed at eliminating the problem of latent tuber infection.

Latent infection of tubers of resistant clones may be related to the apparent instability of the resistance to *P. solanacearum* originating from *S. phureja*. It has been reported that this resistance breaks down under high ambient temperatures (Hayward, 1964; Sequeira and Rowe, 1969; French, 1972). When plants were grown at cool temperatures, no latently infected tubers could be detected. The possibility that very small numbers of bacteria were present in these tubers cannot be ruled out entirely. However, incubation of these tubers at 28°C for 70 days, followed by periodic sampling of the tubers by bacterial enrichment techniques, did not

detect any bacteria. Since the warm temperature regime should have enhanced multiplication of *P. solanacearum,* the tubers apparently were free of the bacterium.

The high incidence of latently infected tubers from resistant clones grown at high temperatures suggests that the use of these clones in tropical areas may result in inadvertent movement of highly virulent strains of the pathogen. Harris (1972) has shown that potato tubers infected by *P. solanacearum* may not rot in storage. Plants grown from these tubers, however, succumb to the pathogen in the field. The ability of the bacterium to survive as a latent infectious agent is the most insidious aspect of this disease (Graham *et al.,* 1979). Although low temperature (4°C) storage may offer a possible method of eliminating latent infections, because it had a deleterious effect on survival of the pathogen in latently or artificially infected tubers, it is evident that small numbers of bacteria may remain after protracted storage. Our results confirm those of Nielsen (1963) who found that two out of 10 infested tubers still had virulent bacteria after 200 days of cold storage.

Although the tuber injection method was developed specifically for evaluating the pathogenicity of soft rot bacteria (De Boer and Kelman, 1978) we have shown that it can be adapted to measure the survival of *P. solanacearum.* Although *P. solanacearum* primarily is a vascular pathogen, it can multiply and rot cortical tissues. The technique has potential as tool for detecting clones that are resistant to bacterial wilt and to determine how environmental conditions affect populations of the bacterium within the tubers.

The multiplication and distribution of the *P. solanacearum* that we detected in stem-inoculated potato plants was in agreement with previous results with tobacco (Wang, 1937; Copeman, 1969). Bacteria moved upward or downward from the site of inoculation in the stem, but the tissues close to this site generally supported the largest populations of bacteria. The important contribution from our work was the finding that resistant plants could support very large populations of bacteria, but show no overt symptoms of wilting. Evidently, resistance originating from *S. phureja* depends, to a large extent, on a high degree of tolerance to the presence of the bacterium.

As expected, there were differences among strains of *P. solanacearum* in their ability to multiply in stems of *S. phureja.* This cultivated diploid potato is native to the highlands of the Andean region, where race 3 (biotype II) of *P. solanacearum* is common (Thurston, 1963; Hayward, 1964; Thurston and Lozano, 1968; Hayward, 1976). Resistance in *S. phureja,* therefore, could be expected to be more effective against race 3 strains, such as S206, than against those of race 1, such as K60 and S123, which are from other geographical areas. When absence of wilting symptoms was used as the only criterion, resistance in clone 1386.15 was effective against all three strains, as reported previously (Sequeira *et al.,* 1969). In terms of bacterial multiplication, however, it is evident that this clone was resistant only to strain S123, which did not grow as rapidly as the other two strains. This was not the result of inherent differences in growth rate between K60 and S123. When inoculated on tobacco leaves, for example, strain S123 grew more rapidly than K60 during the first 12 h (Granada and Sequeira, 1975).

Race 3 strains, on the other hand, generally grow more slowly in defined media than those of other races (Hayward, 1976).

The large numbers of bacteria present at the base of the plant of the resistant clone within a few days after stem-inoculation indicate that the pathogen could move readily into the roots and tubers via the vascular system. When initial infection occurs via the roots, as with the inoculation method used by French (1972), tuber infection may occur even more rapidly. Our results indicate that, under warm temperatures, tubers of inoculated, resistant clones become infected, although they show no visual symptoms of the disease. Some strains of *P. solanacearum* are able to reach and survive in tubers of resistant clones more effectively than others. These differences may be related to the fact that resistant clones react differently when inoculated with different strains of *P. solanacearum*. Different genes determine the response of a clone to different strains of the bacterium (Rowe and Sequeira, 1970; Rowe *et al.*, 1972).

When susceptible potato cultivars are infected in the field most of the plants die before they can produce tubers. Also, roguing can eliminate most of the plants that show symptoms up to the time of harvest. With resistant clones, on the other hand, infected plants and their tubers remain symptomless. Thus, virulent strains are the most likely to be moved via tubers of resistant cultivars from one geographical location. This problem would be particularly serious in underdeveloped countries where certified seed is not available. With the increasing demand for seed of resistant cultivars (International Potato Center, 1979), latent infections in seed tubers enhance the possibility of long-distance movement of virulent strains of the pathogen.

Our results indicate that latent infection is not a characteristic of all resistant clones; one clone (BR 53.1) did not yield infected tubers although the plants were stem inoculated. Thus, there appear to be genetic factors which might be useful in breeding for resistance to bacterial wilt in potato.

Literature Cited

1. Ciampi, L.; L. Sequeira and E. R. French. 1980. Latent infection of Potato tubers by *Pseudomonas solanacearum* Am. Potato J. 57:377-386.
2. Copeman, R. J. 1969. Histological and cytochemical changes in tobacco infected by *Pseudomonas solanacearum* Ph.D. Thesis. Univ. of Wisconsin. 94 pp.
3. De Boer, S. H. and A. Kelman. 1978. Influence of oxygen concentration and storage factors on susceptibility of potato tubers to bacterial soft rot (*Erwinia carotovora*). Potato Res. 21:65-80.
4. French, E. 1972. Progress and problems in selecting resistance to bacterial wilt. Pages 212-214, in: Prospects for the Potato in the Developing World; E. R. French (ed) International Potato Center, Lima, Perú.
5. González, C. 1977. Determinación de niveles de infección por *Pseudomonas solanacearum* en tubérculos de semilla de papa. Fitopatología 12:101-104.
6. González, L. C., L. Sequeira and P. R. Rowe. 1973. A root inoculation technique to screen potato seedlings for resistance to *Pseudomonas solanacearum*. Am. Potato J. 50:96-104.

7. Graham, J., D. A. Jones and A. B. Lloyd. 1979. Survival of *Pseudomonas solanacearum* race 3 in debris and latently infected potato tubers. Phytopathology 69:1100-1103.

8. Granada, G. A. and L. Sequeira. 1975. A hypersensitive reaction induced in tobacco leaves by a compatible (race 1) isolate of *Pseudomonas solanacearum*. Phytopathology 65:731-733.

9. Grieve, B. J. 1943. Studies in the physiology of host-parasite relations. III. Factors affecting resistance to bacterial wilt of Solanaceae. Proc. R. Soc. Victoria, N. S. 55:13-40.

10. Harris, D. 1972. The significance of bacterial wilt in the development of potatoes in Kenya. pp. 200-205. in: Prospects for the Potato in the Developing World. E. R. French (ed) International Potato Center, Lima, Perú.

11. Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol 27:265-279.

12. Hayward, A. C. 1976. Systematics and relationships of *Pseudomonas solanacearum*. Pp. 6-21, in: Proc. 1st. Int. Planning Conf. and Workshop on the Ecology and Control of Bacterial Wilt Caused by *Pseudomonas solanacearum*; L. Sequeira and A. Kelman (eds.). North Carolina State University, Raleigh, U.S.A.

13. International Potato Center. Lima, Perú. 1979. New variety Molinera reaches the farmer. CIP Circular 7(10). 3 pp.

14. Kelman, A. 1953. The bacterial wilt caused by *Pseudomonas solanacearum*. North Carolina Agric. Exp. Sta. Tech. Bull. 99, 194 pp.

15. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

16. Nielsen, L. W. 1963. Longevity of *Pseudomonas solanacearum* in potato tubers and culture in cold storage. Am. Potato J. 40:344-348.

17. Rowe, P. R. and L. Sequeira. 1970. Inheritance of resistance to *Pseudomonas solanacearum* in *Solanum phureja*. Phytopathology 60:1499-1501.

18. Rowe, P. R., L. Sequeira and L. C. González. 1972. Additional genes for resistance to *Pseudomonas solanacearum* in *Solanum phureja*. Phytopathology 62:1093-1094.

19. Sequeira, L., and P. R. Rowe. 1969. Selection and utilization of *Solanum phureja* clones with high resistance to different strains of *Pseudomonas solanacearum*. Am. Potato J. 46:451-462.

20. Thurston, H. D. 1963. Bacterial wilt of potatoes in Colombia. Am. Potato J. 40:381-390.

21. Thurston, H. D. and J. C. Lozano. 1968. Resistance to bacterial wilt of potatoes in Colombian clones of *Solanum phureja*. Am. Potato J. 45:51-55.

22. Wallis, F. M. and S. J. Truter. 1978. Histopathology of tomato plants infected with *Pseudomonas solanacearum* with emphasis on ultrastructure. Physiol. Plant Path. 13:307-317.

23. Wang, G. W. 1937. The entrance and migration of *Bacterium solanacearum* Smith in tobacco plants. Ann. Phytopath. Soc. Japan 7:14-23.

Acknowledgment

Variability in Epiphytic Populations of *Pseudomonas syringae* pv. *savastanoi* from Olive

G.L. Ercolani

Instituto di Microbiologia Agraria e Tecnica
Bari, Italy

Abstract

Epiphytic populations of *Pseudomonas syringae* pv. *savastanoi* were subjected to cluster analysis, based on 20 phenotypic characters. The isolates from leaves of a given age differed from those from leaves of a different age at the same time of the year. The isolates from leaves of the same age at different times of the year were also different. Factor analysis showed that epiphytic populations of pv. *savastanoi* were influenced by the cambial activity, the temperature of the air, summer rains, winter rains, the time of blossoming, and warm southerly winds.

Introduction

The bacterium *Pseudomonas syringae* pv. *savastanoi*, that causes the disease known as olive "knot" or "tubercle," is usually found as an epiphyte on olive leaves. Several studies have indicated that bacterial populations on the phylloplane are controlled by recurrent events in their habitat (1, 2, 3), but the factors accounting for the success of pv. *savastanoi* in colonizing the surface of leaves of different ages under a broad range of conditions remain unknown. This paper reports the characteristics of several isolates of pv. *savastanoi* from the surface of leaves of different ages at different times of the year.

Materials and Methods

Sampling and isolations were carried out as before (2, 3) with a few modifications indicated below. Unfolding leaves were mapped on olive trees, cv. Coratina, at the beginning of April, July, and October each year, 1974 to 1980. Five samples, each with a total leaf surface area of 1,000 cm², were taken approximately 1, 4, 7, 10, and 13 months later. Random samples were also taken from unmapped leaves. Counts of pv. *savastanoi* were taken on each sample. Random isolates identified as pv. *savastanoi* were kept for further study.

Total organic matter was determined in the wash water from each sample. The air temperature, rainfall, winds along two vectors, and leaf turnover were also recorded.

The following 20 phenotypic characters, combining a low probability of error and a high separation figure, were determined on 50 isolates from each sample: transparency of growth on agar, production of levan, colony diameter >2 mm, growth at 35°C, tolerance to pH 5.0, tolerance to pH 7.8, tolerance to NaC1 3.0%, use of malonate as sole carbon source, acid from mannose, lactose, maltose, sucrose, *meso*-inositol and sorbitol, degradation of urea, tyrosine and tween 40, resistance to oleandomycin and dihydrostreptomycin, and induction of a hypersensitive response in tobacco leaves with suspensions containing 10^6 colony forming units (CFU) ml^{-1}.

The simple matching coefficient and single linkage cluster anlysis (5) were used to compare the isolates. The clusters were formed at the >70% similarity level ($\underline{S}$-level). The cluster procedure was also performed after replacing the individual isolates from a given sample with a hypothetical median organism (HMO; 2) to represent them.

The data were factor analyzed (4), using the four environmental parameters listed above, plus total organic matter in the wash water, leaf turnover, and the following 10 microbiological parameters: populations of pv. *savastanoi* on random samples and on samples of 1, 4, 7, 10, and 13-month old leaves, and frequency of occurrence of four phenotypic characters, i.e., acid from lactose, production of levan, resistance to oleandomycin, and HR in tobacco from 10^6 CFU ml^{-1}.

Results

Fig. 1 shows that only the HMOs representing the isolates obtained in January formed a cluster with more than two components. The results did not change appreciably when the data were analyzed year by year. The observed pattern of similarities and differences between the HMOs did not change regularly with the age of the leaves, nor with the time of the year when the leaves attained that age.

Six factors were identified for the environmental and microbiological parameters and characterized as "cambial activity," "air temperature," "summer rains," "winter rains," "time of blossoming," and "warm southerly winds."

Conclusions

The results show that population of pv. *savastanoi* on olive leaves of a given age at a given time of the year share a greater number of physiological, cultural, and biochemical characteristics with one another than with the populations colonizing the leaves of a different age at the same time of the year or the leaves of the same age at a different time of the year.

Factor analysis demonstrated that the distribution of pv. *savastanoi* on the phylloplane of the olive is affected by plant-related and environment-related factors.

```
01-JL
04-JL   0
01-OC   $+                          KEY  {%}
10-JL   =:+                         0  80-85
13-JL   ::+0                        Ⅱ  75-79
13-AP   :$...                       $  70-74
13-OC   ++::.0                      =  65-69
04-JN   ++.+:+.                     +  60-64
07-JN   :+..:$:0                    -  55-59
10-JN   :+:..$:$$                   :  50-54
01-AP   ++++++:...                  .    <50
07-AP   -==-==--==Ⅱ
10-AP   =:+$::+:..$-
04-OC   ..=...-..-..-
07-OC   ...=-..-...:=+
```

Fig. 1. Sorted similarity matrix for the hypothetical median organisms representing the isolates of *Pseudomonas syringae* pv. *savastanoi* obtained from olive leaves of a given age at four different times of the year in 1974-1980. The hypothetical median organisms are identified by the age of the leaves from which the isolates represented were obtained (04-13 Months) and the time of the year when the leaves attained that age (JN = January, AP = April, JI = July, OC = October).

Literature Cited

1. Ercolani, G.L. 1970. Presenza epifitica di *Pseudomonas savastanoi* (E.F. Smith) Stevens sull'olivo, in Puglia. Phytopathol. Medit. 10:130-132.
2. Ercolani, G.L. 1978. *Pseudomonas savastanoi* and other bacteria colonizing the surface of olive leaves in the field. J. Gen. Microbiol. 109:245-257.
3. Ercolani, G.L. 1979. Distribuzione di *Pseudomonas savastanoi* sulle foglie dell'olivo. Phytopathology Medit. 18:85-88.
4. Rosswall, T., and E. Kvillner. 1978. Principal component and factor analysis for the description of microbial populations. Pp. 1-48 in: M. Alexander, ed. Advances in Microbial Ecology, vol. 2. Plenum Press, New York, NY. 297-pp.
5. Sneath, P. H. A., and R. R. Sokal. 1973. Numerical Taxonomy. Freeman and Co., S. Francisco, CA. 573 pp.

Interaction Between Bacteria Inhabiting Cotton Leaves

J. P. Verma, P. P. Sinha, R. P. Singh
Indian Agricultural Research Institute
New Delhi, India

Abstract

Species of *Aeromonas, Flavobacterium,* and *Pseudomonas* comprising 14.2% of total phylloplane bacterial (PlB) population of cotton (*Gossypium hirsutum*) leaves, on preinoculation (8-20 h), protected the leaves from infection by a virulent isolate of *Xanthomonas campestris* pv. *malvacearum* (Xcm). They gave protection not only on a highly susceptible cultivar, but also on differentials (isogenic lines with different bacterial blight resistant genes) used for race characterization. The presence of bacterial blight resistant genes did not alter significantly the preinoculative protective effect (PPE) of the PlBs. Heat-killed (HK) cells of PlB and their streptomycin resistant (to 1000 ppm streptomycin) mutants (Str' M) afforded similar PPE (50-60%). At 20:1 (PlB:*Xcm*) ratio the protection was complete on a few cultivars, while on others it was 80-93%; further, at this ratio a fair degree of protection (about 40%) was obtained immediately upon inoculation.

Introduction

Premunity (16) was defined (15) as "an acquired nonspecific immunity manifesting itself in plants pretreated with one bacterium that immunizes or protects the plant from infection by another pathogen." Such preinoculative protective effect (PPE) is known (3, 17, 29, 33) for several bacteria, including saprophytic phylloplane bacteria (PlB). A few PlBs, heat-killed (HK) and avirulent cells of *Xanthomonas campestris* pv. *malvacearum* (Xcm), possessed this PPE against a challenge dose of virulent Xcm (27, 29, 31). The production of protection factor (PF) or a protection factor inducing principle (PFIP=elicitor?) by the PlB has been implicated (29). These researches were extended and the results are reported in this paper.

Materials and Methods

Preinoculation of PlBs (PlB-5, -6, -12 & -51) and challenge *Xcm*R-32 (*Xcm* race-32) was done following procedures previously described (29), using about 0.1 ml bacterial suspension containing equal number of cells (callibrated by O.D. and viable plant count for both PlB and *Xcm* separately). Controls were kept as usual with sterile water, non-protecting PlBs, etc.

The details of cotton cultivars used, PlBs, and races of Xcm have also been described (29, 30).

Results

PPE of HK-P1B. HK—CELLS OF *Xcm* are known to have PPE against infection of *Xcm* (27). There are certain other reports that HK-cells of unrelated bacteria also affect the pathogenic/hypersensitive reaction (33). The present experiment was designed to study the activity of protective P1Bs after heat killing. HK-cells were prepared by exposing the bacterial cells (a dilute suspension of P1B containing 1.2×10^7 CFU^{-1}) to 100°C for 10 min. Viability tests were done by viable plate count to confirm the inactivation of bacterial cells. Inoculations were made as usual (29) using a hypodermic syringe, creating watersoaking by HK-P1B and challenging with an equal amount of suspension of XcmR-32 containing 1.2×10^7 CFU^{-1} at 8 h. Results (Table 1) indicated that, by and large, the HK cells were as effective as the live cells, although slightly lower values were in cases of P1B-6 and P1B-12. The results also established that the protection factor (elicitor? produced by the P1B) was thermostable.

PPE of Str^r M (Streptomycin Resistant Strains) of P1B. Antibiotics are generally used for the control of *Xcm* infections (19, 20, 26, 28). The effect of these antibiotics on epiphytes, including P1Bs, is now known. However, several P1Bs were isolated from nature which possessed resistance to streptomycin and penicillin. It was, therefore, thought desirable to study the effect of Str^r M strains of P1Bs and changes, if any, in their PPE. Str^{r}M-P1B were first isolated (32) against 100 ppm streptomycin and then trained to 1000 ppm. These were stable Str^{r}M-100, used for PPE studies. The results (Table 1) showed that both the wild type and the mutants afforded similar protection. It was, therefore, concluded that resistance to streptomycin developed without any effect on the preinoculative protective ability of the P1B strains. Apparently, these two processes are quite unrelated and under the control of noninteracting/interfering genes.

Comparative Growth of Wild Type and Str^r M Strains of P1B and Xcm. As the protection provided by the Str^r strains of P1Bs was equal to that of their parent strains, it was thought desirable to study their growth in broth so that the Str^r M could be used for further studies, i.e. for the isolation of PF as well as in other *in vivo* studies where contaminations could be a problem. Results (Table 2) indicated that the rate of growth of mutants was much slower than that of their respective parent strains. Further, the strains of *Xcm* which were Str^r produced symptoms at a much slower rate (i.e. after a longer incubation period) and of lower grades (32). These, therefore, could not be used for further studies where rapid growth was needed. One may also speculate here that Str^r M of *Xcm* would not pose a big problem in nature, because they can never build high populations in comparison to their parent Str^s strains, simply because of their slow rate of multiplication.

Varietal Response to Protective P1Bs. Previous studies have demonstrated the PPE of P1Bs -5, -6, -12 and -51 to Acala-44 against the infection of *Xcm*. Acala-44 is a highly susceptible cultivar of *Gossypium hirsutum* to *Xcm*. It possesses no genes for bacterial blight resistance. The present studies were conducted with different *G. hirsutum* cultivars, which were developed by Hunter *et al.* (12) and were also used by Verma and Singh (26) as differentials to characterize the races of *Xcm*. These cultivars

Table 1. Population dynamics of bacteria associated with cotton leaves.

Date of sampling	Number of bacteria/g fresh weight of leaf						Wash method
	Maceration method						
	Yellow colonies	White colonies	Xcm	Actino-mycetes	Others	Total	
24/ 6/79	3.0×10^2	6.0×10^2	—	1.0×10^2	3.0×10^2	1.3×10^3	1.2×10^3
16/ 7/79	3.3×10^3	2.4×10^3	5.0×10^2	3.0×10^2	5.0×10^2	7.0×10^3	5.5×10^3
31/ 7/79	5.0×10^4	1.0×10^4	1.1×10^4	—	9.0×10^3	8.0×10^4	7.2×10^4
20/ 8/79	1.4×10^5	4.0×10^4	7.0×10^4	—	—	2.5×10^5	2.1×10^5
04/ 9/79	1.8×10^6	1.2×10^5	4.8×10^5	—	—	2.4×10^6	2.0×10^6
23/ 9/79	1.6×10^6	2.0×10^5	4.0×10^5	—	—	2.2×10^6	1.7×10^6
10/10/79	1.3×10^6	2.0×10^5	5.0×10^5	—	—	2.0×10^6	1.5×10^6
30/10/79	2.8×10^5	6.0×10^4	6.0×10^4	—	—	4.0×10^5	3.7×10^5
30/ 6/80	3.0×10^2	8.0×10^2	—	4.0×10^2	3.0×10^2	1.8×10^3	1.7×10^3
15/ 7/80	5.0×10^4	3.0×10^4	1.5×10^4	—	5.0×10^3	1.0×10^5	8.0×10^4
29/ 7/80	2.0×10^6	2.0×10^5	8.0×10^5	—	—	3.0×10^6	2.8×10^6
17/ 8/80	5.0×10^6	5.0×10^5	2.5×10^6	—	—	8.0×10^6	7.0×10^6
02/ 9/80	5.0×10^6	5.0×10^5	3.0×10^6	—	—	8.5×10^6	7.3×10^6
18/ 9/80	2.1×10^6	4.0×10^5	1.5×10^6	—	—	4.0×10^6	3.7×10^6
3/10/80	1.8×10^6	2.0×10^5	1.0×10^6	—	—	3.0×10^8	2.5×10^6
28/10/80	6.0×10^5	2.0×10^5	2.0×10^5	—	—	1.0×10^6	8.0×10^5

—, not significant; the average temperature ($^{\circ}$C) in the week preceding sampling was 28.5 ($\pm$ 3) and 29.0 ($\pm$ 2) in 1979 and 1980 respectively, while the mean R.H. was 65.8 ($\pm$ 13) and 69.9 ($\pm$17) in 1979 and 1980 respectively.

Table 2. Bacterial population on different parts of cotton plants

Plant part	Number of bacteria/g fresh weight					
	Yellow colonies	White colonies	Xcm	Actino-mycetes	Others	Total
Buds	2.5×10^6	1.0×10^6	$-^a$	$-$	2.0×10^5	4.0×10^6
Bolls	1.5×10^5	6.0×10^4	$-$	$-$	1.0×10^4	2.0×10^5
Leaves	4.8×10^6	1.2×10^6	9.0×10^5	$-$	1.0×10^5	7.0×10^6
Petioles	3.5×10^5	1.2×10^5	3.0×10^4	$-$	$-$	5.0×10^5
Twigs	2.0×10^5	8.0×10^4	2.0×10^4	$-$	$-$	3.0×10^5
Stems	8.0×10^4	3.0×10^4	1.0×10^4	$-$	$-$	1.2×10^5

^{a} not significant

have the same background (isogenic lines), but differ from each other in the presence of different blight resistant genes. The main idea was to determine the interaction of PPE of P1Bs and the expression of resistance genes. Preinoculations and other steps were taken as described before (29). The results (Table 3) indicated that PPE of the P1Bs was operative on all the cultivars which were susceptible to *Xcm*. The degree of protection ranged between 50 and 60% with some exceptions. It was concluded that the presence of bacterial blight resistance genes was ineffective as far PPE was concerned, and did not interfere with the protection mechanism of the host (induced by the P1Bs). The preinoculation of P1Bs also did not affect hypersensitive reaction (i.e. rapid browning, a resistant reaction) as evidenced from the reaction of 101-102-B, which is resistant to *XcmR*-32.

PPE of P1B-6 at different concentrations. About 60 to 70% protection was obtained by P1B-6 in previous experiments when 2.4×10^6 CFU^{-1} were used for preinoculation per lesion. The present studies were conducted to determine if the degree of protection varied with the concentration of P1B-6. Preinoculation/challenge inoculations were as described earlier, using 0.1 ml bacterial suspension containing 2.4×10^6, 12×10^6, and 24×10^6 CFU^{-1} of P1B and 1.2×10^6 CFU^{-1} of *Xcm* per lesion. The lesions were also photographed in transmitted light to count the number of water-soaked/necrotic cells in order to determine the extent/spread of infection. The experiment was conducted with only P1B-6 and *XcmR*-32 and the eight differentials used for race characterization (12, 26).

Results (Table 4) indicated that the degree of protection increased with the increasing concentration of P1B and at 20:1 (P1Bx*Xcm*) it was 100% in Acala-44 and Mebane-B-1, while in other varieties it varied from 80 to 93%. It was concluded that P1B-6 afforded greater protection at higher concentrations. However, higher concentration of P1B did not alter the HR on the immune cultivar 101-102-B. The data on the extent of infection also corroborated that protection increased with increasing concentration of P1B, because the number of cells infected at 20:1 was zero in the cases of Acala-44 and Mebane-B-1.

Table 3. Eight hour preinoculative protective effect of phylloplane bacteria against *X. campestris* pv. *malvacearum* on different cotton cultivars.

Phylloplane bacteria	Differential hosts and genes for resistance							
	Acala 44 (none)	Stoneville 2B-S9 (polygenes)	Stoneville 20 (B_7+poly-genes)	Mebane B-1 (B_2+poly-genes)	1-10B (B_{In}+poly-genes)	20-3 (B_N+poly-genes)	101-102B (B_2B_3+ unknown)	Gregg (unknown)
PLB-5								
A	1.83	1.66	1.66	2.00	1.83	1.66	HR	2.33
B	4.83	4.00	3.33	4.83	4.83	3.66	HR	4.33
C	62.12	59.50	50.16	50.32	62.12	54.65	—	46.19
PLB-6								
A	1.83	2.33	1.83	2.33	1.66	1.66	HR	1.83
B	5.00	4.66	4.00	4.50	3.83	4.00	HR	3.66
C	63.40	50.00	54.25	48.25	56.66	58.50	—	50.00
PLB-12								
A	2.00	2.64	1.66	2.66	1.50	1.83	HR	1.66
B	4.50	4.00	3.66	4.50	3.16	3.66	HR	3.66
C	55.66	34.00	54.60	40.89	52.54	50.00	—	54.65
PLB-51								
A	1.83	1.83	2.00	2.66	1.50	1.50	HR	2.00
B	4.50	4.83	4.33	4.16	4.50	4.00	HR	4.16
C	53.34	62.12	53.82	36.06	66.66	62.50	—	51.90

A = Average grade on PLB pre-inoculated leaves; B = Average grade on water pre-inoculated leaves;
C = Per cent protection.

Table 4. Preinoculative protective effect of different concentrations of PLB-6 on the infection of *Xanthomonas campestris* pv. *malvacearum*.

Differential lines	Ratio of PLB-6 to XMR-32										
	2:1			10:1			20:1			0:1	
	No. of cells infected	Average grade	Per cent control*	No. of cells infected	Average grade	Per cent control*	No. of cells infected	Average grade	Per cent control*	No. of cells infected	Average grade
Acala-44	17	1.83	62.12	7	1.16	75.99	0	0.0	100.00	66	4.83
Stoneville 2 BS. 9	16	2.16	52.0	8	1.33	70.67	4	0.5	88.89	50	4.50
Stoneville 20	22	1.83	54.25	9	1.33	66.75	3	0.5	87.50	50	4.0
Mebane B-1	25	1.83	62.12	7	1.33	72.43	0	0.0	100.00	48	4.83
1-10B	33	1.83	57.74	15	1.16	73.21	7	0.83	80.84	46	4.33
20-3	26	2.0	53.82	9	1.16	73.21	6	0.66	84.76	57	4.33
101-102B	0	HR		0	HR		0	HR		0	HR
Gregg	15	2.0	53.82	4	1.16	73.21	0	0.33	92.38	35	4.33

Cv — Acala-44; * Calculated on the basis of grades obtained 10 days after challenge.

The quantitative assessment of the disease development indicated that the number of cells infected ranged between 35 and 66 per lesion, and Acala-44, which possessed no genes for bacterial blight resistance showed the greatest infection, i.e. an area covered by 66 cells (Table 4). The cultivar with resistance genes showed 46 to 50 infected cells per lesion, thereby indicating fairly low variation and also demonstrating that they were fairly tolerant and inhibited free spread of *Xcm* as in the case of Acala-44.

Discussion

The knowledge available so far on the interaction between bacteria inhabiting cotton (*G. hirsutum*) leaves consisted of the fundamental fact that certain P1Bs had preinoculative protective effect (PPE) on the challenge dose of active *Xcm* on a highly susceptible cultivar, Acala-44 (27, 29). In our investigation the varietal response to P1Bs growth and their preinoculative protective effect was studied with the use of four P1Bs (P 1B-5, -6, -12, -51) identified respectively as *Flavobacterium* sp., Aeromonas sp., and Pseudomonas sp.) and eight cotton differentials (cultivars of *G. hirsutum*, used for race identification), which differ particularly in the presence of different bacterial blight-resistant genes. All the P1Bs used in the present studies possessed preinoculative protection effect against *Xcm* infection on all the varieties, which were susceptible to *Xcm;* and, interestingly, under properly controlled and replicated conditions, the degree of protection varied within a narrow range of 50 to 60%. It was, therefore, obvious that the presence or absence of bacterial blight resistant genes did not alter significantly the protective effect of the P1Bs. The same was true for hypersensitive reaction on cv 101-102-B, which was not altered in any significant manner by the preinoculation of P1Bs.

Heat killed (HK) cells of P1Bs were almost equally effective. HK cells of pathogenic or other strains of *Pseudomonas tabaci* or even other species of plant pathogenic bacteria protected tobacco leaves against wildfire disease (17). Verma and Singh (27) demonstrated that HK-cells of *Xcm* possessed PPE against *Xcm* infection, while Sequeira and Hill (24) observed that tobacco leaves developed systemic, non-specific resistance to a wide variety of pathogens following infiltration of HK cells of *P. solanacearum*. The protection response was detected by a challenge inoculation with a compatible strain of the same bacterium at 24 h.

Str′M (streptomycin resistant mutants) of all the P1Bs also gave 50% protection, which indicated that the adaptation to Str did not affect the protection activity/ability. However, their rate of multiplication *in vitro* was significantly reduced. One isolate among the several hundred Str′M isolates of XcmR-32, a highly virulent race pathogenic to at least 5 bacterial blight resistant genes (32), lost its pathogenicity.

When cultured on acridine orange, *Xcm* did not lose its pathogenicity; there was only a delay in symptom development and the disease developed to lower grades only (unpublished observations). Str resistance also was not lost on acridine orange or ethidium bromide medium. These facts indicated that pathogenicity and Str resistance were not plasmid controlled

but governed by genes located on the main bacterial chromosome. However, the delay in symptoms and lower grades did indicate that some virulence/aggressiveness factors were plasmid controlled, directly or indirectly. More work is needed to confirm this.

Resistance mutants of *P. solanacearum* against 20 ng^{-1} acridine orange lost their virulence toward tomato, their ability to produce hypersensitive reaction on tobacco, and their altering ability with heat killed cells to induce protection against hypersensitive reaction (18). Pathogenicity in some has been demonstrated to be plasmid controlled (6, 14, 22). Kelman (13) had earlier described a spontaneous avirulent mutant of *P. solanacearum* on tetrazolium chloride medium. Loss in virulence on synthetic media is fairly well known (27), but loss in virulence associated with resistant mutants is a rare phenomenon. The transfer of streptomycin resistance from P1Bs to *Xcm* has still to be shown, but is known between several other bacteria including saprophytic and phytopathogenic ones (28). However, the present studies clearly demonstrate that neither the pathogenicity of *Xcm* nor the PPE of P1B^S, with rare exceptions, is changed by streptomycin resistance.

The ratio of P1B to *Xcm* had a profound effect on the preinoculative protective ability of P1B, and the degree of protection was almost directly proportional to the concentration of P1B. There was complete protection at 20:1 ratio of P1B:*Xcm* in certain cultivars; at this ratio there was some protection (about 40%) even at the time of inoculation. Apparently, at higher initial concentration, the critical concentration of P1B required for protection was reached earlier; or at certain low concentrations this critical concentration may never be reached or reached only slowly, necessitating a short/long incubation period.

Saprophytic bacteria generally died within 3 or 4 days when inoculated into leaves (25). Chowdhury and Verma (2) demonstrated that P1B did not multiply (on the basis of net population) either in susceptible or resistant cultivars when inoculated at 10^3 bacteria/cm^2, and the inoculated level was maintained for about 2 weeks. However, when P1B was coinoculated with *Xcm*, a higher population of P1B was achieved (the population of P1B increased from 10^3 to 10^8/cm^2), and it was concluded that the P1B multiplied only in the presence of *Xcm* (2). In nature, therefore, as demonstrated in the present study, *Xcm* was present on apparently healthy leaves (Sinha and Verma, this proceeding), at a population level which was insufficient to cause infection; but this might be sufficient to support/activate the multiplication of the P1Bs. For example, the P1Bs, as such, might not be multiplying, but in the presence of low level of *Xcm* they might multiply and reach the critical levels needed for protection against the infection of *Xcm*. Interestingly, when P1B was mixed with *Xcm in vitro* in nutrient dextrose broth the *Xcm* could not be recovered after 8 h incubation from the mixture, even at P1Bs ratio to *Xcm* of 1:10 (unpublished observations).

Habish (8, 9) showed that *Flavobacterium* sp. isolated from bacterial blight infected cotton trash could delay the symptom production when inoculated with *Xcm* (1:1 ratio). The reduction of *X. campestris* pv. *oryzae* was more pronounced when the cell number of *Erwinia herbicola* was increased in the inoculum mixture (10). Similar reduction in disease

severity with increase or protective/antagonistic bacteria was obtained in *X. campestris* pv. *vesicatoria*-system (23). Such inhibition by saprophytic bacteria was attributed to the production of large amounts of acid, which made the enviroment unfavorable for the growth of plant pathogenic bacteria (4, 7, 10). Incompatible pathogens induced plant response which inhibited the growth of the compatible pathogens (11).

On the other hand, Garrett and Crosse (5) concluded from mixed inoculation experiments with plum and cherry strains of *P. syringae* pv. *morsprunorum* and other pseudomonads on cherry, that a reduction of canker could not be explained by lysogenicity, bacteriocinogenicity, or other antagonistic effects observed *in vitro*, but was presumably host mediated. The analysis of interaction between saprophytic bacteria and higher plants is difficult, because usually they do not involve pronounced physiological or biochemical changes (21).

The exact nature of inhibition of *Xcm* by P1B has not yet been demonstrated properly. However, preliminary results have shown that the culture filtrate (CF) of P1B possessed PPE and an 8 to 16 h CF afforded maximum protection at 8 h preinoculation. The CF was also inhibitory to the growth of *Xcm in vitro*. Heat inactivated CF retained its activity. Of the two main protection factors (elicitors?), at least one was a polypeptide, which was capable of inducing phytoalexin production in cotton (unpublished results). Capsular slime of *Xcm* are capable of inducing appreciable phytoalexin production in resistant varieties, but little or no synthesis in susceptible varieties (1). Elicitors may, however, be proteinaceous or glycan in nature (1).

Literature Cited

1. Bell, A. A., 1974. Biochemical bases of resistance of plants to pathogens. In: Biological control of plant insects and diseases, Eds. F. G. Maxwell and F. A. Harris, the University Press of Mississippi, Jackson, pp. 403-461.

2. Chowdhury, H. D., and J. P. Verma. 1980. Multiplication of *Xanthomonas malvacearum* and a phylloplane bacterium in leaves of *Gossypium hirsutum*. Indian Phytopath. 33:245-248.

3. Crosse, J. E., 1965. Bacterial canker of stone fruits. IV. Inhibition of leaf scar infection of cherry by a saprophytic bacterium from leaf surface. Ann. appl. Biol. 56:149-160.

4. Farbee, G. J., and J. L. Lockwood. 1958. Inhibition of *Erwinia amylovora* by Bacterium sp. isolated from fire blight cankers. Phytopathology 48:209-211.

5. Garrett, C. M. E., and J. E. Crosse. 1975. Interaction between *Pseudomonas morsprunorum* and other pseudomonads in leaf scar infection of cherry. Physiol. Plant Pathol. 5:89-94.

6. González, C. F., and A. K. Vidaver. 1978. Analysis of plasmids of syringomycin producing strains of *Pseudomonas syringae*. Proc. 4th Int. Conf. Plant Path. Bact., Angers, 31-38.

7. Goodman, R. N. 1965. *In vitro* and *in vivo* interaction between components of mixed bacterial cultures isolated from apple buds. Phytopathology 55:217-221.

8. Habish, H. A. 1968 a. Role of antagonistic bacterium in the decline of *Xanthomonas malvacearum* in wet cotton debris. Cott. Gr. Rev. 45:36-41.

9. Habish, H. A. 1968 b. Effect of an antagonistic bacterium on degree of infection of cotton with *Xanthomonas malvacearum*. Cott. Gr. Rev. 45:137-140.

10. Hsieh, S. P. Y., and I. N. Buddenhagen. 1974. Suppressing effect of *Erwinia herbicola* on infection by *Xanthomonas oryzae* and on symptom development in rice. Phytopath. 64:1182-1185.

11. Hsu, S. I., and R. S. Dickey. 1972. Interaction between *Xanthomonas phaseoli, Xanthomonas vesicatoria, Santhomonas campestris,* and *Pseudomonas fluorescens* in bean and tomato leaves. Phytopathology 62:1120-1126.

12. Hunter, R. E., L. A. Brinkerhoff, and L. S. Bird. 1968. The development of a set of upland cotton lines for differentiating races of *Xanthomonas malvacearum*. Phytopathology 58:830-832.

13. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

14. Kerr, A. 1978. The Ti plasmid of Agrobacterium. Proc. 4th Int. Conf. Plant Path. Bact., Angers, 101-108.

15. Klement, Z. 1972. Development of hypersensitive reaction induced by plant pathogenic bacteria. Proc. 3rd Int. Conf. Plant Path. Bact., 157-164.

16. Klement, Z., and R. N. Goodman. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Annu. Rev. Phytopathol. 5:17-44.

17. Lovrekovich, L., and G. L. Farkars. 1965. Induced protection against wildfire disease in tobacco leaves treated with heat killed bacteria. Nature, Lond. 205:823-824.

18. Message, B., P. Boistard, M. Pitrat, J. Schmit, and C. Boucher. 1978. A new class of fluidal avirulent mutants of *Pseudomonas solanacearum* unable to induce a hypersensitive reaction. Proc. 4th Int. Conf. Plant. Bact., Angers, 823-833.

19. Nayak, M. L., and J. P. Verma. 1975. Uptake and translocation of chemicals in cotton seedlings. Z. Pflkrankh. PflSchutz 82:587-592.

20. Nayak, M. L., R. P. Singh, and J. P. Verma. 1976. Effective chemical sprays to control bacterial blight of cotton. Z. Pflkrankh. PflSchutz 83:407-415.

21. Rudolph, K. 1976. Models of interaction between higher plants and bacteria. In: Specificity in plant diseases, Eds. R. K. S. Wood and A. Graneti, Plenum Press, N. Y., pp. 109-126.

22. Sands, D. C., G. Warren, D. F. Mayers, and A. L. Scharen. 1978. Plasmids as virulence markers in *Pseudomonas syringae*. Proc. 4th Int. Conf. Plant Path. Bact., Angers, pp. 39-45.

23. Sekhawat, P. S., and B. P. Chakravarti. 1977. Epiphytic bacterial and actinomycetes population and control of bacterial leaf spot of chillies by an antagonistic bacterium associated with bacterial lesions. Indian Phytopath. 30:358-360.

24. Sequeira, L., and L. M. Hill. 1974. Induced resistance in tobacco leaves: the growth of *Pseudomonas solanacearum* in protected tissues. Physiol. Plant Pathol. 4:447-455.

25. Soding, H. 1959. Uber das Verhalten von Bakterien in lebenden Blattern. Arch. Mikrobiol. 34:103-131.

26. Verma, J. P., and R. P. Singh. 1974. Recent studies on the bacterial diseases of fibre and oilseed crops in India. In: Current trends in plant pathology, Eds. S. P. Raychaudhuri and J. P. Verma, Lucknow University, Lucknow, pp. 134-145.

27. Verma, J. P., and R. P. Singh. 1976. Races of *Xanthomonas malvacearum*, loss in virulence and the protective effect of avirulent strains heat killed cells and phylloplane bacteria. Z. PflKrankh. PflSchutz 83:748-75 7.

28. Verma, J. P., and R. P. Singh. 1976b. Chemical control of bacterial diseases -of plants. Chemical concepts (special no. on pesticides) 4:31-50.

29. Verma, J. P., H. D. Chowdhury, and R. P. Singh. 1978. Interaction between phylloplane bacteria and *Xanthomonas malvacearum*. Proc. 4th Int. Conf. Plant Path. Bact. Angers, pp. 795-802.

30. Verma, J. P., H. D. Chowdhury, and R. P. Singh. 1979. Interactions between different races of *Xanthomonas malvacearum* in leaves of *Gossypium hursitum*. Z. PflKrankh. PflSchutz. 86:460-464.

31. Verma, J. P., H. D. Chowdhury, and R. P. Singh. 1981. Biological control of bacterial blight of cotton. Proc. Natl. Symp., Calcutta (In press).

32. Verma, J. P., R. P. Singh, S. G. Borkar, P. P. Sinha, and R. Prasad. 1980. Management of bacterial blight of cotton with particular reference to variation in the pathogen, *Xanthomonas malvacearum*. Ann. agric. Res. 1:98-107.

33. Verma, J. P., R. P. Singh, and H. D. Chowdhury. 1980. Relationship of epiphytic bacteria to bacterial diseases of plants. In: Recent advances in the biology of microorganisms, Eds. K. S. Bilgrami and K. M. Vyas, Prof. S. B. Saxena felicitation volume, pp. 95-106.

Acknowledgment

Fluorescent Antibody Technique for Detection of *Xanthomonas campestris* **pv.** *Manihotis* on Cassava Leaves

J. F. Daniel and B. Boher

Laboratoire de Phytopathologie
Office de la Recherche Scientifique et Technique
Outre-Mer Centre de Brazzaville, République Populaire du Congo

Abstract

During its disease cycle, *Xanthomonas manihotis* is known to have a "resident" phase on the above ground parts of the cassava plant. The adaptation of the indirect immunofluorescence method allows detection, specifically *X. manihotis* in place on the leaf surface. Two methods are proposed: (1) The impression method, that uses a leaf disc collodion print stuck on adhesive tape and affixed to glass slides on which the immunofluorescence reaction is carried out; and (2) the direct leaf disc method, where leaf discs stuck on adhesive tape are placed on glass slides and stained by the fluorescent antibody technique.

Observations of prints and leaf discs are made with a microscope, using reflected light fluorescent microscopy. A schedule of operations is presented. Preliminary results of cassava leaf surface colonization by *X. manihotis* in fields and after artificial inoculations are given and the limitations of these methods are discussed.

Introduction

The epiphytic phase of *X.c.* pv. *manihotis* on the phylloplane of cassava has been reported previously (5, 11). The study of this phylloplane bacteria has involved plating and counting methods or detection by leaf infiltration methods. These methods have shown that the pathogen is able to survive on the cassava leaf surface for extended periods. However, leaf characteristics have not been considered in relation to these methods. A better understanding of the leaf-bacteria interaction may be gained by methods using direct observation of the cassava blight bacteria "in situ." Direct examination of phylloplane bacteria has been attempted using light (4, 9) and scanning electron microscopy (10) but relatively few studies concern the detection "in situ" of plant pathogenic bacteria on the leaf surface (3).

In this paper, we propose two methods of direct observation of cassava blight bacteria on the leaf surface that use the sensitivity and specificity of the fluorescent antibody technique.

Materials and Methods

Preparation of Antisera

Antisera were prepared against whole washed heat killed cells (1 hour, 100°C) of a strain of *X. c.* pv. *manihotis* (A 104-2) isolated from infected cassava stem in Mbé (People's Republic of Congo) by injecting rabbits as described by Coleno *et al.* (2). Gammaglobulin fraction of antisera was precipitated with ammonium sulfate (8). Specificity of antisera was tested against 80 strains isolated from cassava phylloplane and soil.

Preparation of Leaf Samples for Detection of *X. c.* pv. *manihotis* "in situ"

Discs from intercoastal areas of naturally or artifically contaminated leaves were excised using a 1 cm diameter paper punch. These discs were treated according to the following methods:

Impression method. An adaptation of the technique described by Dickinson *et al.* (6) is used. Each leaf disc (upper and lower face) was sprayed with a solution of collodion (5%) mixed gelatin - Rhodamine conjugate (1:480, v/v) prepared according to the method of Bohlool *et al.* (1). The spray of collodion was left to dry for about 20 min. Then, the film was carefully peeled off and stuck (contact surface uppermost) on adhesive tape (Scotch Magic), that was itself affixed to a glass slide.

Direct leaf disc method. The leaf disc was directly stuck on adhesive tape, upper or lower face uppermost and as described above placed on a glass slide.

Prior to the fluorescent antibody staining, the leaf discs and prints were pretreated by soaking them in a mixture of Tween 80 and phosphate buffered saline (one drop of Tween 80 in 100 ml of saline) for 5 minutes and gently washing (7). This increased the wettability of samples.

Fluorescent antibody staining. The fluorescent antibody technique (FAT) was conducted according to indirect method. The print or leaf disc surface was covered with the antiserum anti-*X.c.* pv. *manihotis* (previously passed through 0.45 nm filter and incubated for 30 min at room temperature. The preparation was washed in 0.1 M phosphate buffered saline (PBS) pH 7.2, for 20 min. Then, the commercial fluorescent antirabbit globulin (fluorescein isothiocyanate conjugated antirabbit globulin, Institut Pasteur Production) was applied over the surface of the print or disc and allowed to stain again for 30 min. The slide was gently rinsed in PBS for 20 min. The preparation was mounted with a cover slip using glycerolphosphate buffer solution as mounting fluid.

Fluorescence microscopy. Stained preparations were examined under an X 40 objective fitted to a Leitz Orthoplan microscope with incident fluorescence illumination from an HBO 200 OSRAM mercury light source (BG 12:OG-1 filter system).

Results

The impression and leaf disc methods associated with fluorescent antibody technique (FAT) allow detection and give clear images of spatial distribution of *X.c.* pv. *manihotis* on the cassava leaf surface (Fig. 1-2).
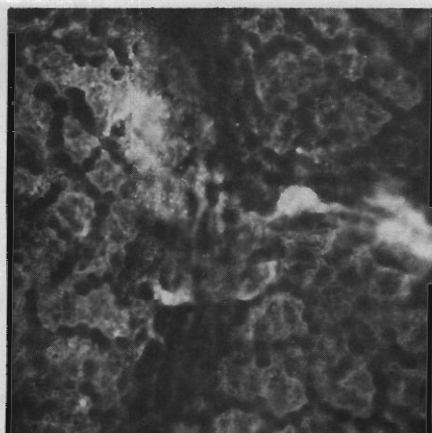
Fig. 1. Undersurface of cassava leaf showing clustered cells of *X. c.* pv. *manihotis* on lamina areas contiguous to veins (impression method).

Fig. 2. Undersurface of cassava leaf showing cells of *X. c.* pv. *manihotis* on epidermal cells of vein (leaf disc method).

The impression method, which has been tested on upper and lower leaf faces of 10 cultivars, was used with some success. The mixture of gelatine-rhodamine conjugate with collodion solution allows us to obtain a suitable background (orange-brown) for epifluorescence observations. The use of unconjugated gelatin, as described by Buhlool *et al.* (1), prior to the fluorescent antibody staining, may be useful to limit nonspecific fluorescence due to absorption of stain by mechanisms other than immunologic reactions.

The direct leaf disc method was more satisfactory for visualizing the geographical localization of bacteria on the leaf surface structures (epidermal cells, veins, guard cells of stomata). For the cultivars tested, the background was red to brown, in good contrast to the green fluorescein labeled bacteria. As previously noted the treatment of discs with the unconjugated gelatine prior to FAT provided a good control of nonspecific staining.

Fluorescent antibody staining of leaf discs after the collodion film had been peeled off revealed that bacteria were retained on the leaf surface, particularly on the lower face where the surface is uneven and in areas where the bacteria were densely clustered.

The cassava blight bacteria were not distributed homogeneously throughout the leaf lamina (Fig. 3). The bacterial cells occurred generally in depressions between epidermal cells and were clustered in small (5 to 20) groups. Single cells were rarely detected, except after rains. These microcolonies were preferentially detected on veins and on the adjacent lamina areas. The observation studies carried out on 10 cultivars naturally infested suggested that the micro-colonies of pathogen were more numerous on lower than on upper leaf surfaces. On the areas of lower

178
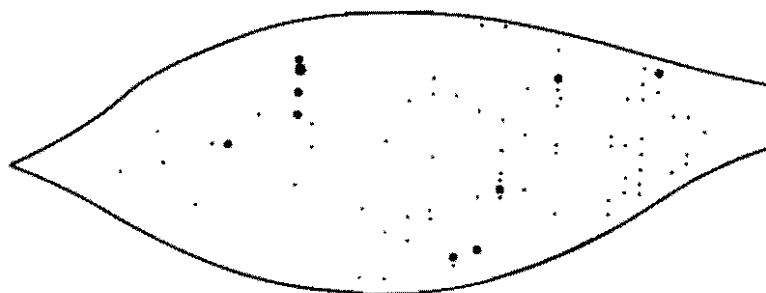
Fig. 3. Example of distribution of micro-colonies of *X. c.* pv. *manihotis* on the undersurface of lobe of cassava leaf (leaf disc method).

surfaces contiguous to veins, the bacteria were frequently absorbed to digitated epidermal cells that surround the stomata. Observations have shown the presence of bacteria clustered on the guard cells of stomata. On the same leaves, by the same methods, bacteria could be detected on some lobes.

Preliminary field experiments with artificial inoculations by pulverization of 3 months old cassava plants, demonstrated that, initially, the bacteria occurred throughout the lamina as single cells and sometimes as clusters due to the low degree of wettability of the surface. About two weeks after inoculation only clustered bacteria were detected with a preferential location on the lower face of the leaf.

Discussion

The sensitivity of the fluorescent antibody technique associated with the impression or leaf disc method allows us to detect, specifically, the cassava blight bacteria in place and to give clear images of its spatial distribution on the leaf surface. The leaf disc method is simpler than the impression method and overcomes some of the problems associated with it, particularly the retention of bacteria on the leaf surface after peeling off the collodion film. Moreover, this method using the epifluorescence system allows us to clearly visualize the bacteria in place on the leaf structures such as epidermal cells, veins, and guard cells of stomata and to detect early, the yellowish microlesions in good contrast with the red fluorescence of the leaf lamina. For the cultivars tested, autofluorescence of leaf tissues was not a problem. However, in cultivars where autofluorescence might interfere with fluorescent antibody detection of *X.c.* pv. *manihotis*, the method described by Daft & Leben (1966) (4) for bleaching leaves with chlorine gas might overcome this inconvenience. The printing method may be used with success for examination of fruits, seed, and stem surfaces.

The first results of the use of these methods with 10 cultivars, tested in natural conditions, suggest that the distribution of *X.c.* pv. *manihotis* on the cassava leaf is not homogeneous. The cassava blight bacteria seemed to occur more preferentially on lower than on upper faces of the leaves and were distributed in groups and, more rarely, as single cells. In other parts of

the same contaminated leaf, taking the leaf shape into account (5-6 lobes per leaf), we have heterogenous contamination of lobes, with some being bacteria free. The bacteria were often localized in depressions between epidermal cells of lamina, in particular, in the areas contiguous to veins and on veins.

On the lower leaf face, the bacteria were found on the digitate epidermal cells that surround the stomata along the veins. Some preparations showed the presence of the pathogen clustered on the guard cells of stomata.

The fact that the micro-colonies of *X.c.* pv. *manihotis* seem to occur preferentially on the undersurface of the cassava leaf where the stomata are numerous, suggests that this face may constitute the most suitable ecological niche for the development, maintenance, and acitivity of the populations of *X.c.* pv. *manihotis* on the cassava phylloplane. However, the role of the pathogen on the upper face of the leaf must not be neglected. These methods could be used with some success for the detection of surface-borne cassava blight bacteria "in situ." However, they have limitations in that they do not separate living and dead bacteria and do not permit counting due to distribution of the bacterial cells in dense clusters.

Literature Cited

1. Bohlool, B.B., and E. L. Schmidt. 1968. Nonspecific staining: Its control in immunofluorescence examination of soil. Science 162:1012-1014.
2. Coleno, A., L. Hingand, and M. R. Barzic. 1970. Contribution á l'étude sérologique de *Pseudomonas phaseolicola* (burk) Dowson. Annales de Phytopathologie 2.199-207.
3. Coleno, A., L. Hingand, and B. Rat. 1977. Une tecnique d'étude de la phase épiphyte de *Xanthomonas vesicatoria*. (Doidge) Dowson. Annales de Phytopathologie: 79-83.
4. Daft, G.C., and C. Leben. 1966. A method for bleaching leaves for microscopy investigation of microflora on the leaf surface. Plant Dis. Reptr. 50:493.
5. Daniel, J.F., and B. Boher. 1978. Ecology of cassava bacterial blight: Epiphytic survival of *Xanthomonas manihotis* on aerial parts on the cassava plant. Proc. 4th Int. Conf. Plant Path. Bact. Angers. 2:763-771.
6. Dickinson, C. H., J. Watson, and B. Wallace. 1974. An impression method for examining epiphytic microorganisms and its application to phylloplan studies. Trans. Brit. Micol. Soc. 63:616-619.
7. Dupouey, P. 1977. L'exécution des réactions d'immunofluorescence directes et indirectes sur lame. Pages 67-72 in 'Les techniques de l'immunofluorescence et les réactions immunoenzymatigues'. Cours de l' Institut Pasteur. Maloine S.A. Editeur, Paris. 566 pp.
8. Hebert, B. A., B. Pittman, R. M. McKinney, and W.B. Cherry. 1972. The preparation of physiological characterization of fluorescent antibody reagents. Pp. 9-16 in Annu. Rep. U.S. Dep. Health Educ. Welfare, Center for Disease Control, Atlanta, GA. 41 pp.
9. Leben, C., and G. C. Daft. 1967. Population variations in epiphytic bacteria. Can. J. Microbiol. 13:1151-1156.
10. Leben, C. 1969. Colonization of soybean buds by bacteria: observation with the scanning electron microscope. Can. J. of Microbiology 15:319-320.
11. Persley, G. J. Epiphytic survival of *Xanthomonas manihotis* in relation to disease cycle of cassava bacterial blight. Proc. 4th Int. Conf. Plant Path. Bact. Angers. 2 773-777.

Loss in Mango Fruit Due to Bacterial Canker *Xanthomonas mangiferaeindicae*

Ram Kishun

Indian Institute of Horticultural Research
Bangalore, India

Abstract

Field trials were conducted to find out the losses in Alphanso, Pairi, Totapuri, and Local mango varieties caused by bacterial canker. Per cent drop of fruit from trees in Alphanso, Pairi, Totapuri, and Local varieties were 20 to 80, 10 to 70, 10 to 40, and 10 to 55, respectively. Maximum fruit drop was recorded in all varieties 30 to 60 days after fruit set. Fruit drop from the tree was more where stem end and fruit surface were injured and inoculated with the bacteria. Fruit rot in storage was 5 to 80, 10.6 to 66.6, 10 to 100, and 5 to 80 per cent in Alphanso, Pairi, Totapuri, and Local varieties, respectively. Maximum rot was in surface injured fruit and least in uninjured fruit. There was no storage rot in uninoculated fruit.

Introduction

Bacterial canker caused by *Xanthomonas mangiferaeindicae* (Patel, Moniz and Kulkarni; Robbs *et al.*) is one of the important diseases of mango. The disease was first reported by Patel *et al.* (7) from India in 1948. Subsequently, the disease was noticed in other parts of the world (1, 2, 3, 4, 5, 8, 10, 11, 12). It infects leaves, petioles, branches, and fruit and causes heavy crop loss when the fruit are infected (9). Recently, the author noticed bacterial canker in severe form on varieties of mango in Southern India. Most of the commercial varieties were susceptible to bacterial canker and the percentage of fruit infection under natural conditions ranged from 10 to 70 (Ram Kishun, unpublished).

Field experiments were conducted to estimate the losses in yield in four commercial varieties of mango.

Materials and Methods

Trials were conducted at the Indian Institute of Horticulture Research Farm, Hessaraghatta, Bangalore during February to June, 1980 on mango varieties Alphanso, Pairi, Totapuri, and Local. Fruit were inoculated with or without injury at different stages of development, i.e. 30, 45, 60, 75, and 90 days after fruit set. Fruit were injured at the stem end, surface, and stem end + surface at all the stages of inoculation. The injured and

uninjured fruit were spray inoculated with 48-hr-old culture of bacterium suspended in water (10^7 cells/ml). Control fruit were sprayed with water alone. Sixty fruit per treatment per variety were used at different stages of fruit development. Data on fruit drop were recorded separately for all the treatments and undetached fruit were harvested and stored at room temperature (22 to 28°C) for ripening and recording of storage rot.

Results

Per cent fruit drop in varieties Alphanso, Pairi, Totapuri and Local varied from 20 to 80, 10 to 70, 10 to 40, and 10 to 55, respectively (Table 1). Maximum fruit drop in all the varieties was recorded when fruit were inoculated at the age of 30 to 60 days after fruit set. In early stages, fruit drop was also recorded in the control but it was less than that of inoculated fruit. In later stages of fruit development, drop was recorded only in inoculated fruits. Fruit drop from the tree was more in all the varieties when both stem end and fruit surface were injured and inoculated. Uninjured inoculated fruit had least fruit drop. Fruit drop in the control was observed up to 45 days in Pairi, Totapuri, and Local, whereas in Alphanso it was recorded up to 30 days.

Storage rot varied from 5 to 80, 10.6 to 66.6, 10 to 100, and 5 to 80 per cent in varieties Alphanso, Pairi, Totapuri, and Local, respectively. Maximum storage rot was found in surface injured fruit. Minimum storage rot was observed in uninjured inoculated fruit of all the varieties. Alphanso had noticeable storage rot when fruit were inoculated 30 days after fruit set. Pairi and Totapuri had storage rot when fruit were inoculated at the very late stage of 60 and 75 days after fruit set, respectively, whereas Local variety had storage rot when fruit were inoculated at 45 days after fruit set. There was no storage rot in uninoculated control fruit .

Discussion

Fruit drop due to bacterial canker was observed in all the varieties tested; however, it was less in Totapuri. Young fruit (30 to 60 days old) were more susceptible than old ones. The reasons are not known. Fruit drop was more where both stem and fruit surface were injured and inoculated. It is possible that the stem end becomes weak due to infection of bacterial canker, causing fruit to drop from trees with only a slight jerk. It is also possible that due to surface infection other microorganisms attack the fruit , causing decay and fruit drop. Similar observations were made by Shekhawat and Patel (9). They observed 20 to 26 per cent decaying of mango fruit before harvest.

Storage rot of 5 to 100 percent was recorded in different varieties of mango. Other workers have observed storage rot in mango due to bacterial canker disease. Shekhawat and Patel (9) have reported 20 to 47 percent rot during ripening in storage. Severe bacterial rot in mango fruit after 3 days of storage at room temperature was observed by Palaniswami *et al.* (7).

Table 1. Loss of mango fruit in four varieties due to bacterial canker.

| Name | Treat-ment | Manual inoculation days after fruit set | | | | | | | | | |
| | | 30 | | 45 | | 60 | | 75 | | 90 | |
		Percent fruit drop	Percent storage rot	Percent fruit drop	Percent storage rot	Percent fruit drop	Percent storage rot	Percent fruit drop	Percent storage rot	Percent fruit drop	Percent storage rot
Alphanso	A	70.0	77.7	80.0	50.0	40.0	16.6	20.0	50.0	20.0	50.0
	B	80.0	25.0	70.0	0.0	60.0	25.0	40.0	16.6	25.0	20.0
	C	70.0	33.3	80.0	0.0	50.0	50.0	40.0	77.7	25.0	40.0
	D	45.0	27.7	33.3	5.0	30.0	0.0	30.0	0.0	20.0	0.0
	E	45.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Pairi	A	70.0	66.6	60.0	50.0	30.0	50.0	20.0	50.0	10.0	55.5
	B	70.0	27.7	60.0	0.0	50.0	30.0	30.0	0.0	10.0	22.2
	C	70.0	66.6	60.0	50.0	70.0	66.6	40.0	50.0	10.0	50.0
	D	60.0	0.0	60.0	0.0	20.0	0.0	21.6	10.6	10.0	0.0
	E	50.0	0.0	10.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Totapuri	A	30.0	50.0	25.0	22.0	10.0	100.0	0.0	90.0	0.0	100.0
	B	40.0	0.0	30.0	0.0	10.0	0.0	10.0	22.2	0.0	40.0
	C	40.0	66.6	30.0	21.2	10.0	100.0	20.0	100.0	0.0	100.0
	D	35.0	0.0	30.0	0.0	0.0	45.0	10.0	50.0	0.0	0.0
	E	30.0	0.0	35.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Local	A	50.0	40.0	50.0	80.0	33.3	80.0	10.0	50.0	0.0	55.0
	B	55.0	0.0	50.0	0.0	50.0	60.0	50.0	40.0	5.0	26.3
	C	55.0	48.1	50.0	33.3	50.0	80.0	40.0	25.0	10.0	50.0
	D	30.0	0.0	33.3	25.0	33.3	5.0	10.0	0.0	0.0	0.0
	E	40.0	0.0	30.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0

A - Surface injury   B = Injury at stem end   C = Injury at stem end + surface
D - Without injury   E - Control (without inoculation)

Literature Cited

1. Ciferri, R. 1960. Report of bacterial disease of mango in the Juba Valley, Somaliland. Att. Ist. bot. Univ. Pavia Ser. 5, 17:167-170.
2. Daniel J. F., L. Gardan, J. Lusetti, and J. P. Prunier. 1975. Identification of the causal agent of black spot disease of mango (*Mangifera indica*). Fruits. 30 (1):625-630.
3. Khan, A., and M. Kamal. 1966. Studies on bacterial spot of mango in Pakistan. West Pak. J. Agric. Res. 4:126-139.
4. Liao, C. H. 1972. Studies on mango fruit spot. I. Symptoms and causal organism. J. Taiwan Agric. Res. 21 (2):146-150.
5. Moffett, M. L., R. A. Peterson, and B. A. Wood. 1979. Bacterial black spot of mango. Australian Plant Pathol. 8 (4):54-56.
6. Palaniswami, A., S. Muthuswamy, and C. S. Krishnamurthy. 1972. Bacterial rot of stored mangoes. South Ind. Hort. 20 (1/4):102-103.
7. Patel, M. K., L. Moniz, and Y. S. Kulkarni. 1948. A new bacterial disease of *Mangifera indica* L. Curr. Sci. 27 (6):189-190.
8. Robbs, C. F." P. L. D. Ribeiro, D. Kumura, and F. Akiba. 1973. Studies on the white Xanthomonads in Brazil. 2nd. Intern. Congr. Plant Path. Minneapolis, Minnesota, Abst. Pap. Abstr. 1046.
9. Shekhawat, C. S. and P. N. Patel. 1975. Studies on bacterial canker of mango. Zeitschrift fur Pflanzenkrankeiten und Pflanzenschutz. 82 (3):129-138.
10. Steyn, P.L., N. M. Viljoen, and J. M. Kotze. 1974. The causal organism of bacterial black spot of mango. Phytopathology 64:1400-1404.
11. Tarr, S. A. J. 1957. Recent observations on plant disease in the Sudan. FAO Pl. Prot. Bull. 5(12) 188-190.
12. Viljoen, N. M., P. L. Steyn, and J. M. Kotze. 1972. Bacterial black spot of mango. Phytophylactica. 4(3):93-94.

Distribution of *Pseudomonas solanacearum* in Tropical Soil of Peru

C. Martín, E. Torres, U. Nydegger

International Potato Center (CIP)
Lima, Peru

Abstract

The presence and distribution of *P. solanacearum* at three soil depths (0-30, 30-60 and 60-90 cm) was determined in two infested potato fields in the tropics of Peru (Huanuco 2400 m and San Ramon, 1150 m elevation) at harvest time and 140 days later after bare fallow.

Potatoes cv. Ticahuasi, tomatoes cv. Huando and a new selective medium developed at the University of Wisconsin were used to assay bacterial populations. The selective medium plated with soils collected at harvest time indicated that *P. solanacearum* was present at all three depths at both locations. However, populations of bacteria were much greater at 0 to 30 cm for both fields, and also much higher at San Ramon than at Huanuco. These results agree partially with those using host plants because at San Ramon tomato was readily infected when planted in soils from 0 to 60 cm depth but not at 60 to 90 cm, and potato failed to demonstrate bacteria at 30 to 90 cm depths. At Huanuco, both host plants demonstrated the presence of bacteria at all depths. Populations of *P. solanacearum* were markedly decreased at all three soil depths after 140 days of fallow at both locations.

Although the number of bacteria detected by the selective medium was much lower, the percentage wilted tomato and potato plants was in general high. The use of both the selective medium and host plants is recommended for determination of presence of *P. solanacearum* in infested soils.

Introduction

Bacterial wilt caused by *Pseudomonas solanacearum* is a very important limiting factor in production of several important food crops, particularly potato, tomato, eggplant, and bananas in tropical, subtropical, and warm temperate regions of the world (7). The ecology of that bacterium, especially its survival in naturally infested soils, is not yet well understood despite numerous efforts (1, 7). Recent research has, however, explained some of the factors involved in the survival of the bacterium under different ecological conditions (2, 3, 9, 10, 13). The concept developed by Graham and Lloyd (2) of "sheltered sites" where the bacterium might survive is of great interest, especially their findings of the pathogen in the deeper soil layers. The studies by Mc Carter *et al.* (9) on the distribution of *P. solanacearum* in soils of Georgia have also contributed substantially to the understanding of the ecology of this organism.

With the exception of a few cases, such as those reported by Navarro (10) and Sequeira (13), most studies on the ecology and survival of *P. solanacearum* have been done outside the tropical zone, in the Americas. The purpose of the research reported here was to examine the presence and distribution of *P. solanacearum* in infested soil at two tropical locations at harvest time and after 140 days of subsequent fallow, a period long enough for the establishment of deep roots by another crop in the constant cropping system prevalent there.

Materials and Methods

Two fields representative of different ecological zones in which potatoes are grown in Peru, were selected on the basis of previous infestation with *P. solanacearum.* The field at Umari, Huanuco has an elevation of 2400 m. and is representative of mid-elevation potato farming (2,270 mm annual rainfall, 6.3°C minimum and 20.5°C maximum, average). It had been used for two years as a site for the final field screening of potato clones developed for resistance to bacterial wilt at the International Potato Center (CIP) installations in Lima. Wilt incidence in the susceptible potato "Ticahuasi" interplanted with the test plants was close to 80%. The field at San Ramon, 1150 m elevation, is on the first slopes to the Amazon jungle (2700 mm rainfall, 18.5°C minimum and 29.5°C maximum, average).

Potatoes are not normally grown at San Ramon; they were introduced to this area in 1974 by CIP as part of their adaptation studies. In 1978 one of these fields, previously in pineapples, was planted with potatoes and a large incidence of bacterial wilt resulted. Later studies have shown that the bacterium is native in this soil (C. Martin and E. R. French, unpublished). This field has been used as a testing site for resistance under higher temperatures and to (possibly) different strains.

Soils at both locations were sampled during 1980 at potato harvest time and after 140 days of fallow, using the methods described by Mc Carter *et al.* (9). A square hole was dug and soil samples were collected from each 30 cm increment down to a depth of 90 cm. Ten holes were dug in each field at sites previously occupied by infected plants. Tools used in collecting soil were rinsed in 95% alcohol and flamed after each layer of soil was collected. Soil sampled from each layer at each location was collected separately, placed in plastic bags, mixed by hand and then potted in 15 cm diameter clay pots. A sample of soil from each layer was kept for soil analysis.

Fields at both locations were bare fallowed and 140 days later soil samples were again collected as described above. The fallow corresponded for both locations with the dry season, although scattered rains usually fall during this time at San Ramon.

To detect *P. solanacearum* in the soil, two susceptible hosts and a selective medium were used. Two healthy tomato plants (*Lycopersicon esculentum* "Huando"), about 12 cm tall, were transplanted to each pot. One plant was later removed. One small potato tuber (*Solanum tuberosum* "Ticahuasi") was also planted at the same time in a different pot. Ten pots for each host plant, per soil layer, and per location were used. A non-

infested, sterilized soil control was added to each experiment. Pots were arranged in a complete randomized block design. Soil samples from San Ramon collected at 30 to 60 and 60 to 90 cm depth were mixed with sterilized vermiculite. Because of their high clay content, they were mixed at the rate of 3 parts of soils to 1 of vermiculite.

Plants were maintained up to the end of the experiment in a glasshouse with a night/day temperature of $26/31 \pm 1°C$. Soil moisture was maintained at near field capacity by repeated watering. As plants wilted they were cut and a section of the stem was placed in a test tube containing sterile water and examined for streaming of bacterial ooze from the vascular system (7). A number of bacterial suspensions were streaked on TZC medium (8) for their identification and some of them were also inoculated to tomato and potato plants.

A random sample of the isolates obtained on the TZC medium were purified and their biotype was determined as described by Hayward (5). At the end of the experiment all remaining living plants were assayed for bacterial streaming. When negative, the suspension water in which the stem end was placed was streaked on TZC medium.

In addition to tomato and potato plants, a selective medium developed by Granada and Sequeira (4) for recovery of *P. solanacearum* from the soil was used. Two grams of soil were added to 8 ml of sterile water. After 1 h of slow agitation in a rotary shaker a dilution series was done. On the basis of previous observations it was determined that the best dilutions were 10^{-3} and 10^{-4}. Then, 0.05 ml of each of the two dilutions was spread over the selective medium, which consisted of the following ingredients added to the TZC medium: crystal violet (50 ppm), merthiolate (0.005%), polimyxin B sulphate (100 ppm), tyrothricin (20 ppm), and actidione (50 ppm). Five plates were done per each dilution and then repeated. Plates were incubated for 48 h at 30°C and then observed for *P. solanacearum* colonies.

Results

Soil Samples Collected at Harvest Time

The bacterium was detected at all three layers and at both locations by the selective medium. Colonies of *P. solanacearum* were clearly distinguished from other colonies and in a few cases of doubtful identification they were transferred to TZC and/or inoculated to tomato or potato plants. The number of colonies was much greater for the three soil layers in samples collected at San Ramon than for those collected at Umari (Fig. 1). The results obtained by the use of the selective medium were in agreement with those obtained from the use of host plants for Umari (Fig. 2); tomato and potato plants wilted in a large percentage when they were planted in soils collected at all three layers. On the other hand, despite the large number of colonies detected at San Ramon, both host plants failed to detect the bacterium in some of the soil layer (Fig. 3); tomato plants failed to detect the bacterium when they were planted in soil collected at 60 to 90 cm depths, and potato wilted only when planted in soil collected at the 0 to 30 cm depth.

The study of the isolates collected from the soil samples at both locations indicates they were all biotype II (race 3, the potato strain), according to
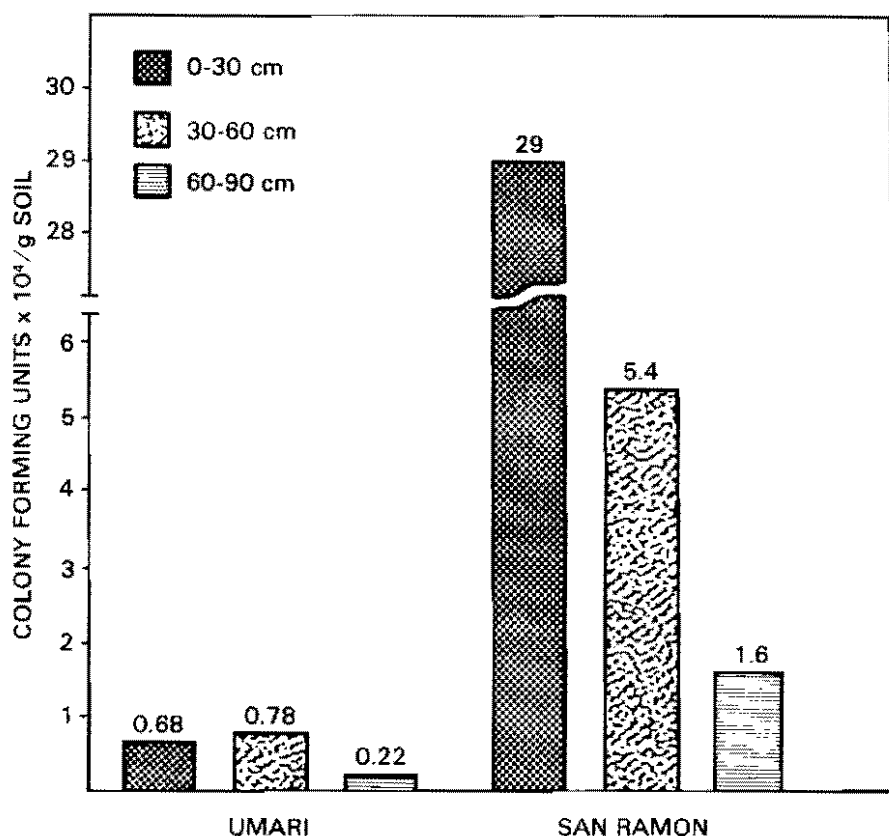
Fig. 1. Initial bacterial populations of *Pseudomonas solanacearum* at two locations and three soil depths in Peru. Samples taken at harvest time, April 1980.

Hayward's classification (5). The analysis of the soil samples collected at both locations indicated that there were only small differences between them in texture and chemical components (Table 1). Probably one of the biggest differences between them was the higher clay content of the samples collected at 30 to 60 and 60 to 90 cm at San Ramon.

Soil Samples Collected 140 Days After Bare Fallow

P. solanacearum was detected on the selective medium in soils collected at both locations and at all but the 0 to 30 cm soil layer at Umari (Table 2). The populations of the bacterium detected at both locations were substantially lower than those detected at harvest time. Despite the drastic reduction, both tomato and potato plants detected the bacterium at all three soil layers at both locations with the exception that tomato plants failed to detect the bacterium in soil sample collected at 60 to 90 cm depth at Umari (Figs. 4 and 5). On the other hand, although the selective medium did not detect the bacterium in soil samples collected at 0 to 30 cm depth in Umari soil, tomato and potato plants did (Fig. 4).
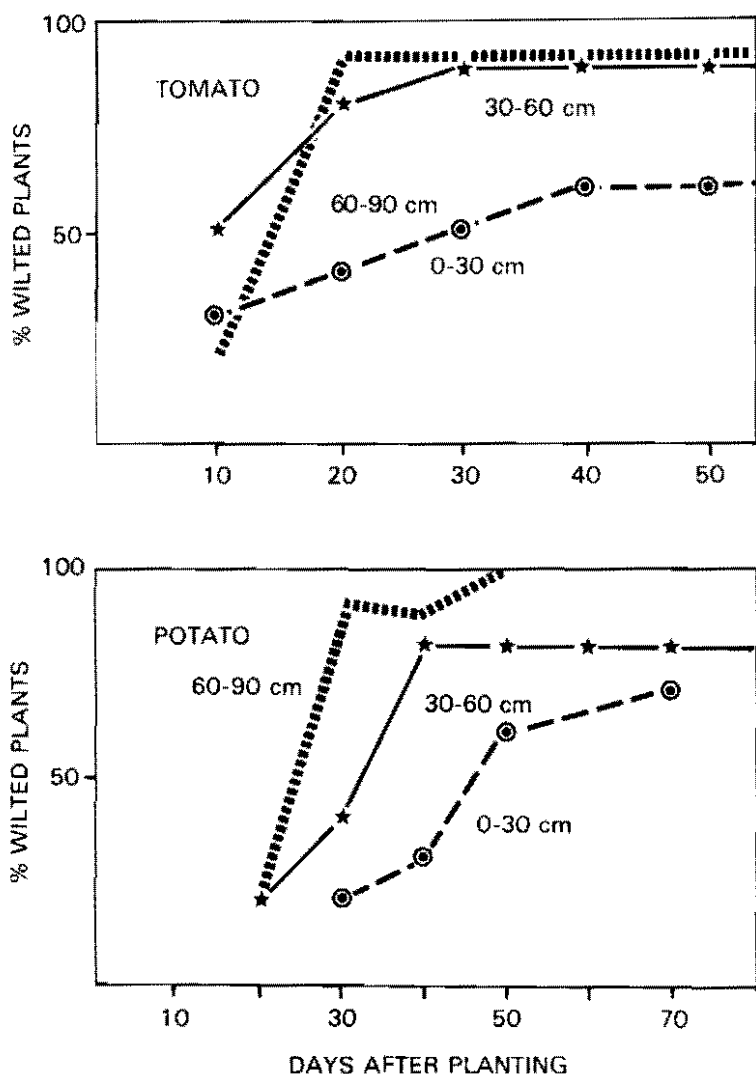
Fig. 2. Percent wilted tomato and potato plants, planted in soil collected at three depths in Umari. Test conducted under glasshouse conditions at 26 to 31°C.

Discussion

P. solanacearum was detected in soils up to a depth of 90 cm at two different tropical locations by the use of a selective medium and/or indicator host plants. In similar type of work only tomato plants were used to detect the presence of the bacterium (2, 9). The greater usefulness of host plants in addition to the culture medium is well indicated in these results. In the case of Umari the medium failed to detect the organism in the
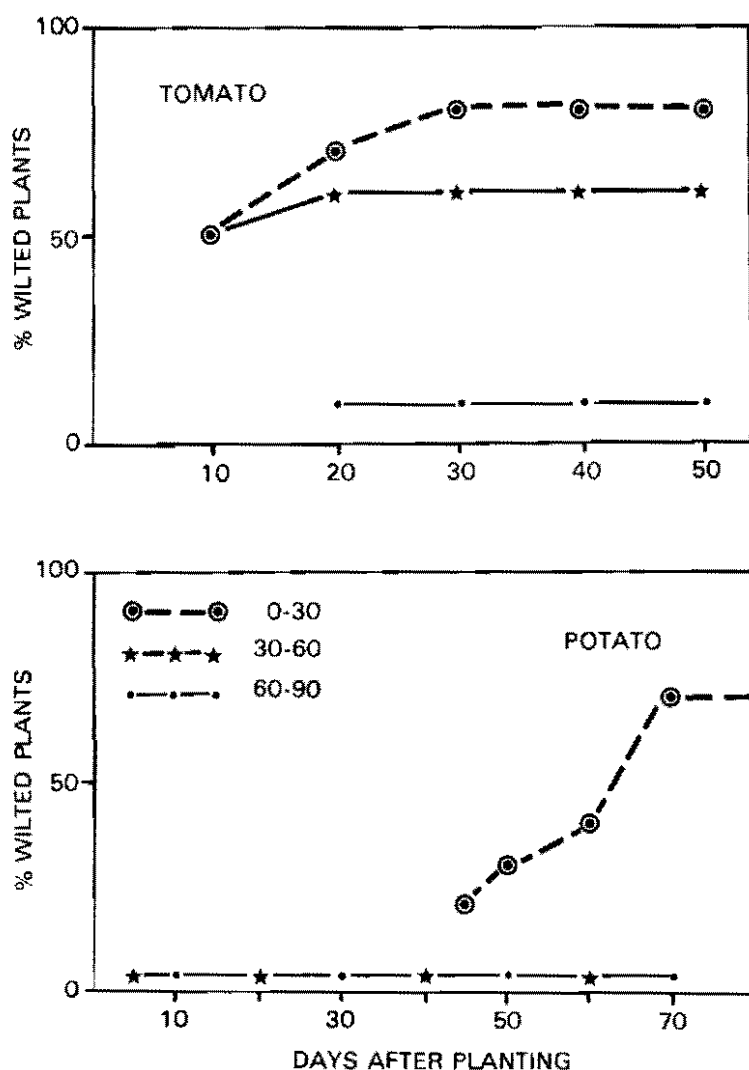
Fig. 3. Percent wilted tomato and potato plants, planted in soil collected at three depths in San Ramon. Test conducted under glasshouse conditions at 26 to 31°C.

0 to 30 cm soil layer after 140 days of fallow, but both test plants detected it. Apparently, the bacterial population had fallen below the limits of detectability by the plating technique.

Although the bacterium was detected at the three soil layers at both locations, there were some differences in the bacterial populations and their distribution. The bacterial populations were much greater at San Ramon than at Umari both at harvest time and 140 days later. The highest bacterial population was at the 0 to 30 cm soil layer at San Ramon but at

Table 1. Principal characteristics of soils collected at three depths at Umari (Huanuco) and San Ramon (Junin), Peru.

Location and Soil Depth (cm)	Texture	pH	Organic Matter (%)	Electr. cond. (mmhos/cm)	Al (me/100 g)
UMARI					
0-30	Loam-clay	5.1	3.0	0.6	0.82
30-60	Loam-clay	5.0	2.3	0.4	1.47
60-90	Clay	5.2	2.1	0.2	1.36
SAN RAMON					
0-30	Sandy loam	4.8	4.0	0.3	1.75
30-60	clay	4.7	2.2	0.5	1.45
60-90	clay	4.8	1.6	0.3	0.63

Table 2. *Pseudomonas solanacearum* populations at Umari (2400 m) and San Ramon (1150 m) at three soil depths at harvest time and 140 days later after bare fallow as determined by plating on selective medium.

Soil Depths (cm)	Cells × 10^4 per gram of soil			
	Umari		San Ramon	
	Harvest	140 days	Harvest	140 days
0-30	0.68[1]	0.00	29.90	6.15
30-60	0.78	0.22	5.40	0.65
60-90	0.22	0.08	1.60	0.23

[1] Average of two repetitions (5 plates/repetition).

Umari the highest was in the 30 to 60 cm layer. These differences and also the extremely low population found at the 0 to 30 cm soil layer after 140 days of fallow at Umari might be a result of less rainfall, heavier soil, and a difference in root development. Dessication has been named repeatedly as one of the major factors in reducing soil populations of *P. solanacearum* (2, 3, 7, 9, 14). During the fallow period rains were absent at Umari. At San Ramon scattered showers fell regularly; thus the top soil did not dry out.

McCarter *et al.* (9) found that the highest population of *P. solanacearum* in soils of Georgia occurred in the top 30 cm layer. In Graham and Lloyd studies on potatoes (2) and those of Okabe (12) and Tanaka (14) on tobacco in Japan, the investigators found the highest concentrations at deeper soil
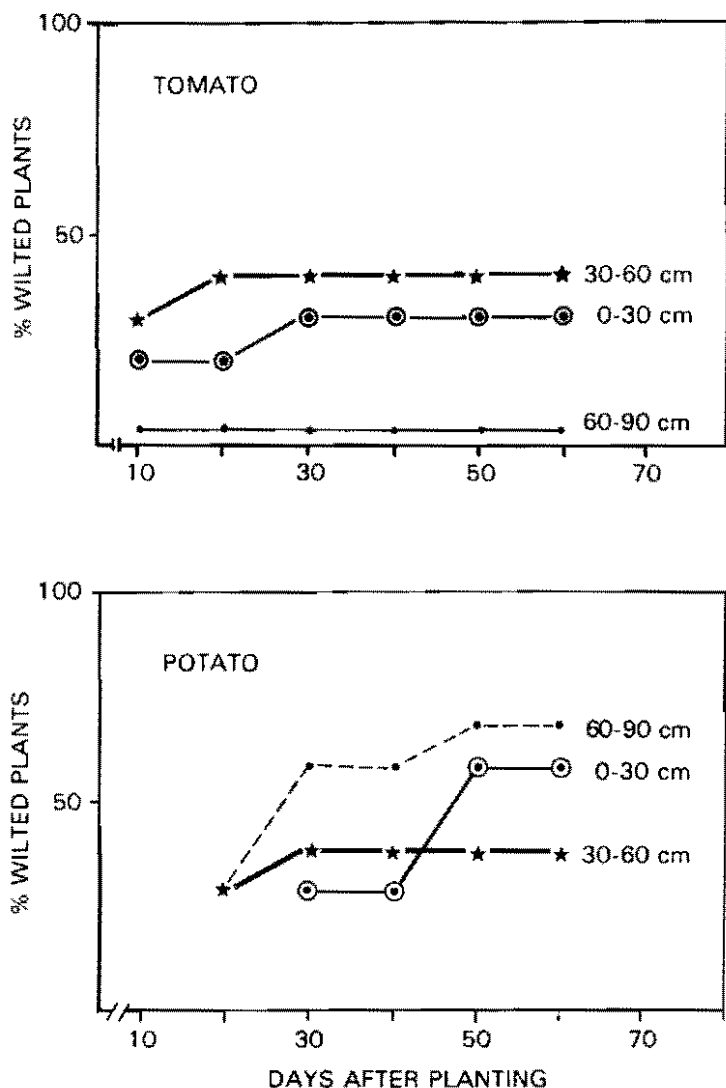
Fig. 4. Percent wilted tomato and potato plants, planted in soil collected at three depths in Umari 140 days after bare fallow. Test conducted under glasshouse conditions at 26 to 31°C.

layers. Our results varied, depending on the location. At Umari *P. solanacearum* survived in higher numbers in the deeper soil layers; at San Ramon the opposite occurred, and large bacterial populations survived in the top soil layers, even 140 days after bare fallow.

Fallow practices have reduced the soil population of *P. solanacearum* in several instances (10, 13, 14). Although our fallow was not very long it caused a drastic reduction of the bacterial population at both locations. Undoubtedly, an integrated control approach that could include fallow,
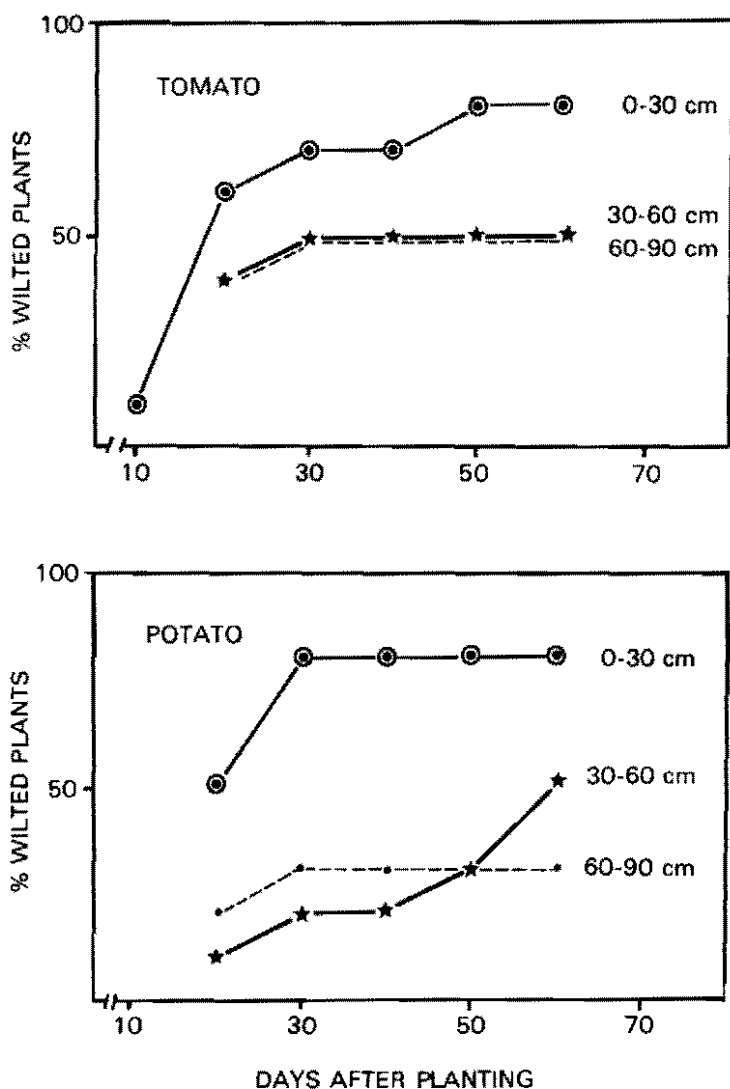
Fig. 5. Percent wilted tomato and potato plants, planted in soil collected at three depths in San Ramon 140 days after bare fallow. Test conducted under glasshouse conditions at 26 to 31°C.

elimination of susceptible weed and infected potato debris, rotation, and use of clean seed would reduce the incidence of bacterial wilt significantly.

The selective medium developed by Granada and Sequeira (4) demonstrated in this work the usefulness and greater sensitivity than previously developed selective media (6, 11). Its ease of preparation and clear distinction of *P. solanacearum* among other organisms make this medium of practical use. However, the use of host plants has been shown

to be more sensitive for detection of *P. solanacearum,* especially when populations are low. A further advantage of the use of host plants is that they provide an indication of the pathogenicity of the strains involved.

Literature Cited

1. Buddenhagen, I. W. and A. Kelman. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum.* Annu. Rev. Phytopathol. 2:203-230.
2. Graham, J. and A. B. Lloyd. 1979. Survival of potato strain (race 3) of *Pseudomonas solanacearum* in the deeper soil layers. Austr. J. Agr. Res. 30:489-496.
3. Graham, J., D. A. Jones, and A. B. Lloyd. 1979. Survival of *Pseudomonas solanacearum* race 3 in plant debris and in latently infected potato tubers. Phytopathology 69:1100-1103.
4. Granada, G. A. and L. Sequeira. 1981. A selective medium for *Pseudomonas solanacearum* (Abstr.). Phytopathology 71:220.
5. Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum.* J. Appl. Bacteriol. 27:265-277.
6. Karganilla, A. D., and I. W. Buddenhagen. 1972. Development of a selective medium for *Pseudomonas solanacearum.* Phytopathology 62:1373-1376.
7. Kelman, A. 1953. The bacterial wilt caused by *Pseudomonas solanacearum* N. C. Exp. Sta. Tech. Bull. 99. 194 pp.
8. Kelman, A. 1954. The relationship of pathogenicity in *Psedomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.
9. Mc Carter, S. M., P. D. Dukes, and C. A. Jaworski. 1969. Vertical distribution of *Pseudomonas solanacearum* in several soils. Phytopathology 59:1675-1677.
10. Navarro, R. A. 1975. Supervivencia de *Pseudomonas solanacearum* E. F. Smith en suelos cultivados con papa. Notas Fitopatológicas 4 (2):160-166. (Colombia).
11. Nesmith, W. C., and S. F. Jenkins, Jr. 1979. A selective medium for the isolation and quantification of *Pseudomonas solanacearum* from soil. Phytopathology 69:182-185.
12. Okabe, N. 1971. Population changes of *Pseudomonas solanacearum* and soil microorganisms in artifically infested natural field soil. Ref. Plant Prot. Res. 4:105-108.
13. Sequeira, L. 1962. Control of bacterial wilt of bananas by crop rotation and fallowing. Trop. Agric. (London) 39:211-217.
14. Tanaka, Y. 1976. Factors affecting survival of *Pseudomonas solanacearum* pp. 122. L. Sequeira and A. Kelman (eds.) Proc. 1st Intern. Plann. Conf. and Workshop on the ecology and control of bacterial wilt caused by *Pseudomonas solanacearum.* July 18-23, NCSU, Raleigh. 166 pp.

Tropical Rainforest Vegetation's Influence on Survival of
Pseudomonas solanacearum

E. R. French, C. Martin, U. Nydegger

International Potato Center (CIP)
Lima, Peru

Abstract

After the tropical rain forest vegetation of altisols in the Amazon basin at Yurimaguas (Loreto Department), Peru, is slashed and burned, bacterial wilt is one of the ever present pathogens of the potato. Two of the most common *Heliconia* spp. (Amarilla and Cresta de Gallo) from the jungle floor were collected and attempts were made to isolate *Pseudomonas solanacearum* without success. Seeds were collected from two common solanaceous shrubs, cocona *Solanum topiro* HBK and Suikawito (unidentified), and from the most common solanaceous weed found among potatoes, *S. nigrum*.

These plant species were grown in a fiberglass house (28-32°C) at Lima and inoculated by soil infestation with 40 ml of 2×10^9 cells/ml per pot of CIP isolates No. 015 (Tea Gardens, Huanuco, 1600 m elevation), 042 (Huancayo, Junin, 3300 m), 077, 141, 108, and 142 (Yurimaguas, Loreto, 180 m). The first four are Bv. I (race 1), the last two Bv. II (race 3). *S. nigrum* was infected by all isolates except 015. Cocona and Suikawito were not infected. One of ten Amarilla were infected by isolates 077 and 141, but Cresta de Gallo was not. When Ticahuasi potato tubers were planted in the pots next to inoculated but healthy looking Cocona, Suikawito, Cresta de Gallo, and Amarilla, the percentages that wilted were 80, 13, 53, and 38%, respectively. *P. solanacearum* infected very few Heliconia but a high percentage of *S. nigrum* and it persisted in differing degrees in the rhizosphere of all plant species tested under artificial conditions. Heliconias and solanaceous plants may be acting as reservoirs of *P. solanacearum* strains that bring about bacterial wilt of potatoes. Such a role is known for heliconias with bacterial wilt of bananas in Costa Rica. Potatoes and bananas share the characteristic of being exotic in the tropical American rain forest.

Introduction

The tropical rain forest vegetation of old, leached, alluvial altisols of the upper Amazon basin in Peru remained mostly undisturbed until population pressure during this century resulted in the full use of the more fertile active flood plain (7). Crops grown on these old lateritic soils in rotation with 8 to 10 years of jungle regrowth have included cassava, rice, plantain, and banana; more recently maize, soybeans, peanuts, cowpea, kudzu, and Castilla grass have been grown at Yurimaguas, utilizing modern soil management technology (1).

Wilt diseases have not been noticed in these crops, but when potatoes were grown in a lowland tropics adaptation program of the International Potato Center, bacterial wilt caused by *Pseudomonas solanacearum* E. F. Sm. biovars (Bv.) I and Bv. II (races 1 and 3, respectively) developed each season in newly cleared land, including one instance of virgin jungle (10). This problem had been anticipated (16) but available resistant clones are not adapted to hot conditions and their resistance is not expressed at high temperatures (4).

Severe outbreaks of bacterial wilt (*P. solanacearum* race 2, strain B) of bananas occured in Costa Rican plantations. *Heliconia latispatha* and *H. caribaea* were found to have, respectively, low and very low incidence of wilt in abandoned banana plantations destroyed by wilt disease. *H. latispatha* and two other species, *H. acuminata* and *H. imbricata,* were occasionally found diseased, with distortion and slow wilting symptoms, in virgin jungle, caused by the D strains of race 2. When repeatedly cut back the disease spread mechanically to other clumps of these heliconias. Bananas planted in cleared jungle developed distortion and slow wilt not characteristic of B strain wilt disease. The D strain from bananas or heliconias caused these symptoms in inoculated plants, whereas the B strain caused rapid wilt. Serial passage of the D strain by inoculation of potted bananas 5 to 8 times at weekly intervals resulted in up to a doubling of the disease index as a result of the selective pressure exerted, which presumably led to the selection of variants arising by mutation. It was concluded that bacterial wilt of bananas arose by a selection pressure exerted by bananas upon strain D, that initially only caused mild symptoms. In the native jungle it is an occasional pathogen of heliconias which, like bananas, are musaceous plants, although the latter are exotic (2, 5, 14, 15).

Race 3 or the "potato" strain is considered to be indigenous to the highland forests of the island of Ceylon and is sometimes endemic in cool climates throughout the world (9, 11), whereas race 1 is indigenous in forests and endemic in cultivated lands throughout the hotter tropics and subtropics (12, 13).

This report encompasses field observations, and inoculations with *P. solanacearum* isolated from potatoes in a tropical rain forest situation to some plants native to that forest, to determine their potential role as sources of inoculum when the forest is cleared and potatoes are planted.

Materials and Methods

The floor of an 8-year jungle regrowth at the Yurimaguas Agricultural Substation (10) next to a recently cleared field in which potatoes had bacterial wilt, was found to be occupied by several *Heliconia* spp. The upper canopy included the fruiting solanaceous shrubs Cocona (*Solanum topiro* HBK) and Suikawito or Suikanito (unidentified). The only common weed in the potato field was *S. nigrum.* No wilt symptoms were observed in any of these plant species. The two most common heliconias, Amarilla (short plant with nearly concealed yellow flowers) and Cresta de Gallo or Cresta (tall plants with a cock's comb-like brilliant red inflorescence), were

uprooted from their respective clumps and flown to CIP's Lima facilities the same day. Seeds of the Solanaceae were harvested.

Prior to potting and five months later, two root segments 2 cm long per heliconia plant were assayed for presence of *P. solanacearum* by first manually shaking them in one tube of sterile water and then placing them in another to permit vascular exudation for 15 min. Both water suspensions were assayed for presence of the bacterium (10). The seed plants were grown in flats, transplanted after 1 month, and the soil was infested 25 days later. By this time the heliconias had been potted for 6 months. Soil consisted of a mixture of 1:1:1 sandy loam soil, sand, and, peat moss with added nutrients. Plants grew in a fiberglass heated house with night lows and daytime temperature high ranging from 26 to 32°C.

Plant Infection

Soil infestation was chosen as the most suitable method of inoculation (3, 8). It was done with 40 ml per 500 ml of 2×10^8 bacteria/ml of Peruvian potato isolates CIP No. 015 from Tea Gardens (1,600 m elevation), Huanuco Department (isolated by I. Herrera); 042 Huancayo (3,300 m), Junin Dept. (H. Torres); and Yurimaguas (180 m), Loreto Dept. isolates 077, 141, 108, and 142. The biovars (6, 10) for these are shown in Table 1. Each pot sat in a clay saucer in which water was constantly maintained and the top of the soil was watered once daily as needed. Symptoms were recorded every 3 days. As plants wilted, the presence of *P. solanacearum* was confirmed by isolation. After 52 days the single stem was aseptically cut at pot-brim level and assayed for presence of the bacterium. The check consisted of potted soil kept moist.

Persistence in Soil

The presence of *P. solanacearum* in the soil or rhizosphere of the test plants was assayed by planting a small tuber seed of Ticahuasi potato next to the test plant the same day their tops were removed. This was not done for *S. nigrum*. Symptoms of wilt in the potato plant or the sprouting original test plant were recorded every 3 days and wilted plants were assayed for the presence of *P. solanacearum*; healthy appearing plants were also assayed after 60 days.

Results and Discussion

Infection

The percentages of infected test plants are shown in Table 1. *S. nigrum* was susceptible to the Bv. I and II Yurimaguas isolates, but more so to the highland Huancayo isolate 042, Bv. I. However, it was not infected by isolate 015. *S. nigrum* has been reported before as a host for *P. solanacearum* Bv. II/race 3 (6). However, it was not regarded as important in maintaining the bacterium in soil of an infested field plot used to screen for resistance to *P. solanacearum* race 3 in Lima (authors, unpublished), and is not likely to compete favorably under the jungle canopy.

Of the jungle hosts tested none developed wilt symptoms. Only *Heliconia* Amarilla were infected (10%) and with only the two Bv. I isolates from

Yurimaguas; stem sections produced streaming in water (as also did root sections after the persistence test). A reduced plant height or dwarfing was apparent. These results suggest it is unlikely that any of these plant species will show wilt symptoms in their natural habitat, unless that environment is more conducive to disease than the one used in these tests. Average temperatures in Yurimaguas are a low of 20 and a high of 30°C, both lower than the respective temperatures used in these tests, so this seems unlikely. This situation thus appears to differ from the one encountered in Costa Rica, when clumps of heliconias wilted in the jungle; they did also when growing in abandoned wilting banana plantations, where presumably the inoculum potential in soil was unusually high.

Persistence

The wilting of potato plants grown to detect the presence of *P. solanacearum* in the soil or rhizosphere of the test plants began around the 19th day after planting for all test plants, a few potatoes developing symptoms as late as the 40th day. Results are shown in Table 1, and illustrated in Fig. 1.

Persistence in soil for Amarilla was not the highest, though it occurred for all isolates, not just the two that caused infection. Persistence was highest for Cocona and it also took place with all isolates. With Suikawito, only the Bv. I Yurimaguas isolates and 042 persisted, whereas with Cresta, the Bv. II isolates did also, but not 042. This may suggest a specificity of association takes place even though disease does not occur. Cresta led to greatest persistence with isolate 141 (Fig. 2), but this difference may not be significant since it was based on only 3 plants. Although persistence was

Table 1.   Percentage of plants infected (Inf.) with isolates of *Pseudomonas solanacearum* 52 days after soil infestation, and of persistance of the bacterium (Per.) determined thereafter by infection of susceptible potatoes planted in the same pots for 60 days more.

Isolates (and their Biovar)	TEST PLANTS[a]										
	S. nigrum		Cocona		Suikawito		Cresta		Amarilla		Check
	Inf.	Per.	Inf.	Per.	Inf.	Per.	Inf.	Per.	Inf.	Per.	Per.
015 (I)	0	—	0	80	0	0	0	50	0	20	0
042 (I)	100	—	0	60	0	40	0	0	0	20	0
077 (I)	30	—	0	100	0	20	0	50	10	50	0
141 (I)	30	—	0	100	0	20	0	100	10	40	0
108 (II)	30	—	0	60	0	0	0	50	0	40	0
142 (II)	20	—	0	80	0	0	0	67	0	60	0
Average	35		0	80	0	13	0	53	3	38	0

[a]Based on 10 plants of each, except for Cresta in which there were 2 or 3.

Fig. 1. Determination of persistence of *P. solanacearum* in soil by planting a potato (P) next to the test plant, the top of which has been cut (CT) at pot-brim level, 52 days after soil infestation. Left, whole plant; center (CT + P), uninoculated cut check and right, soil infested with isolate 042. In this example the emerged potato is wilting after 20 days, demonstrating persistence with Cocona, sooner than with suikawito.

relatively low in percentage and for number of isolates with Suikawito, one each of the two plants that gave persistence with the two Bv. I Yurimaguas isolates became infected without symptoms being apparent; the bacterium was isolated from the regrowth of the cut stem at the end of the experiment. This late infection suggests that the cut back, weakened plant,

Fig. 2. Isolate 141 persistence with Cresta de Gallo heliconia is seen next to an instance of non-persistence with isolate 015. The more rapid sprouting of plant on left may not be a significant difference.

exposed to the added inoculum potential brought about by the wilting potato plant, resulted in overcoming the inherent resistance to invasion.

The two Bv. I Yurimaguas isolates appear to have a greater opportunity to persist in the soil with the hosts tested since (1) they persisted on the rhizosphere of all the jungle species tested, whereas the two Bv. II isolates did not on Suikawito; (2) they caused infection in Amarilla; and (3) they infected Suikawito after it was cut back.

The presence of both races I and 3 that wilt potatoes in the Yurimaguas jungle suggests that a more heterogeneous population of *P. solanacearum* is present than in other forest ecosystems, where either race 1 or 3 has been determined when susceptible crops have been planted (9, 11, 12, 13). Bananas are not infected at Yurimaguas, but race 2 is present about 200 km downstream on the Amazon River's banks (5). This greater heterogeneity may result in the lack of need for an adaptation process to occur as it did when bananas became highly susceptible to the heliconia wilt organism in Costa Rica (2, 14, 15).

Unlike that situation, no obvious plant reservoir for the bacterium can be seen at Yurimaguas, leading to the conclusion that a susceptible host may not be necessary and that the rhizosphere of apparently healthy jungle plants may fulfill this role. Differences were shown between a few plants among the many that conform the jungle flora, as to their capacity to favor persistence of *P. solanacearum*. Whether any of these heliconias or solanaceous plants are actually involved is not certain.

Since both potatoes and bananas are exotic in the lowland jungle of the American continents in which the bacterium is indigenous, it would seem that pathogenicity did not evolve with the host, except for a final adaptive step which took place in bananas.

Literature Cited

1. Bandy, D. E. and R. Benites. 1977. Proyecto Internacional de suelos tropicales. Ministerio de Alimentación (Perú). 32 p.
2. Buddenhagen, I. W. 1960. Strains of *Pseudomonas solanacearum* in indegenous hosts in banana plantations of Costa Rica, and their relationship to bacterial wilt of bananas. Phytopathology 50:660-664.
3. French, E. R. 1972. Evaluación de la resistencia de clones de papa a *Pseudomonas solanacearum*. Phytopathology 62:757-758. (Abstr.)
4. French, E. R. 1978. Pathotypes of *Pseudomonas solanacearum* relative to breeding for resistance in potatoes. Abstracts. III Internat. Congr. Pl. Pathol. Munich, 16-23 Aug. 1978. 290 pp.
5. French, E.R., and L. Sequeira. 1970. Strains of *Pseudomonas solanacearum* from Central and South America: A comparative study. Phytopathology 60:506-512.
6. Hayward, A. C. 1964. Characteristics of *Pseudomonas solanacearum*. J. Appl. Bacteriol. 27:265-277.
7. Lathrap, D. W. 1970. The upper Amazon. Praeger Publishers, New York. 256 pp.
8. Lalimahomed, G. M. and C. Ricaud. 1978. Root versus stem inoculation in pathogenicity tests and in the assessment of resistance to bacterial wilt in tobacco and tomato. Proc. IV Internat. Conf. Pl. Path. Bact. Angers, France. Pp. 857-862.
9. Lloyd, A. B. 1978. Survival of the potato strain of *Pseudomonas solanacearum* in soil. Proc. IV Internat. Pl. Path. Bact. Angers, France. Pp. 875-878.
10. Martin, C., E. R. French, and U. Nydegger. 1981. Bacterial wilt of potatoes in the Amazon Basin. Plant Disease 65:246-248.
11. Seneviratne, S. N. 1969. On the occurrence of *Pseudomonas solanacearum* in the hill country of Ceylon. J. Hort. Sci. 44:393-402.
12. Seneviratne, S. N. 1976. Bacterial wilt in solanaceous crops grown in rice fields. In Sequeira, L. and A. Kelman (eds.). Planning Conference and Workshop on the Ecology and Control of Bacterial Wilt caused by *Pseudomonas solanacearum*. North Carolina State Univ., Raleigh. July 18-23, 1976. Pp. 95-101.
13. Seneviratne, S. N. 1979. Survival in the soil of *Pseudomonas solanacearum*. Proc. II reg. symp. on potato prod. Febr. 5-16, 1978. Los Baños, Laguna, Philippines.
14. Sequeira, L. 1960. *Heliconia* wilt. A progress report. Research newsletter, United Fruit Co. Vol. 7:1-9.

15. Sequeira, L., and C. W. Averre III. 1961. Distribution and Pathogenicity of strains of *Pseudomonas solanacearum* from virgin soils in Costa Rica. Plant Dis. Reptr. 45:435-440.

16. Thurston, H. D. and E. R. French. 1972. Major pathological resistances needed for adapting the potato to the lowland tropics and the possibilities of obtaining them. *In* E. R. French (ed.) Prospects for the potato in the developing world. CIP, Lima-Peru. Pp. 236-246.

Epiphytic Survival of *Xanthomonas manihotis* on Common Weeds in Colombia

F. N. Elango*
International Potato Center
Lima, Peru

J. C. Lozano
CIAT
Cali, Colombia

Abstract

Many common weeds are implicated in the epiphytic survival of *Xanthomonas manihotis*, the cassava bacterial blight pathogen, during the dry season in Colombia. Studies using direct immunofluorescence and ELISA techniques established pathogen survival in or on most weeds at below 10^4 cells/leaf disc, approximately. Studies on weed control in CBB-endemic and non-endemic areas are suggested.

Introduction

Cassava bacterial blight (CBB), caused by *Xanthomonas manihotis*, remains one of the most destructive diseases of cassava (11, 12). In CBB endemic areas, cassava plants rapidly become infected under favorable conditions. For this reason, many attempts have been made to determine the means by which *Xanthomonas manihotis* survives from one season to another (3, 9, 12, 14, 15). Methods for CBB control include the use of disease-free and resistant planting materials (11) and removal, burning, and burial of infected material (9). These methods are considered effective because *X. manihotis* is known to attack only plants belonging to the *Euphorbiaceae*. However, although weeds are a common problem in most cassava fields, their importance as a potential source of inoculum, or as an epiphytic host during the dry season, has not been considered. This paper describes the potential of some common weeds on the survival of the pathogen during the dry season in Colombia.

Materials and Methods

Sample Collection

Two sampling areas were selected, one in Carimagua (in the eastern plains of Colombia) and the other at Media Luna (north coast of Colombia), two widely different ecosystems in which CBB epiphytotics occur. Selected common weeds (Table 1) were identified and samples of them were taken

---

* Portion of a Ph.D. thesis submitted by the first author to McGill University, Montreal, Canada.

Table 1. Weed species[a] used in ecological studies on *Xanthomonas manihotis*.

Species	Family	Common name	Other classification[b]
Solanum nigrum americanum	Solanaceae	Hierba mora	N,D,C.
Sida dictyocarpa	Malvaceae	Escoba	N,D,C.
Emilia sagittata	Compositae	Oreja de alce	B,D,C.
Hyptis mutabilis	Labiatae	Botoncillo mastranto	N,M,C.
Amaranthus dubius	Amaranthaceae	Bledo	B,D,C.
Amaranthus espinosus	Amaranthaceae	?	B,D,C.
Melothria sp.	Cucurbitaceae	Pepinillo	B,D,C.
Euphorbia hirta	Euphorbiaceae	Pimpinela	N,D,C.
Conyza canadiense	Compositae	Venadillo	B,D,C.
Centrosema macrocarpum	Labaceae	Centrosema	B,D,ML
Boerhaavia erecta	Nytaginaceae	rodilla de pollo	B,D,ML
Eleucina indica	Gramineae		N,M,C
Digitaria sanguinalis	Gramineae		N,M,C
Cyperus rotundus	Gramineae		N,M,C

a   Weeds were identified by E. Escobar, Taxonomist, Universidad Nacional, Facultad de Agronomía, Palmira, Colombia.

b   N=Narrow leaved; M=Monocot. weed; C=From Carimagua; D=Dicot. weed; B=Broad leaved; ML=From Media Luna.

once monthly at each location from January to April, periods corresponding with the driest part of the year when CBB symptoms disappear on cassava plants.

Pathogen Detection

Leaf comminution was used for detecting the bacterium. For each sampling, weeds were collected along two diagonals of a cassava field, and each weed was separately put in a plastic bag and used within 24 h. Ten leaf discs/weed obtained along the diagonal of the lamina were removed with a 7 mm diameter flamed cork borer and each sample was comminuted in 5 ml of sterile distilled water. A smear (10 nl) of each crude weed extract and its Kelman's enriched broth (4) was stained with an *X. manihotis* antibody conjugated with fluorescein isothiocyanate (FITC), previously prepared (1, 2). Slides were observed under an U. V. microscope at 1000 magnification. Similarly, crude and enriched weed and cassava leaf extracts were subjected to the enzyme-linked immunosorbent assay technique (6); the enzyme used was horse-radish peroxidase. Results were visually scored as reported previously (4). Another sample of the crude and enriched extract of each weed and control (cassava leaf) was plated on Kelman's tetrazolium chloride agar (10) in order to isolate the pathogen. Verification of the identity of *X. manihotis* was made morphologically and symptomatologically. To relate numbers of cells/microscope field in each weed extract to numbers of cells/ml, a 48 h growth of a pure culture of the pathogen was serially diluted and stained with the fluorescent conjugate. The average number of bright fluorescent cells/10 microscope fields per dilution at 1000 magnification was determined.

Results and Discussion

When tested with a known concentration of *X. manihotis* in suspension, the fluorescent antibody technique detected with certainty cell concentrations of 10^5 cells/ml whereas the ELISA technique detected 10^4 cells/ml. During most of the period under study, broad-leaved (or mostly dicotyledonous) weeds carried the bacterium at concentrations probably below 10^4 cells/leaf disc, as on symptomless cassava leaves, possibly because they remained more succulent. The population of the pathogen on or in weeds remained steady throughout the sampling period, and this made detection of the pathogen in crude extracts difficult. However, Kelman's broth enrichment of crude extracts plus the immunofluorescence or ELISA technique (4) made pathogen detection easier. The two techniques were reliable in showing whether crude extracts carried the pathogen (Table 2). With the advent of the rainy season, in March, the pathogen population increased to 10^5 cells/leaf disc on symptomless cassava leaves but remained below this level on most weeds sampled (Fig. 1). Differences in cell counts of *X. manihotis* surviving on different weeds before and after enrichment were presumably an indirect reflection of population trends in/on the weed samples, assuming that comminution of leaf discs did not promote or inhibit bacterial growth. High cell counts were most often detected on *Amaranthus dubius* while graminaceous weeds rarely carried the pathogen.

Table 2. Effect of enrichment technique on the detection of *X. manihotis* on weeds.

Weed species	Unenriched leaf extract		Enriched leaf extract	
	I.F.[a]	ELISA	I.F.	ELISA
S. nigrum americanum	±[b]	—	+	+
S. dictyocarpa	—	—	—	+
E. sagittata	+	—	+	+
H. mutabilis	+	+	+	+
A. dubius	+	+	++	++
Melothria sp.	±	+	+	+
E. hirta	—	—	—	—
C. canadiense	—	—	+	+
C. macrocarpum	—	—	—	—
C. nitida	—	—	—	—
Cissampelos sp.	±	—	+	+
E. indica	—	—	—	—
D. sanguinalis	—	—	—	—
C. rotundus	—	—	±	—

[a] I F = immunofluorescence test; ELISA = enzyme-linked immunosorbent assay.
[b] ++ = strongly positive reaction; + = positive reaction; ± = weakly positive — = negative reaction.
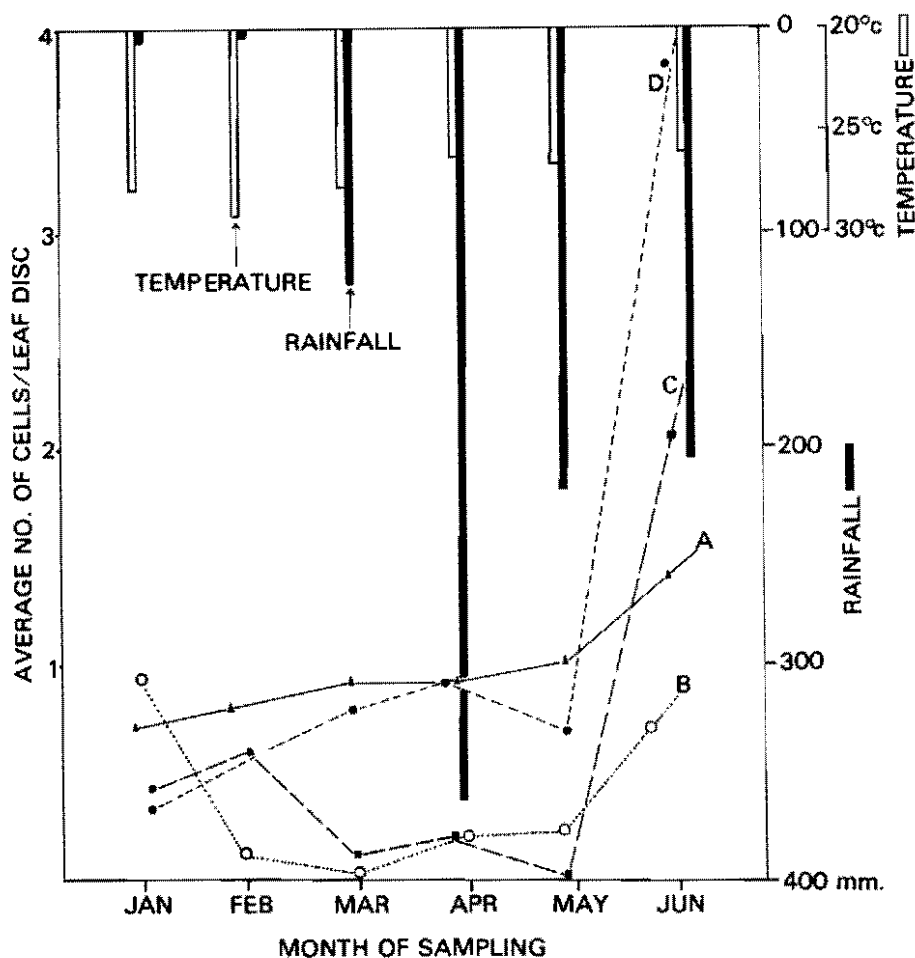
Fig. 1. Population dynamics of *Xanthomonas manihotis* on three weeds during the dry season.

The present findings confirm the results of previous workers that *X. manihotis* survives epiphycally on cassava leaves (3, 14). For the first time, weeds were implicated as an epiphytic survival base. In the epidemiology of foliar pathogens, survival of a few cells on vegetation and on symptomless plants may have a far-reaching significance (7, 8). Many studies have established that only one bacterial cell is required to initiate an infection (13, 16). In a situation where all host materials are eliminated, this study shows that weeds may provide a base for inoculum survival. Since weeds are so plentiful in cassava fields and survive remarkably well throughout the driest period when host materials are not available, it would be

interesting to investigate the effect of regular weed control in the dry season on reduction of the initial inoculum in the next growing season. It might also be of interest to know the effect of eradicating weeds in CBB endemic and non-endemic areas.

Literature Cited

1. Allan, E. and A. Kelman. 1977. Immunofluorescent stain procedures for detection and identification of *Erwinia carotovora* var. *atroseptica*. Phytopathology 67:1305-1312.
2. Cherry, W. B. 1974. Immunofluorescence techniques. *In* Manual of Clinical Microbiology 2nd. Ed. pp 29-44. Lennette, E.H., E.H. Spaulding and J.P. Truant (Eds.) Am. Soc. for Microbiol.
3. Daniel, J. F. and B. Boher. 1979. Ecology of Cassava bacterial blight: Epiphytic survival of *X. manihotis* on aerial parts of cassava plants. pp 762-770. *In* Proceedings of the IV Int. Conf. on "Plant Pathogenic Bacteria," Angers, France.
4. Elango, F. and J. C. Lozano 1980. Transmission of *Xanthomonas manihotis* in seed of cassava (*Manihot esculenta* Crantz) Plant Disease 64:784-786.
5. Elango, F.N. 1980. Relationship between *Xanthomonas manihotis* (Arthaud-Berthet and Bondar) Starr and *Xanthomonas cassavae* (Wiehe and Dowson), and some epidemiological studies on cassava Bacterial Blight. Ph.D. thesis, McGill University, Montreal, Canada. 301 pp.
6. Ellens, D. J., P. W. de Leeuw, P. J. Straver and J. AM. M. Van Balken 1978. Comparison of five diagnostic methods for the detection of Rotavirus antigen in calf feces. Med. Microbiol. Immunol. 166:157-163.
7. Goto, M. 1972. The significance of the vegetation for the survival of plant pathogenic bacteria. *In* Proceedings of the 3rd. Int. Conf. on "Plant Pathogenic Bacteria," 1971. Maas-Geesteranus (Ed.) pp 39-53. Centre for Agricultural publishing and documentation, Wageningen.
8. Hayward, A. C. 1974. Latent infections by bacteria. Annu. Rev. Phytopathology. 12:87-97.
9. Ikotun, B. 1977. Survival of *Xanthomonas manihotis,* the cassava bacterial blight pathogen. pp 24-27. *In* Persley, G., E. R. Terry and R. Mac Intyre (Eds.) Cassava bacterial blight-report of an interdisciplinary workshop, IITA, Ibadan, IDRC 096e.
10. Kelman, A. 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.
11. Lozano, J. C. and R. Booth 1974. Diseases of cassava (*Manihot esculenta* Crantz). PANS 20:30-54.
12. Lozano, J. C. and L. Sequeira 1974. Bacterial blight of cassava in Colombia. Epidemiology and control. Phytopathology 64:83-88.
13. Perembelon, M.C.M. 1972. A quantitative method of assessing virulence of *Erwinia carotovora* and *Erwinia carotovora* var. *atroseptica* and susceptibility to rotting of potato tuber tissue. pp 299-303. *In* Mass-Geesteranus (Ed.). Proc. of the 3rd. Int. Conf. on "Plant Pathogenic Bacteria," 1971. Centre for Agricultural publishing and documentation, Wageningen.
14. Persley, G. J. 1979. Epiphytic survival of *Xanthomonas manihotis* in relation to the disease cycle of cassava bacterial blight. pp 772-776. *In* Proceedings of the IV Int. Conf. on "Plant Pathogenic Bacteria," Angers, France.

15. Terry, E. R. 1974. A mode of survival of *Xanthomonas manihotis,* the cassava bacterial blight pathogen. Nigerian Soc. of Plant Protection. Occasional publication No. 1:19 (Abstr.).
16. Thyr, B. D. 1968. Bacterial Canker of tomato: inoculum level needed for infection. Plant Disease 52:741-743.

Seed Borne Bacterial Tumors in Tobacco

Arun Misra, Vidyanath Jha, Sashinath Jha, B. P. Sharma

L. N. Mithila University, C. M. Sc. College
Darbhanga, India

Abstract

Thick green stem tumors in tobacco (*Nicotiana clevelandii* x *glutinosa*) have been observed to be caused by the bacterium *Corynebacterium fasciens*. Seeds obtained from tumorous plants showed lower percentage of the germination of the seeds, and the development of tumors in plants automatically. The transmission of the tumor-property in tobaccos has been investigated. Transfer and survival of the bacterium through the seeds has been confirmed. Scanning electron microscopy has been utilized to observe bacterial colonies on the surface of the tobacco seeds. Surface sterilization of the seeds to improve the percentage of germination and elimination of tumors has been successfully accomplished.

Introduction

Decreased percentage of seed-germination in tobacco, due to tumorigenesis, and the presence of bacteria on the surface of tumorous seeds, observed by scanning electron microscopy, has been explained in this communication.

Various types of studies done with plant tumors have been well summarized by Braun (1972), and Beiderbeck (1977). We have tried to investigate further the effects of tumorigenecity on germination of tobacco seeds. Seeds obtained from healthy and infected (with tumors) tobacco (*Nicotiana glutinosa* x *clevelandii*) plants were sown separately in sterilized soil and the emergence of plumule over the surface of the soil was noted. Percentage of the seeds that germinated, out of the total number of seeds sown, was also counted until six weeks after sowing. In addition to the healthy and infected seeds, a third group of seeds that were taken from infected (tumorous) plants and surface sterilized by serial passage through 2% Pril (10 minutes), 0.5% Chlorox (2 minutes), 96% ethyl alcohol (15 seconds), and distilled water (10 minutes) were also utilized for tests. The results of the germination experiment are shown in Table 1.

Results and Discussion

It is obvious that tumorigenecity reduces the germination percentage, and delays the process of emergence of plantlets. Nienhaus and Gliem (1973) have indicated the seed-borne nature of the tumors in tobacco. Lacey (1948) had also studied the effects of the "leafy-galls" on the

Table 1. Differences in the germination behavior of healthy and tumorous seeds.

Type of seed	Emergence of plumule above soil in days	Percentage of seeds germinated by six weeks after sowing
healthy	10	90
tumorous	20	35
surface sterilized	15	70

Based on 200 seed samples in each case.

seedlings of bean, sweet-potato and lettuce. Far earlier, Tilford (1936) had mentioned that bacteria responsible for "fasciation-disease" are carried through the exterior of the seed-coat of sweet-peas. This superficial presence of bacteria is supported by our work in tobacco, as the surface sterilized seeds germinate more frequently and quicker in comparison to the tumorous seeds.

The presence of bacteria on the surface of the seeds was further confirmed by scanning electron microscopy. A Leitz-AMR 1000 model of the microscope was used to observe the surface pattern of seeds. Fig. 1 shows the surface structure of healthy and tumorous seeds. Numerous bacterial colonies were noted, attached to the surface of infected seeds. The healthy seeds had clean surfaces.

Literature Cited

1. Beiderbeck, R. 1977. Pflanzentumoren. Ulmer, Stuttgart.
2. Braun, A. C. 1972. Plant Tumor Research. S. Karger, Basel.
3. Lacey, M. S. 1948. Studies of *Bacterium fascians*. V. Further observations on the pathological and physiological reactions of *Bacterium fascians*. Ann. Appl. Biol. 35:572-580.
4. Nienhaus, F. and G. Gliem. 1973. Evidence for a new mechanically transmissible tumor-inducing principle in tobacco. Phytopathology Z. 78:367-368.
5. Tilford, P. E. 1936. Fasciantion of sweet peas caused by *Phytobacterium fascians* n. sp. J. agric. Res. 53:383-385.

Fig. 1. Scanning electron microscope photographs (at 20 Kilo Volt) of seed with germ pore (x 185); B, healthy seed with clean surface (x 460); and C, infected or tumorous seed with bacterial colonies (x 460). Each magnified 2.8 times further by enlarged printing.
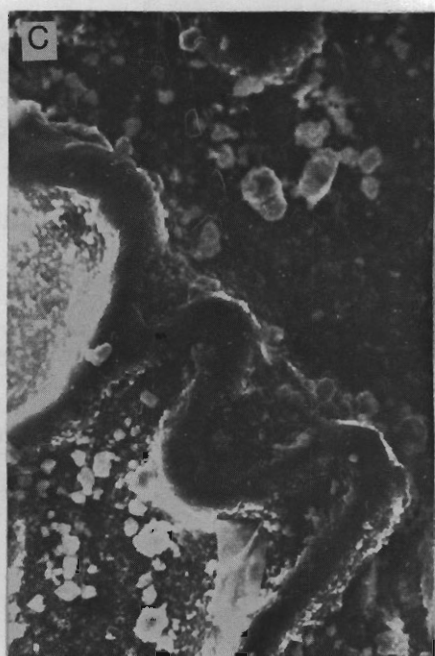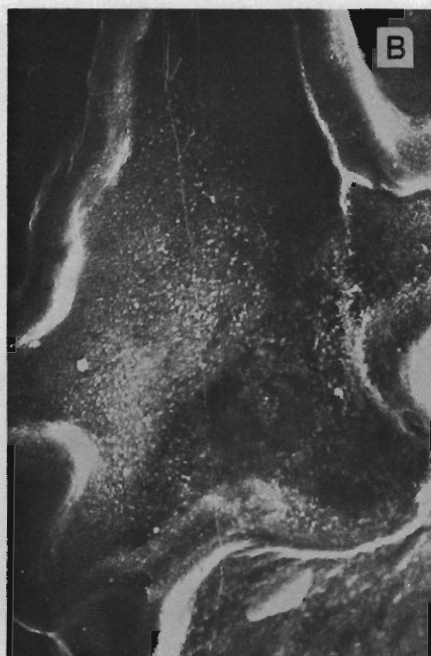
Abstracts

INFLUENCE OF TEMPERATURE ON VIRULENCE OF RACE 3 STRAINS OF *Pseudomonas solanacearum.* Ciampi, L., and L. Sequeira. Facultad de Ciencias, Universidad Austral de Chile, Valdivia, Chile, Dept. of Plant Pathology, University of Wisconsin-Madison, Madison 53706, U.S.A. respectively.

The bacterial wilt of patatoes caused by race 3 of *Pseudomonas solanacearum* can be a severe problem at high elevations in Colombia and Perú, where relatively low mean temperatures predominate. Because race 3 exists within wide geographical areas, attempts were made to determine if there are specific strains that are adapted to, and can cause wilting at, these low temperatures. Russet Burbank potato plants were stem-inoculated with eight race 3 strains and then were maintained in growth chambers at 16, 20, 24 and 28°C for 15 days. At the lowest temperature (16°C) only one strain (s206) caused considerable wilting. S206 was more virulent than the other seven strains at both 16 and 20°C. At 28°C, however, no differences in virulence could be detected; all strains caused complete wilting by 15 days. These results were confirmed in a second experiment in which 10 strains were used, five of race 1 and five of race 3. Only two race 3 strains (s206 and s257) caused wilting at the lower temperature. At 28°C all strains were equally virulent. Differences in virulence were not correlated with growth rates of the strains in liquid culture at 16°C. Some strains grew at rates similar to those of s206 or 257 *in vitro,* but did not cause symptoms when grown in plant at this temperature.
(Paper presented at the Conference; complete text being published elsewhere.)

SURVIVAL OF *Pseudomonas solanacearum* **IN PRESUMED NON-HOST PLANTS: A NEW CONCEPT.** Granada, G.A., and L. Sequeira. Dept. of Pathology, University of Wisconsin-Madison, Madison, Wisconsin 53706, U.S.A.

The bacterial wilt pathogen *Pseudomonas solanacearum* is thought to survive in soil for several years, but there are no consistent correlations between survival and soil type, pH, moisture, previous cropping histories, etc. The highly variable incidence of the disease in the field indicates that many factors affect survival of the bacterium. Of these, multiplication of the bacterium in the rhizosphere or roots of certain plants may be important. This has not been given much attention, perhaps due to lack of a suitable selective medium. We devised a medium with high selectivity and good plating efficiency for *P. solanacearum* (Phytopathology 71:220), and then used it to measure survival of the bacterium in greenhouse soil maintained at 56% water-holding capacity at 28°C. These studies confirmed the well established notion that Race 1 survives longer than Race 2, and Race 2 longer than Race 3. The relatively short survival in soil (maximum of 20 wk for Race 1) suggests that, in nature, the bacterium probably survives on or in plant roots. Protracted survival in the soil occurred only when naturally infested soil was stored in plastic bags at 4°C. In this case, low populations (10^2 CFU/g soil) were maintained for as long as 673 days. For rhizosphere

studies, we inoculated host (pepper, castor bean, tomato) and presumed nonhost (bean, corn, sugarcane) plants by the root-dipping method. The rhizosphere population dropped to undetectable levels in most cases. Large populations of the bacterium were recovered from rhizosphere soil only when systemic infection had taken place, in the absence of external symptoms. These results suggested that infection might be a common phenomenon and a prerequisite for long-term survival of the bacterium. To confirm that infection had taken place, roots were surface-sterilized by dipping them in 70% ethanol for 5 min after the soil was removed by washing. Large bacterial populations were recovered from pepper and tomato plants inoculated with the three races, or from castor bean and presumed nonhost plants inoculated with Race 1. That these results were not affected by the inoculation procedure used was confirmed in experiments in which presumed nonhost plants (such as corn, rice sorghum, bean, peas, soybean) were planted in infested soil. All plants, except rice, became infected with a Race 1 strain, but remained symptomless. This previously unreported ability of a plant pathogenic bacterium to attack roots of a wide diversity of plants suggests that infection is essential for long-term survival. The results indicate that we must modify our concept as to long-term survival of *P. solanacearum* in the soil. The implications of this revised concept of survival on control of the bacterium by crop rotation are obvious.

(Paper presented at the Conference; complete text being published elsewhere.)

SURVIVAL OF *Xanthomonas manihotis* **IN HOST TISSUES.** Takatsu, A. Universidade de Brasília, Depto. de Biología Vegetal. 70910, Brasília, DF, Brazil.

The survival of *Xanthomonas manihotis* in the tissue of susceptible cultivars of cassava (*Manihot esculenta* Crantz) was determined at two different temperature conditions: 1) field temperature conditions of Brasilia (approximately 17 and 27°C averages of minimum and maximum temperatures from October to March, respectively, and 14 and 26°C from April to September) and glasshouse conditions (about 19 and 34°C from October to March and 15 and 30°C from April to September). The test plants were developed from young shoots inoculated before rooting and grown in 2000 cc capacity plastic bags. All plants that showed external symptoms of disease during development were eliminated. The presence of bacteria in the internal tissue of apparently healthy plants was determined by isolation in agar media from 6, 12, and 18 month old plants. *X. manihotis* was recovered from approximately 50% of 6 month old plants. The number of healthy plants recovered increased at higher temperature conditions. At 12 months, the bacterium was present in only 3% of plants grown in the glasshouse at higher temperature conditions, whereas 17% of plants developed outside at lower temperature conditions were still infected. At 18 months, the bacterium was recovered from only 2% of plants grown outside. Infected plants without external symptoms sprouted diseased shoots when the stems were pruned at ground level.

(Abstract provided but paper was not presented at the Conference.)

SURVIVAL OF *Xanthomonas campestris* **pv.** *oryzae* **AND ITS PHAGE IN FIELD WATER INCUBATED AT DIFFERENT TEMPERATURES.** Thri Murthy, V. S. and S. Devadath. Central Rice Research Institute, Orissa, India.

Incubation temperature influenced the viability of the pathogen and its phage in field water. Viability of the pathogen and its phage was prolonged at lower temperatures (15-25°C) than at higher temperatures (30 -45°C). Antagonistic effect of a fungal species and a bacteriophage present in the field water seemed to influence the viability of the pathogen at favorable temperatures. At 15 and 20°C phage survived longer than the pathogen. At 25, 35, 40, and 45°C the phage and the pathogen survived for equal periods and at 30°C the pathogen survived longer than the phage. Much of the earlier work equated phage presence and population with pathogen presence and epidemiological potential but the evidence gathered in these experiments suggests that presence of phage does not necessarily indicate the presence of the pathogen at certain temperatures.

(Paper presented at the Conference; complete text being published elsewhere.)

EFFECT OF NITROGEN NUTRITION ON THE AGGRESSIVENESS OF *Xanthomonas campestris* **pv.** *oryzicola* **ISOLATES.** Satpathy, B. and S. Devadath. Central Rice Research Institute, Orissa, India.

Aggressiveness of *Xanthomonas campestris* pv. *oryzicola* isolates was influenced differentially by the nitrogen nutrition. Aggressive isolates 6 and 7 failed to differ in their aggressiveness when grown in arginine, cystine, and glutamic acid. But when grown in asparagine, leucine, ammonium dihydrogen orthophosphate, di-ammonium orthophosphate, ammonium oxalate, and calcium nitrate, isolate 7 was more aggressive than isolate 6. On the contrary, when grown in tryptophan isolate 6 was more aggressive than isolate 7. The less aggressive isolates 15 and 17 failed to differ when grown in any one of the nitrogen sources except calcium nitrate, wherein isolate 17 was more aggressive than isolate 15. Aggressiveness of all four isolates was less when grown in organic nitrogen sources (except tryptophan) than when grown in inorganic nitrogen sources. Aggressiveness of the bacterial isolates grown in different nitrogen sources interacted differentially on resistant (BJ 1), tolerant (T (N) 1), and susceptible (IR 8) rice cultivars. On BJ 1 and T(N) 1, four causal bacterial isolates failed to differ in aggressiveness when grown in glutanic acid but they differed on IR 8. Aggressiveness of these four isolates, however, did not vary on any one of the rice cultivars when grown in cystine.

(Paper presented at the Conference; complete text being published elsewhere.)

SUMMARY OF SESSION III

Leopold Fucikovsky, Chairman

Some important conclusions were drawn from the papers presented. These included:

1. Some Race 1 and Race 3 strains of *Pseudomonas solanacearum* produced wilt symptoms in resistant potato clones, when these were grown in infested soil. Latent infections occurred in some cases; however, no tuber infection was present when the plants were grown in infested soil at 12-22°C. Bacteria inoculated into tubers were not eliminated completely even at 4°C after 40 days. At low temperatures (16°C) some strains did not cause symptoms in potatoes, but at 28°C all strains caused wilting within 15 days.

2. The majority of *Pseudomonas syringae* pv. *savastanoi* isolates from young and older olive leaves, at the same time of the year, differed in several characteristics. The isolates of pv. *savastanoi* also differed consistently from leaves of the same age at different times of the year. Bacterial isolates from different tubers in the field during a period of 18 months did not differ. Factor analysis of all the pv. *savastanoi* isolates identified the plant and environment factors that had ecologically discriminating properties.

3. *Xanthomonas manihotis* has a known "resident" phase on the aerial surfaces of the cassava plant. Immunofluorescent method detected, specifically, *X. manihotis* and showed its nonhomogenous distribution.

4. By the use of either selective medium or cvs. of potatoes and tomatoes, *Pseudomonas solanacearum* was detected at three depths in the soil in two infested potato fields in the tropics of Peru, at harvest time and 140 days later after bare fallow. After fallow, populations decreased, but were still able to produce wilting of potato and tomato plants.

5. A heterogenous population of Race 1 and 3 of *P. solanacearum* was detected in Amazon jungle at Yurimaguas, Peru, and it appears that the rhizospheres of *Solanum nigrum*, heliconias, and other jungle plants may be hosts to the bacterium, not always showing visible symptoms, but favoring the persistence of *P. solanacearum*. These plants may play a role in bringing the disease to potatoes and bananas, plants that are exotic to Amazon tropics.

6. With a newly devised selective medium for *P. solanacearum*, it was confirmed that Race 1 survives longer than Race 2 and Race 2 longer than Race 3 in greenhouse soil. In population studies, the bacterium was recovered from rhizosphere soil only when systemic infection had taken place, even in nonhost plants, but without external symptoms. This indicates that *P. solanacearum* may survive in soil for very long periods.

7. Using immunofluorescence and ELISA techniques it was found that *Xanthomonas campestris* pv. *manihotis* could survive during dry season in Colombia on or in most weeds, at below 10^4 cells/leaf disc, approximately.

Session IV

Host Pathogen Interactions

Klaus Rudolph, Chairman

Host-Pathogen Interactions in Phytobacteriology

PROBLEMS AND PROSPECTS

Introductory Remarks

by K. Rudolph, Chairman, Session IV

The field of host-pathogen interactions has attracted the interest and involvement of plant pathologists for many years.During the first phase of plant pathology at the end of the 19th Century, the vast majority of the studies were naturally directed toward the description of plant diseases, the isolation and characterization of the causal organisms, and the development of methods for control. However, even in this early phase, physiological and biochemical interactions between host and parasite were reported. Thus, DeBary (6) described the action of a toxin from *Sclerotinia*, and Woods (31) measured high increases in peroxidase in chlorotic tissue.

Studies with phytopathogenic bacteria in 1909 (13) revealed that pectinolytic enzymes were responsible for tissue maceration. Also, other typical symptoms, induced by phytopathogenic bacteria, led to very early physiological and biochemical investigations, for example the toxin-induced symptom of wildfire of tobacco and crown-gall caused by *Agrobacterium tumefaciens* (2). After investigations at many different laboratories in the following years, only a few mechanisms by which bacteria cause diseases are now generally accepted. These include, for instance, the role and nature of several pectinolytic enzymes which participate in soft rot caused by *Erwinia* species (1, 20) or the induction of tumors by plasmid-DNA from *Agrobacterium tumefaciens*, (4).

In most of the cases where bacterial diseases have been studied, however, the role and composition of biochemical products of the host or the parasite, and the nature of interactions are not fully elucidated. Thus, there still exist different opinions on the chemical structure of toxins (21, 19), or the proposed structures have not yet been generally accepted; for example, the glycopeptides of *Corynebacteria* (29). The mode of action of many toxins is only partially understood (5) and the role of toxins in pathogenesis is mostly unkown. Also, the question as to whether certain toxins were early or late products during pathogenesis, could not always be answered (25).

Some of the controversy regarding bacterial toxins may be due to the false assumption that *one* dominant *factor of virulence* has to be demonstrated. On the contrary, it is quite possible that such dominant factors of virulence do not exist in certain host-parasite interactions. Toxins, enzymes, as well as other products of the pathogenic bacteria probably act together during pathogenesis, with changing importance at different stages of disease development. We have to assume multifactorial

interactions between the bacteria and the host plant. In other words, the bacteria possess a *group* of virulence factors of different nature. Similarly, the plants can react with several and different mechanisms toward bacterial invasion. This does not imply, however, that these different factors or mechanisms cannot be clarified in the future.

A different approach seems to be necessary in order to study the phenomenon of host-specificity, shown by many phytopathogenic bacteria. Leaf spot causing pseudomonads and xanthomonads, especially, are characterized by a very narrow host-specificity, often on only one plant species. This characteristic is in sharp contrast to the ability of these bacteria to grow on very simple media, without any specific growth requirements. Taxonomists have therefore grouped many former independent species of pseudomonads and xanthomonads into one species with different "pathovars" (7), since all the available taxonomic data did not seem to justify independent species status. Whether this new system will be practicable has to be seen. Up to now it seems to be more confusing than helpful.

The goal of phytobacteriologists should now be to elucidate the underlying mechanisms of narrow host specificity. The taxonomists can probably only be persuaded to rank independent species according to host-specificity when the physiological and biochemical factors involved in host specificity have been clarified, as well as genetically localized. Independent from toxonomic considerations, however, the explanation of host-specificity of phytopathogenic bacteria on a molecular level should be one of the ultimate goals in phytobacteriological research. The suggested participation of bacterial extracellular polysaccharides or lipopolysaccharides (9, 10) has to be investigated further in this respect.

In addition to species-specific bacteria, many different pathotypes or races of bacteria have also been described which often differ in their aggressiveness toward different plant cultivars. Also, herein lies an explanation of the observed differences in terms of physiological or biochemical characters often missing.

A better knowledge of the bacterial factors for virulence and host specificity is also required in order to understand the *resistant reaction.* Thus, many leaf spot causing pseudomonas induce the so-called hypersensitive reaction (HR) in leaves of non-host plants (15); tobacco leaves often have been used to study this reaction; since saprophytic pseudomonads do not induce this reaction, the induction of HR must be regarded as the effect of a virulence factor present in all of these phytopathogenic bacteria. Only on the host plant is the effect of this factor retarded such that the host tissue disintegrates much slower, allowing the bacteria to multiply to high concentrations. In other words: Only on the host plant are the bacteria able to retard the hypersensitive reaction by the action of a host specific principle.

In addition to this basic differential reaction of hosts and non-hosts, a resistance toward different virulence factors of the pathogen must also be assumed. This implies that the host also possesses different factors or mechanisms of resistance. Therefore, it is not a question as to whether phytoalexines, oxidative enzymes, inhibitiors, agglutinines or other principles are responsible for the resistant reaction, rather it is a question

of how and under which conditions different mechanisms of resistance play a role, or possibly have additive effects. This assumption of different factors for resistance present in one plant should not, however, lead to the compromise of explaining resistance with a little bit of everything.

Thus, for example, the phenomenon of the attachment of bacteria to cell walls (11, 28, 14) should be investigated further to explain why the attached bacteria do not multiply. Also, the isolation and chemical characterization of bacteriostatic or bactericidal compounds seems to be necessary. The typical phytoalexins are mostly lipophilic, whereas the bacteria are more or less surrounded by water and could be more easily inhibited by hydrophilic compounds.

Also, other host-pathogen interactions which have been described for plants and fungi may not be applicable to bacterial diseases. Fungal hyphae can grow without water, whereas bacterial infections cannot proceed if water is not available (27). Thus, when bacterial attachment on cell surfaces is established in a resistant reaction, the possibility that only evaporation of water from the plant tissue caused the attachment and immobilization of the bacteria should always be evaluated (12). In contrast to the situation in plants (with the exception of the vascular bundles) which are mostly dry on the outer and inner cell surfaces, bacteria causing human and animal diseases need attachment, because they could otherwise be rinsed off by water.

An attachment of the bacteria to the host plant seems only to be necessary in the specific cases of *Agrobacterium* and *Rhizobium,* where the host cell cannot be transformed without immediate contact between plant cell and bacterium (17). Also in these systems are   mechanisms of attachment not fully understood, since earlier reports on specific reactions between plant lectins and bacterial polysaccharides could not always be confirmed (22).

Another difference between fungal and bacterial infections is the dependence of the final reaction of the number of bacteria introduced. Only high concentrations of bacteria induce the visible hypersensitive reaction. Although low numbers of introduced bacteria are also inhibited in the incompatible host, this inhibition may be due to mechanisms other than the visible hypersensitive reaction (26). Similarly, low numbers of bacteria may not always cause disease in a compatible host (24).

A group of bacteria which is attracting more and more attention, and which was nearly unknown a few year ago is the *fastidious prokaryotes* (18). Several of the pathogens cannot yet be cultivated 'in vitro,' and the whole class is characterized by rather specific requirements for the nutrient medium (30). Nevertheless, some of these organisms can infect many different host plants and thus show much less host specificity than the "classical" bacteria which have no specific nutrient requirements.

All the studies on host-pathogen interactions with bacterial diseases have thus shown this relationship cannot easily be appointed to one important interaction. The bacteria seem to possess several factors of virulence and, similarly, the plants, several factors of resistance. A better understanding of these factors and mechanisms may eventually help in the control of bacterial diseases by the application of the different principles;

for example, in the chemical control of bacteria (8), induction of resistance, biological control, or breeding for resistance.

The most successful method in *breeding* for *resistance* is still the selection of plants or crosses by artificial inoculations or field infections. However, if the most important principles for virulence or pathogenicity could be applied instead of whole bacteria, the screening method might be speeded up considerably. Theoretically, at least, broader potentials of differing genetic material can be evaluated by screening tissue or cell suspension cultures (23).

Intrinsic difficulties of such systems have impeded their application on a broader scale for practical purposes till now, and only a few successful attempts have been reported (3). However, the potentialities of these techniques, as well as those of "genetic engineering" have been increased considerably by the development of new methods. This approach implies the ability to work with isolated bacterial products which are decisive in host pathogenicity. The recent progress in bacterial genetics seems to justify this possibility (16).

The localization of the coding for specific factors of pathogenicity and virulence in plasmids, as well as chromosomal DNA should be feasible in the future. Therefore, we have reasons to assume that the field of pathophysiology of bacterial diseases, which has sometimes been regarded as merely an academic occupation, may lead to developments of practical importance.

Literature Cited

1. Bateman, D. F., and H. G. Basham. 1976. Degradation of plant cell wall and membranes by microbial enzymes. In: Physiological Plant Pathology, R. Heitefuss and P. H. Williams (eds.). Encyclopedia of Plant Physiology, New Series Vol. 4, pp. 316-355.

2. Braun, A. C., and R. B. Pringle. 1959. Pathogen factors in the physiology of disease - toxins and other metabolites. In: Plant Pathology, Problems and Progress, Holton, C. S., *et al.* (eds), The Univ. of Wisc. Press, pp. 88-99;

3. Carlson, P. S., 1973. Methionine sulfoximine-resistant mutants of tobacco. Science 180:1366-1368.

4. Chilton, M. D., M. H. Drummond, D. J. Merlo, D. Sciaky, A. L. Montoya, M. P. Gordon, and E. W. Nester. 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown-gall tumorigenesis. Cell 11:263-271.

5. Crosthwaite, L. M., and S. J. Sheen. 1979. Inhibition of ribulose 1,5-biphosphate carboxylase by a toxin isolated from *Pseudomonas tabaci*. Phytopathology 69:376-379.

6. DeBary, A., 1886. Uber einige Sclerotinien und Sclerotinienkrankheiten. Bot. Zeit. 44:377-474.

7. Dye, D. W., J. F. Bradbury, M. Goto, A. C. Hayward, R. A. Lelliott, and M. N. Schroth. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Rev. Plant. Pathol. 59:153-168.

8. Egli, T., and E. Sturm. 1980. Bacterial plant diseases and their control. In: Chemie der Pflanzenschutz- und Schadlingsbekampfungmittel, Band 6, edt. by R. Wegler, pp. 345-388.

9. El-Banoby, F. E., and K. Rudolph. 1979. Induction of water-soaking in plant leaves by extracellular polysaccharides from phytopathogenic pseudomonads and xanthomonads. Physiol. Plant Pathol. 15:341-349.

10. Graham, T. L., L. Sequeira, L., and T. S. R. Huang. 1977. Bacterial lipopolysaccharides as inducers of disease resistance in tobacco. Appl. + Environm. Microbiology 34:424-432.

11. Goodman, R. N., P. Y. Huang, and J. A. White. 1976. Ultrastructural evidence for immobilization of an incompatible bacterium, *Pseudomonas pisi*, in tobacco leaf tissue. Phytopathology 66:754-757.

12. Hildebrand, D. C., M. C. Alosi and M. N. Schroth. 1980. Physical entrapment of pseudomonads in bean leaves by films formed at airwater interfaces. Phytopathology 70:98-109.

13. Jones, L. R., 1909. The bacterial soft rots of certain vegetables II. Pectinase, the cytolytic enzyme produced by *Bacillus carotovorus* and certain other soft-rot organisms. Vermont Univ. Agr. Exp. Sta. Bull. 147 pp. 283-360.

14. Klement, A., 1977. Cell contact recognition versus toxin action in induction of bacterial hypersensitive reaction. Acta Phytopathol. Acad. Sci. Hung. 12:257-261.

15. Klement, A., and R. N. Goodman. 1967. The hypersensitive reaction to infection by bacterial plant pathogens. Ann. Rev. Phytopath. 5:17-44.

16. Lacy, G.H., and J. V. Leary 1979. Genetic systems in phytopathogenic bacteria. Ann. Rev. Phytopath. 17:181-202.

17. Lippincott, J. A., and B. B. Lippincott. 1980. Microbial Adherence in Plants. In: Bacterial Adherence (Receptors and Recognition, Series B, Volume 6), edt. by E. H. Beachey, pp. 375-398, Chapman and Hall, London.

18. Maramorosch, K., 1979. How mycoplasmas and rickettsias induce disease. In: Plant Disease, an Advance Treatise. Vol. IV, edt. by Horsfall, J. G. and Cowling, E. B. pp. 203-217, Academic Press, New York, San Francisco, London.

19. Mitchell, E. R., 1976: Isolation and structure of a chlorosis-inducing toxin of *Pseudomonas phaseolicola*. Phytochemistry 15:1941-1947.

20. Mount, M. S., 1978. Tissue is disintegrated. In: Plant Disease, and Advanced Treatise, Vol. III. J. G. Horsfall and E. B. Cowling (eds.) pp. 279-297, Academic Press New York, San Francisco, London.

21. Patil, S.S., P. Youngblood, P. Christiansen, and R. E. Moore. 1976. Phaseotoxin A an antimetabolite from *Pseudomonas phaseolicola*. Bioch. Biophy. Res. Comn. 69:1019-1027.

22. Pueppke, S. G., T. G. Freund, B. C. Schulz, and H. P. Friedman. 1980. Interaction of lectins from soybean and peanut with rhizobia that nodulate soybean, peanut, or both plants. Canad. J. Microbiol. 26:1489-1497.

23. Reinert, J., and Y. P. S. Bajaj. (edts.) 1977. Applied and fundamental aspects of plant cell, tissue, and organ culture. Springer-Verlag, Berlin, Heidelberg, New York.

24. Rouse, D. I., S. S. Hirano, J. Lindemann, E. V. Nordheim, and C. D. Upper. 1981. A conceptional model for those diseases with pathogen population multiplication independent of disease development:

Abstracts of papers, No. 522, 1981 Annual Meeting, American Phytopathological Society.

25. Rudolph, K., 1976. Non-specific Toxins. In: Encyclopedia of Plant Physiology, New Series, Volume 4: Physiological Plant Pathology, edit. by R. Heitefuss and P.H. Williams, pp. 270-315, Springer-Verlag, Berlin, Heidelberg, New York.

26. Rudolph, K., 1976. Models of interaction between higher plants and bacteria. In: Specificity in Plant Diseases, edt. by R. K. S. Wood and A. Graniti. pp. 109-126, Plenum Press, New York and London.

27. Rudolph, K., 1980. Multiplication of bacteria in leaf tissue. Angewandte Botanik 54:1-9.

28. Sequeira, L., G. Gaard and G. A. DeZoeten, 1977. Interaction of bacteria and host cell walls: Its relation to mechanisms of induced resistance. Physiol. Plant Pathology 10:43-50.

29. Strobel, G. A., 1974. Phytotoxins produced by plant parasites. Ann. Rev. Plant Physiol. 25:541-566.

30. Whitcomb, R. F., and J. G. Tully. (eds.). 1979. The Mycoplasmas - Plant and Insect Mycoplasmas. Vol. III, Academic Press, New York.

31. Woods, A. F., 1899. The destruction of chlorophyll by oxidizing enzymes. Centrabl. Bakteriol. Parasitenk., Abt. II, 5: 745-754.

Multiplication of Virulent and Avirulent Pierce's Disease Bacterial Isolates in Grapevine Tissue

D. L. Hopkins, C. M. Thompson

Agricultural Research Center
University of Florida
Leesburg, FL, U.S.A.

Abstract

Multiplication curves of virulent, weakly virulent, and avirulent isolates of the Pierce's disease bacterium in grape leaves were compared. Populations of bacteria were determined by dilution plating of extracts from 2 cm lengths from the inoculated petioles. With all isolates, there was a 4 to 6 day lag preceding rapid growth which ended 14 to 18 days after inoculation. With virulent isolates, populations were maximal at this time and were 10 to 100 times those of avirulent isolates. Populations of virulent isolates stayed at maximal concentration throughout the remaining 4 to 5 weeks of the tests; whereas, those of avirulent isolates stabilized at a maximum for 2 weeks and then declined. Populations of weakly virulent isolates slowly increased throughout the remaining 4 to 5 weeks of the tests. With virulent isolates, symptoms appeared approximately 28 days after inoculation. All isolates had similar multiplication curves in liquid JD-3 media.

Introduction

Pierce's disease (PD) of grapevine is the principal factor responsible for the failure of the European-type (*Vitis vinifera*) and the American type (*V. labrusca*) bunch grapes in the southeastern United States (5, 8). A small, Gram-negative, xylem-limited bacterium referred to as rickettsia-like was found associated with PD (4, 8). A medium was developed for the isolation and growth of the PD bacterium in pure culture and Koch's postulates were fulfilled (1, 2).

Maintenance of the bacterium in axenic culture makes feasible many studies on the characteristics and taxonomy of the bacterium and on the establishment of the host-pathogen relationship. Techniques for mechanically infiltrating the PD bacterial inoculum into the xylem vessels have been developed (3, 6). The virulence of PD isolates obtained from grapevines in Florida, when expressed as minimum concentration of bacteria in the inoculum that produced visible PD symptoms, was quite variable (6). Also, many PD isolates lose virulence after 6 to 12 months of serial subculture.

This report describes studies on the multiplication of virulent, weakly virulent, and avirulent PD isolates in grapevine tissue. The relationship of bacterial populations in the tissue to symptom production and implications to mechanisms of pathogenesis are discussed.

Materials and Methods

The Pierce's disease (PD) isolates were grown at 28°C on PD 2 medium (2) with 2 g/1 of soluble starch substituted for bovine serum albumin. Inoculum from 4- to 6-day old cultures was prepared in succinate-citrate-phosphate (SCP) buffer (disodium succinate 1.0 g/1; trisodium citrate 1.0 g/1; K_2HPO_4 1.5 g/1; KH_2PO_4 1.0 g/1; pH 7.0). Using O.D. at 600 nm on Spectronic 20, the inoculum was adjusted to 10^7-10^8 cells/ml. PD isolates used in this study included PD-1, a virulent isolate producing symptoms in the indicator within 3 to 5 weeks after inoculation; PD-4, a weakly virulent isolate producing mild leaf marginal necrosis (MN) symptoms 10 to 16 weeks after inoculation; and PD-15, an avirulent isolate that did not produce visible symptoms in the indicator grapevines.

Rooted cuttings of Carignane grapevines were used as hosts for PD bacterium multiplication studies. One drop (0.02 ml) of bacterial suspension was used to inoculate each grape petiole. At various lengths of time after inoculation, populations of PD bacteria were determined from 2 cm samples centered on the inoculation point. The samples were surface sterilized in 1% sodium hypochlorite for 3 min and washed 4 times in sterile water. They were then ground in 10 ml of SCP buffer, filtered through cheesecloth, and centrifuged to sediment the bacteria. The bacteria were resuspended in 2 ml of SCP buffer and quantified by dilution plating on PD 2 medium. Each value given in the figures is the average obtained from 3 petioles. Two-cm samples obtained from the midvein of leaves were processed in the same manner.

Petiole samples were taken 6 weeks after inoculation for the determination of the percentage of bacterial infested xylem vessels. With virulent isolates, the inoculated leaf had marginal necrosis symptoms. Samples were cut into 5 mm pieces centered on the inoculation point, fixed in FAA (formalin-alcohol-acetic acid) for 48 h, dehydrated in tertiary butyl alcohol, and embedded in Paraplast. Fifteen nm sections were cut with a rotary microtome and mounted on slides with adhesive. The sections were stained with Harris' hematoxylin and orange G (10). The percentage of vascular bundles infested with bacteria per cross section, and the percentage of vessels completely plugged with bacteria per cross section were determined. Values given are averages obtained from 60 to 80 petiole cross sections from 3 or 4 inoculated petioles.

Results

With all isolates, live bacterial populations in the petiole declined for the first 4 to 6 days after inoculation and then increased rapidly for 7 to 14 days (Fig. 1). With virulent and avirulent isolates, populations were maximum at 21 days after inoculation. The concentration of the virulent PD-1 was 10^7-
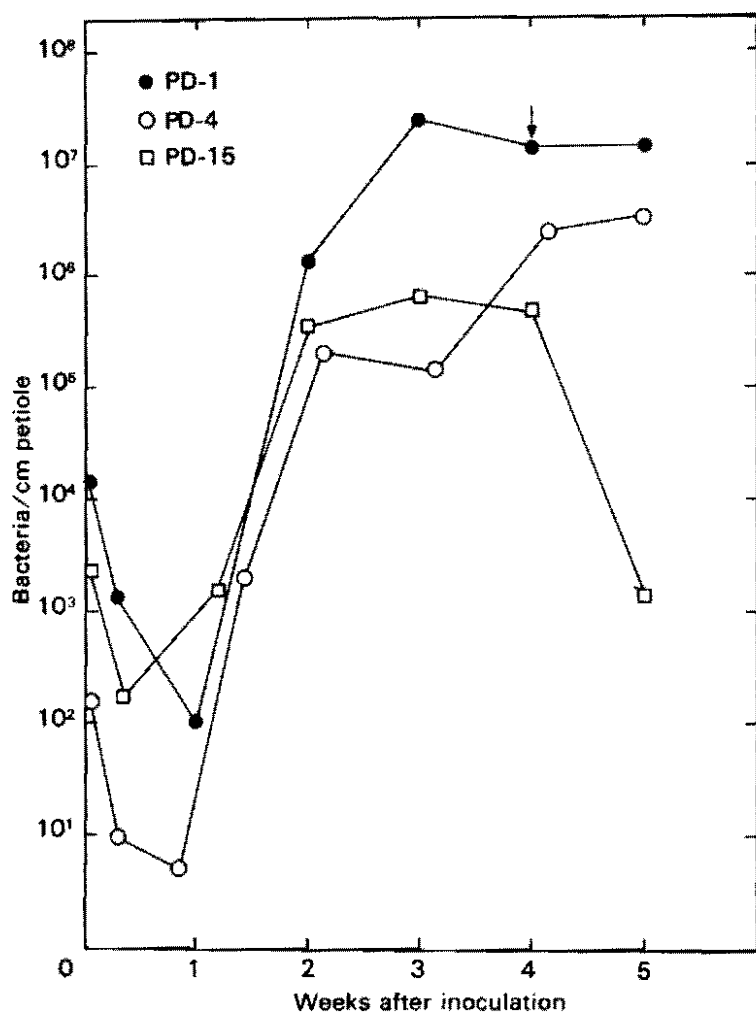
Fig. 1. Growth curves of the virulent (PD-1), weakly virulent (PD-4), and avirulent (PD-15) Pierce's disease bacterium isolates in grapevine petioles. Petioles were inoculated at 0 time with 0.02 ml of a 10^7-10^8 cells/ml suspension. The arrow marks the time of first appearance of symptoms.

10^8 colony forming units per cm of petiole and this was 10 to 100 fold more than the avirulent PD-15. The concentration of weakly virulent PD-4 was similar to PD-15 at 21 days; however, it continued to increase slowly for several weeks until PD symptoms developed 3 to 4 months after inoculation. Marginal necrosis developed approximately 28 days after inoculation with the virulent isolate. Populations of the virulent PD-1 stabilized near the maximum for the life of the inoculated leaf; whereas, those of the avirulent PD-15 started to decline 4 weeks after inoculation.

Other virulent and avirulent PD isolates tested had population curves in grapevine petioles similar to PD-1 and PD-15.

Populations in the midvein of inoculated leaves were determined to evaluate the buildup of the PD isolates at a site removed from the inoculation point. With the virulent PD-1, the growth curve in the leaf vein was nearly identical to that of the petiole with final populations 10 fold less than in the petiole (Fig. 2). Most bacterial population growth in the vein occurred 7 to 14 days after petiole inoculation. With PD-4, the bacterium could not be detected in the leaf vein until more than 28 days after inoculation, with most bacterial growth occurring between 28 and 35 days (Fig. 3). With the avirulent PD-15, bacteria were not generally detected in the leaf vein (Fig. 4). At 28 days, PD-15 had built up in only 1 of 3 leaf veins sampled.

Light microscopy was used to compare the amount of xylem vessel infestation with the virulent and avirulent PD isolates (Table 1). Inoculation with the virulent isolate resulted in approximately 60% of the vascular bundles per cross section having vessels infested with bacteria, compared to only 10% with the avirulent. There were approximately 10 times more vessels infested per cross section with the virulent isolate than with the avirulent. Whereas, almost 5% of the vessels per cross section were plugged by the virulent isolate, very few were plugged by the avirulent one.

Growth curves of the isolates in liquid PD-2 medium were determined using dilution plating techniques. All isolates had multiplication curves similar to those of PD-1 and PD-4 shown in Fig. 5. Maximum cell counts were obtained at 6 days after incubation began. The bacteria then died quickly in the liquid medium

Discussion

Virulence in the PD bacterium does not appear to be related completely with the capability to infest grapevine tissue. Avirulent isolates were able to infect the xylem vessels, multiply, and be reisolated at least 5 weeks later. Rather than the ability to infect grapevine, virulence appeared to depend on the maximum concentration of bacteria attained in the plant tissue. A concentration of 10^7-10^8 bacterial cells per cm of petiole seemed to be required for leaf MN symptom production. Avirulent isolates never reached this concentration before declining, and weakly virulent isolates required a much longer time to reach this concentration than did virulent isolates. The 10 to 100 fold difference in populations of the virulent and avirulent isolates in petioles resulted in bacterial infestation of 10 times as many xylem vessels with virulent bacteria as with avirulent bacteria.

The population of PD bacteria in petioles may be only indirectly related to symptom production, however. It is the PD bacterial concentrations in leaf veins that have been highly correlated with leaf MN symptoms (7). The concentration required in the petioles for symptom production in this study may be important only in providing bacteria for the observed buildup in the leaf veins. A concentration of more than 10^6 bacteria per cm of leaf vein was also correlated with symptom production. Cells of avirulent isolates rarely moved into the leaf veins. Both virulent and weakly virulent isolates
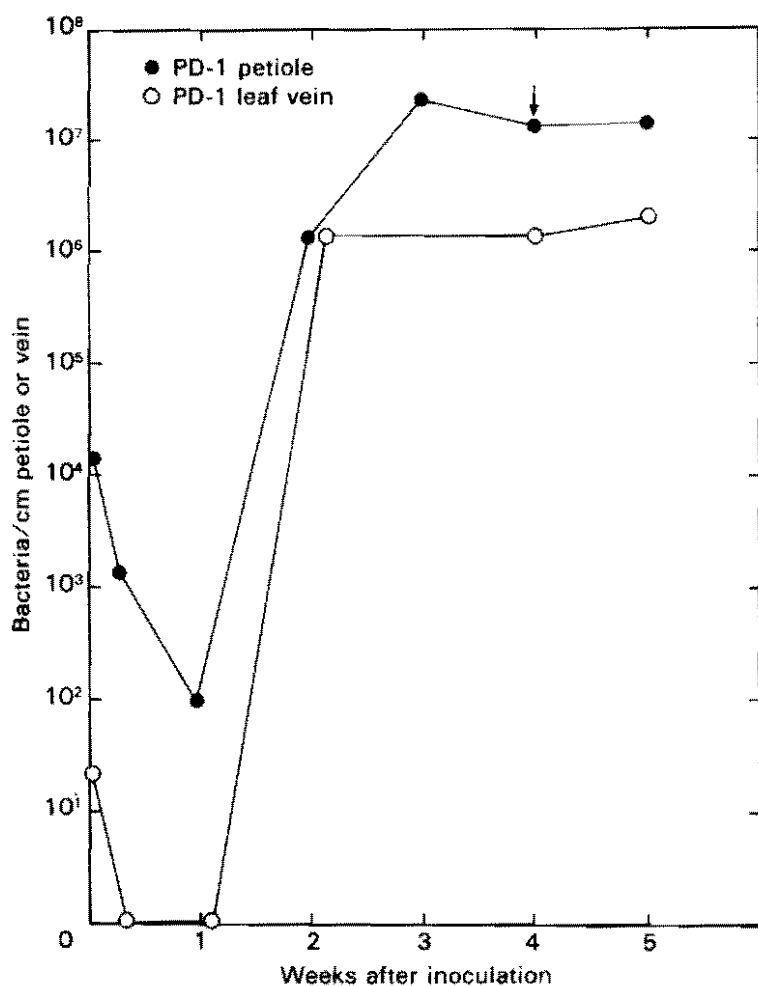
Fig. 2. Growth curves of a virulent Pierce's disease isolate in grapevine petioles and leaf veins. Petioles were inoculated at 0 time with 0.02 ml of a 10^7-10^8 cells/ml suspension. The arrow indicates time of first appearance of symptoms.
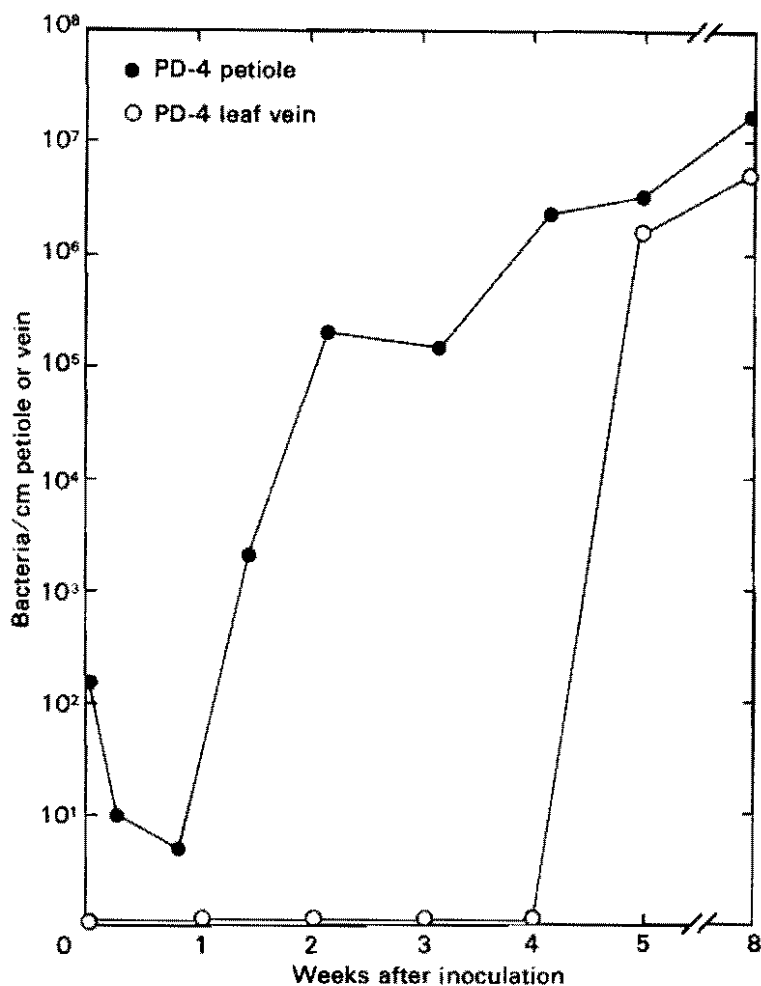
Fig. 3. Growth curves of a weakly virulent Pierce's disease isolate in grapevine petioles and leaf veins. Petioles were inoculated at 0 time with 0.02 ml of a 10^7-10^8 cells/ml suspension.
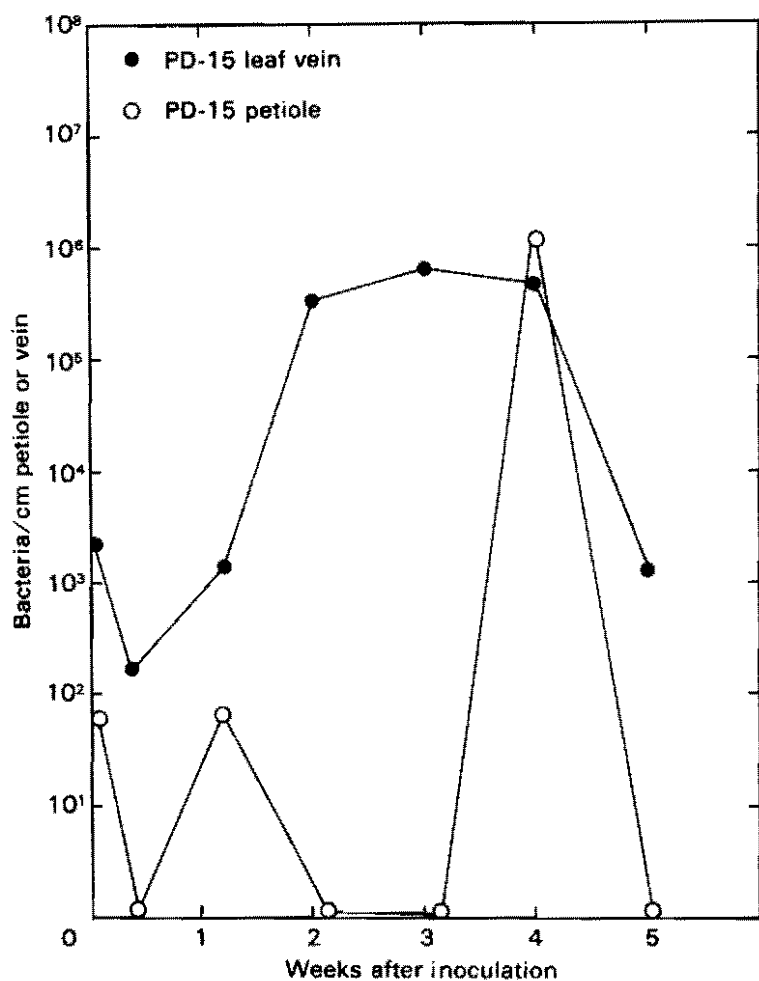
Fig. 4. Growth curves of an avirulent Pierce's disease isolate in grapevine petioles and leaf veins. Petioles were inoculated at 0 time with 0.02 ml of a 10^7-10^8 cells/ml suspension.
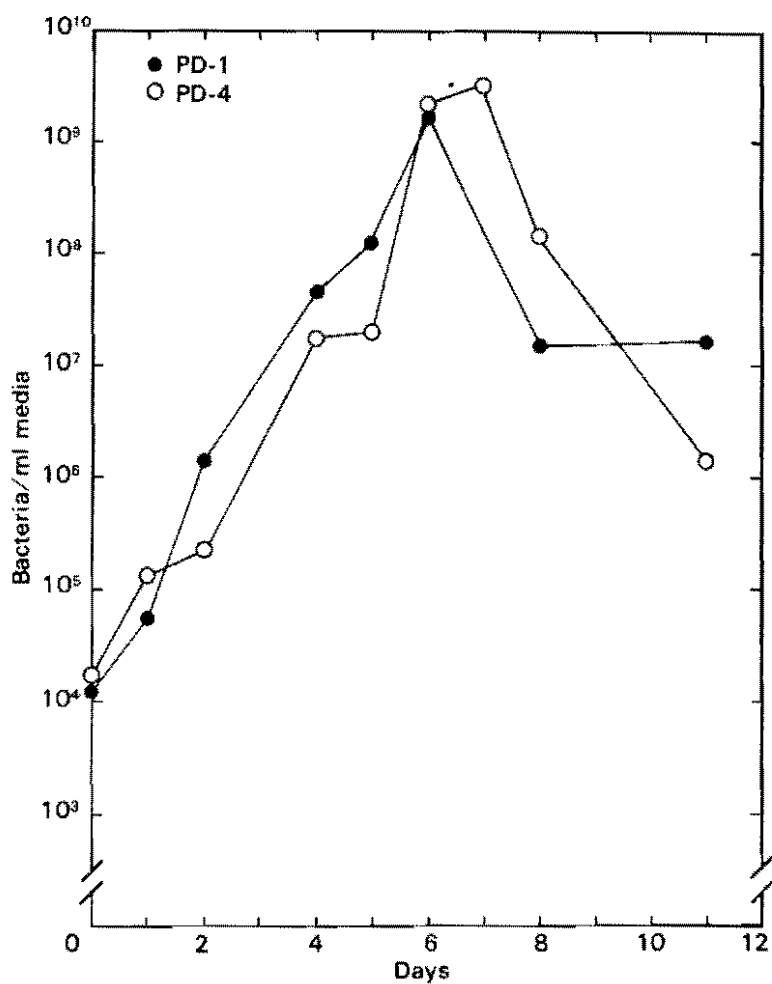
Fig. 5. Growth curves of the virulent (PD-1) and avirulent (PD-15) Pierce's disease isolates in liquid PD-2 medium.

Table 1. Comparison of the concentration of a virulent and avirulent strain of the Pierce's disease bacterium in grapevine petioles.

Strain	Vascular bundles infested with bacteria[a] (o/o)	Vessels infested with bacteria[a] (o/o)	Vessels plugged with bacteria[a] (o/o)
Virulent	61.1	23.6	4.6
Avirulent	11.0	2.5	0.1

[a]Data are given as the average percentage of bacterial infested vascular bundles per cross section or the percentage of infested or completely plugged xylem vessels per cross section. Each value is the average obtained from counting 60-80 petiole cross sections. Samples were taken 6 weeks after inoculation.

appeared to need to reach a threshold of 10^6 cells per cm of petiole before the bacteria moved into the leaf veins. Avirulent isolates never reached this level.

The results of this study tend to support the hypothesis that the leaf marginal necrosis symptoms of PD are caused by the blockage of xylem vessels with bacterial aggregates producing water stress in the leaf (7), rather than by toxin (9). If a potent, transportable toxin were producing the symptoms, one would expect that the populations of avirulent and weakly virulent isolates attained would be sufficient to produce toxin. If the ratios used to convert from infested or plugged vessels per cross section to infested or plugged vessels per 0.5 cm length of petiole (7) are used on the data in Table 1, they indicate the virulent isolate infested over 90% of the vessels and blocked over 20% of them within a 0.5 cm length of petioles. Using the same ratios, the avirulent isolate infested about 13% of the vessels and blocked less than 1%. Leaf veins farther out toward the leaf margins usually have a higher percentage of plugged vessels than the petioles have. This difference in bacterial infestation and plugging of xylem vessels could easily explain the failure of the avirulent isolates to produce symptoms. However, a weak or immobile toxin could be involved in the leaf symptoms, with a threshold population of bacteria in the leaf petiole or vein being required to produce sufficient toxin for symptom development. Further studies on the mechanism of pathogenesis are being conducted.

Literature Cited

1. Davis, M. J., A. H. Purcell, and S. V. Thomson. 1978. Pierce's disease of grapevine Isolation of the causal bacterium. Science 199:75-77.
2. Davis, M. J., A. H. Purcell and S. V. Thomson. 1980. Isolation medium for the Pierce's disease bacterium Phytopathology 70:425-429.
3. Davis, M. J., S. V. Thomson and A. H. Purcell. 1980. Etiological role of the xylem-limited bacterium causing Pierce's disease in almond leaf scorch. Phytopathology 70,472-475.

4. Goheen, A. C., G. Nyland and S. K. Lowe. 1973. Association of a rickettsia-like organism with Pierce's disease of grapevine and alfalfa dwarf and heat therapy of the disease in grapevines. Phytopathology 63:341-345.

5. Hopkins, D.L. 1977. Diseases caused by leafhopperborne, rickettsia-like bacteria. Ann. Rev. Phytopathology 17:277-294.

6. Hopkins, D.L., 1980. Use of the pin-prick inoculation technique to demonstrate variability in virulence of the Pierce's disease bacterium. Proc. 7th Meeting of the International Council for the Study of Viruses of the Grapevine (In Press).

7. Hopkins, D.L., 1981. Seasonal concentration of the Pierce's disease bacterium in grapevine stems, petioles, and leaf veins. Phytopathology 71:415-418.

8. Hopkins, D.L. and H.H. Mollenhauer. 1973. Rickettsia-like bacterium associated with Pierce's disease of grapes. Science 179-298-300.

9. Lee, R.F., B.C. Raju and G. Nyland. 1978. Phytotoxin(s) produced by the Pierce's disease organism grown in culture. (Abstr.) Phytopathology News 12:128.

10. Sass, J. E. 1958. Botanical Microtechnique. 3rd. ed. The Iowa State University Press, Ames, Iowa. 228 pp.

Relation of Water Activity to Growth and Extracellular Pectate Lyase Production by *Erwinia chrysanthemi*

J. P. Mildenhall
Univ. of Fort Hare
Alice, South Africa

B. A. Prior
Univ. of the Orange Free State
Bloemfontein, South Africa

J. M. Brand
Univ. of Fort Hare,
Alice, South Africa

Abstract

The growth of *Erwinia chrysanthemi* pv *zeae* was investigated in a glucose-yeast extract-salts (glucose-YS) medium adjusted to various water activities (A_w's) with either NaCl or mannose. The specific growth rate (n) was similar between 0.998 A_w and 0.990 but declined rapidly thereafter. Extracellular pectate lyase (PL) production decreased significantly between 0.998 and 0.980 A_w when either mannose, sorbitol, or D-arabinose was used to adjust A_w of the sodium polypectate (NaPP)-YS medium.

A similar reduction in enzyme production occurred when *E. chrysanthemi* was grown in galacturonic acid-YS medium adjusted with NaCl. PL activity declined rapidly in cell-free shake culture when the NaPP-YS medium, only, was adjusted to 0.990 (lactose) or 0.980 (mannose) but not at 0.990 (NaCl). Under stationary conditions, however, PL was stable in these media. When *E. chrysanthemi* was grown in these media under stationary culture, PL production was similar to that observed in shake culture. Decrease in PL production with lowered A_w could not be ascribed to enzyme deactivation in viscous media. The significance of these findings to pathogenicity will be discussed.

Introduction

Studies on the ecology of the soft rot erwinias have shown that free water is invariably required for the development of soft rot (9). The role of free water in the infection process has been ascribed to its effect on the turgidity of tissue and to the depletion of oxygen under saturated conditions. The availability of water, however, may also influence the growth and physiology of the pathogen itself. Although the importance of

water in soft rot development has been documented, there have been few quantitative studies on the moisture requirements of phytopathogenic bacteria (1, 15).

Bacterial stalk rot of maize (*Zea mays* L) caused by a pathovar of *Erwinia chrysanthemi* (Burkholder, McFadden and Dimock) characteriscally occurs on sprinkler irritated maize (4, 6, 7). The association of free water with the development of stalk rot prompted us to investigate the water relations of this pathogen.

The term water activity (A_w) as defined by Scott (14) has been widely accepted for determining the effect of solute or water removal on the growth and physiology of micro-organisms. The A_w is directly related to the relative humidity (RH):

$$A_w = \frac{P}{P_o} = \frac{\% \, RH}{100}$$

where P = vapor pressure of solution
Po = vapor pressure of pure water

Water activity is also directly related to water potential, a term that has been used in several studies on fungi (3).

$$\psi = \frac{-RT}{\bar{V}} \ln A_w$$

where ψ = water potential
R = ideal gas constant
T = temperature ($^\circ$K)
$\bar{V}$ = mole volume of water

We selected liquid media for the investigation of the water relations of *E. chrysanthemi* because, first, the results appear to be similar to those obtained on solid surfaces (15) and, second, liquid media facilitate quantitative measurements of growth and enzyme production.

Pectate lyase (PL) or polygalacturonic acid trans-eliminase E.C 4.2.2.2 (2) is an important enzyme in tissue maceration. The growth *in vivo* of *E. chrysanthemi* may be profoundly influenced by factors which affect the production of PL. The objective of this study was to determine the effect of A_w upon growth and extracellular PL production by *E. chrysanthemi*. Growth and extracellular PL production are profoundly influenced by the A_w of the medium.

Materials and Methods

Organism

A local isolate of *E. chrysanthemi* (7) was used. This has been deposited in the collection of Prof. A. Kelman, Department of Plant Pathology, University of Wisconsin, Madison, U.S.A.

Inoculum Preparation

In all experiments, late logarithmic phase cells from a culture which had been seeded with log phase cells were used. One millilitre of inoculum was added to each flask. In all experiments the inoculum was grown on the same carbon source as that being investigated except for galaturonic acid-yeast extract salts (YS) medium where the inoculum was cultured in sodium polypectate (NaPP)-YS medium.

Medium

The YS medium was similar to that described by Scott (14) and contained the following ingredients (g/1000 g H_2O): Na_2HPO_4, 1.42; KH_2PO_4, 0.27; $MgSO_4 7H_2O$, 0.24; $NH_4 NO_3$, 0.40; yeast extract, 0.90; carbon source, 1.80. This yielded a medium of pH 7.6 and no further adjustment was necessary. Either D-galacturonic acid, glucose or NaPP was used as carbon source. The galacturonic acid-YS medium was titrated with 1N NaOH to pH 7.5 and filter sterilized. D-galacturonic acid was obtained from Sigma Chemical Corp., NaPP from Nutritional Biochemical Corp., yeast extract from Difco Laboratories and mannose and D-arabinose from Riedel de Haën, Hannover, W. Germany. All other chemicals were obtained from E. Merck, Darmstadt, W. Germany.

Water Activity Adjusters

The A_w for growth studies was adjusted with either NaCl or mannose. NaCl was autoclaved in the glucose-YS medium. Mannose and glucose were filter sterilized into the autoclaved YS medium. Water lost during autoclaving was replaced. The amount of solute required to prepare media of various A_w's (Table 1) was determined for NaCl from data of Robinson and Stokes (13) or by using Wecor psychometer (10).

Table 1. Water activity of solutes at 25°C.

A_w	Lactose (monohydrate)	Mannose (anhydrous)	NaCl	D-arabinose	Sorbitol
0.995	0.35	0.26	0.15	0.20	0.28
0.990	0.70	0.52	0.30	0.45	0.56
0.985	1.04	0.81	0.46	0.72	0.84
0.980	1.38	1.10	0.61	1.00	1.13
0.975	1.72	1.40	0.76	1.24	1.41
0.970.	2.08	1.70	0.91	1.50	1.69

The heading "Molality" spans the five solute columns.

Growth and Enzyme Studies

All cultures were grown in 250 ml side-arm Erlenmeyer flasks and growth was measured on either a Klett-Summerson colorimeter with a no.

64 filter or at 620 nm on a Spectronic 20 spectrophotometer (Bausch & Lomb). One millilitre samples removed at suitable intervals for extracellular PL assays were assayed on a Beckman model 35 spectrophotometer with a recorder. Details of growth conditions and enzyme substrate preparation have been described elsewhere (8).

Enzyme Stability Studies

The stability of PL in the media adjusted to various A_w's using either NaCl (0.990 A_w), lactose (0.990 A_w), or mannose (0.980 A_w) was investigated in three different ways. First, 2 ml of a sterile dialyzed supernatant fluid (≈20 units PL/ml) was added to 25 ml cell-free medium and shaken as described previously (8). Second, the organism was grown (shake culture, 8) in double strength NaPP-YS medium to early stationary phase (absorbance 0.60) and then the A_w adjusters were added. Third, flasks were prepared as in the first instance but were not shaken. At suitable intervals samples were removed and assayed for PL. The cell-free dialyzed supernatant fluid was prepared as previously described (8).

Results

Evaluation of A_w Adjusters

Difficulties were encountered in selecting a suitable A_w adjuster that is freely soluble in water, is not used as a nutrient, neither represses nor induces PL production, and is not toxic to *E. chrysanthemi*. NaCl was selected as an ionic adjuster and among the organic solutes, sorbitol, D-arabinose, mannose, and lactose were used (Table 2).

Effects of A_w on Growth

The growth rate of *E. chrysanthemi* in glucose-YS medium was similar at 0.998 A_w and 0.990 A_w but declined rapidly when the A_w was lowered to 0.970 (Figs. 1-2). *E. chrysanthemi* failed to grow at 0.970 A_w (NaCl, Fig. 1) within 16 h but upon prolonged incubation growth was observed in some flasks. At 0.970 A_w mannose was less inhibitory to growth (Fig. 2) than NaCl.

Effect of A_w on Extracellular PL Production

Lowering the A_w of the NaPP-YS medium with either sorbitol (Fig. 3) or D-arabinose (Fig. 4) resulted in a significant decline in PL levels although the final cell concentration was similar (Table 3). A similar trend was observed in galacturonic acid-YS medium adjusted with NaCl (Fig. 5).

Effect of A_w on Enzyme Stability

Pectate lyase was unstable in cell-free shaken media (Fig. 6A) but not under still conditions (Fig. 6C). The enzyme was most stable in the medium containing NaCl (0.990 A_w) and least stable in the medium containing mannose (0.980 A_w) and lactose (0.990 A_w) (Fig. 6A, C). Addition of these A_w adjusters to an early stationary phase culture resulted in a similar

pattern of enzyme inactivation (Fig. 6B). When *E. chrysanthemi* was grown to an absorbance of 0.25 in shake culture and subsequently incubated in still culture, extracellular PL production declined with a lowering of the A_w (Fig. 7).

Table 2. Evaluation of solutes for adjusting the A_w of growth media for *Erwinia chrysanthemi*.

A_w adjuster	Growth rate as a o/o of the rate on NaPP	Remarks
NaCl	0	NaPP but not galacturonic acid precipitates below 0.990 A_w
Lactose	55	Poorly soluble at 0.985 A_w PL not repressed
PEG 400	0	Toxic
Sucrose	57	Metabolized PL repressed
Mannose	29	Slowly metabolized PL not repressed
D-arabinose	0	Not metabolized PL not repressed
Glycerol	71	Rapidly metabolized
Sorbitol	0	PL not repressed
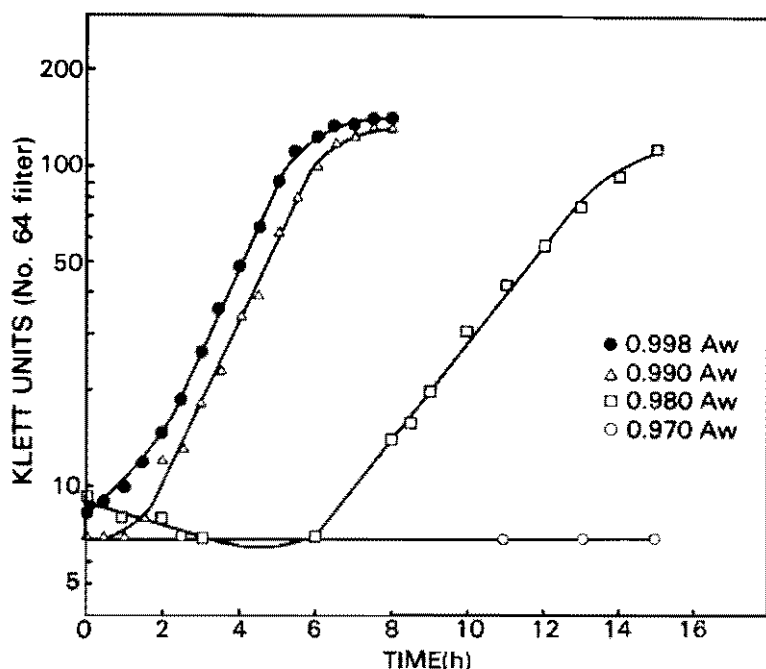
Fig. 1. Effect of A_w (NaCl) on growth of *Erwinia chrysanthemi* in glucose-YS medium at 0.998 A_w, 0.990 A_w, and 0.970 A_w. Each point represents the mean of six determinations. The specific growth rates (h[-1]) at each A_w were 0.63, 0.58, 0.35, and 0.24, respectively.

Table 3. Maximum specific activities of extracellular pectate lyase attained by *E. chrysanthemi* during exponential growth at various water activities.

Adjuster	Water activity					
	0.998	0.995	0.993	0.990	0.985	0.980
Lactose[a]	11.05[c]	7.40	7.48	3.06	ND[d]	ND
Mannose[a]	5.98	1.66	ND	0.37	ND	0.43
D-arabinose[a]	5.52	0.83	ND	0.27	0.24	0.14
Sorbitol[a]	5.46	1.08	ND	0.47	ND	ND
Sodium chloride[a]	13.81	5.29	11.60	20.44	ND	ND
Sodium chloride[b]	1.76	ND	ND	1.32	0.70	0.70

[a]Sodium polypectate as principal carbon source
[b]Sodium galacturonate as principal carbon source
[c]Specific activity expressed as micromol unsaturated uronide released per min per mg cell protein
[d]Not determined.
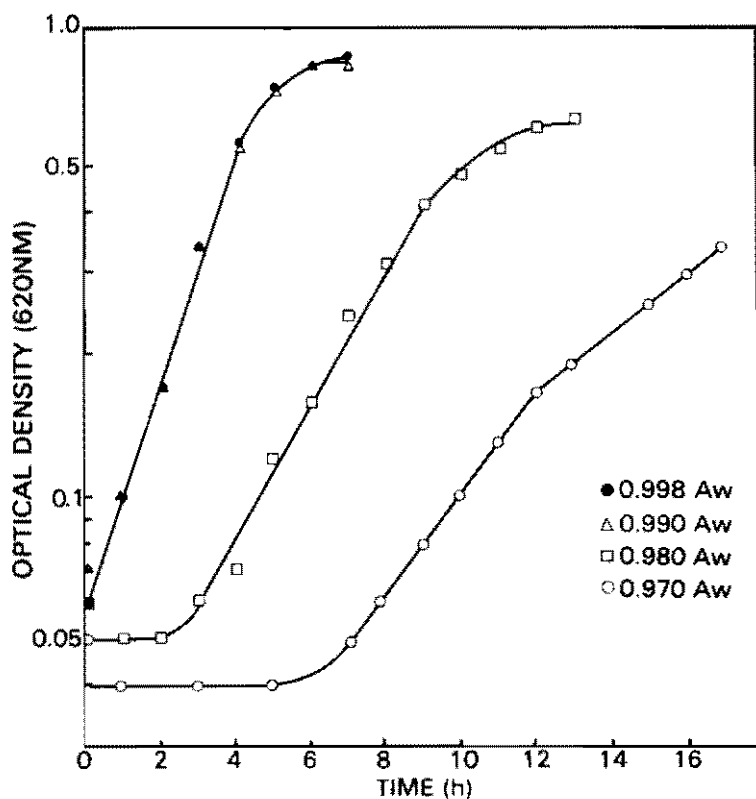
Fig. 2. Effect of A_w (mannose) on *Erwinia chrysanthemi* in glucose-YS medium at 0.998 A_w, 0.990 A_w, 0.980 A_w, 0.970 A_w. Each point represents the mean of three determinations. The specific growth rates (h^{-1}) at each A_w were 0.55, 0.32, and 0.24, respectively.
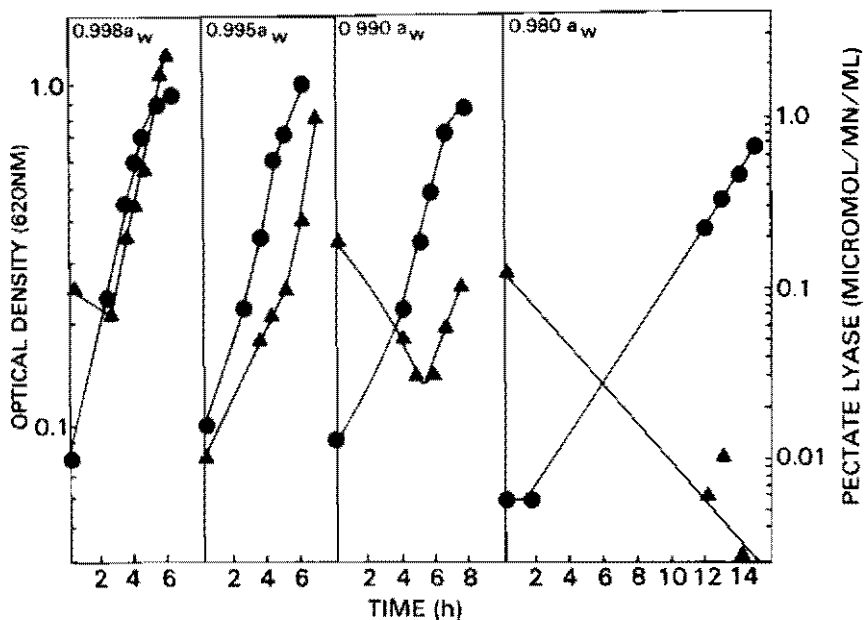
Fig. 3. Relation of growth (●) to pectate lyase production (▲) by *Erwinia chrysanthemi* in NaPP-YS medium at various Aw's adjusted with sorbitol. Each point represents the mean of three determinations.
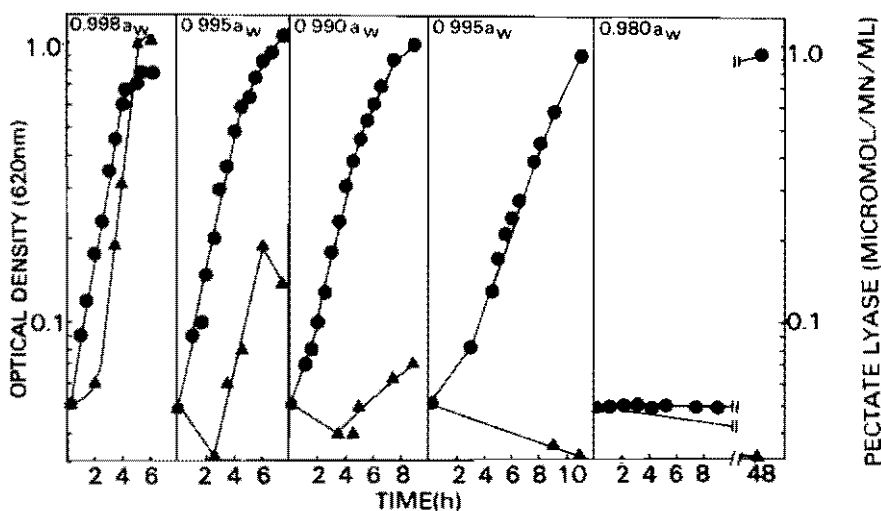
Fig. 4. Relation of growth (●) to pectate lyase production (▲) by *Erwinia chrysanthemi* in NaPP-YS medium at various Aw's adjusted with D-arabinose. Each point represents the mean of three determinations.
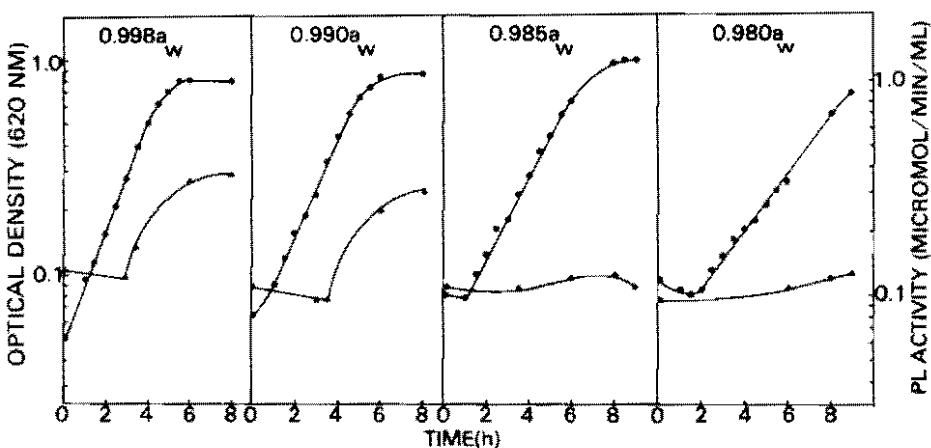
Fig. 5. Relation of growth (●) to pectate lyase production (▲) by *Erwinia chrysanthemi* in galacturonic acid—YS medium at various A_w's adjusted with NaCl. Each point represents the mean of three determinations.
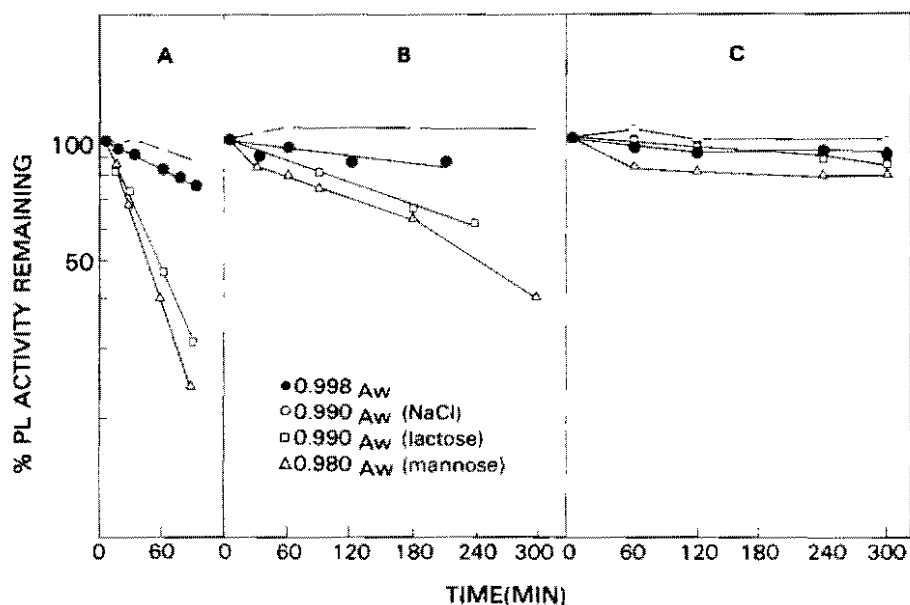
Fig. 6. Stability of pectate lyase of *Erwinia chrysanthemi* at 0x998 A_w, 0.990 A (NaCl), 0.990 A_w (lactose), and 0.980 A_w (mannose). A=cell-free shake medium; B=organism grown to early stationary phase, adjuster added to desired A_w, and culture shaken; C=cell-free still medium.
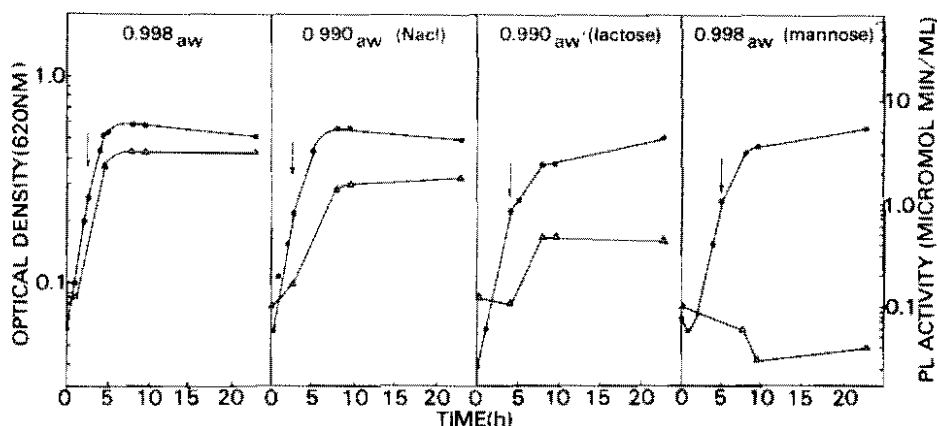
Fig. 7. Effect of agitation on growth (●) and pectate lyase production (△) by *Erwinia chrysanthemi* at various A_w's. Arrows indicate when shaking of cultures ceased.

Discussion

Growth and extracellular PL production by *E. chrysanthemi* are profoundly influenced by lowering the A_w of the medium. The specific growth rate (n) was similar at 0.998 A_w and 0.990 A_w but further lowering of A_w resulted in a rapid decrease of n. A decline in PL production was associated with the lowering of the A_w of both the NaPP-YS medium, adjusted with either sorbitol or D-arabinose (Figs. 3 and 4) and the galacturonic acid-YS medium adjusted with NaCl (Fig. 5). Similar results were obtained when either mannose or lactose was used as A_w adjusters of the NaPP-YS medium (8). An anomaly of these results was the higher levels of PL observed in the NaPP-YS medium adjusted with NaCl (Table 3). Because NaCl precipitated NaPP at A_w's less than 0.990 (Table 2), we were unable to investigate this effect at lower A_w's.

The data presented in Figs. 6 and 7 may be interpreted as follows: First, the addition of NaCl (0.990 A_w) to the NaPP-YS medium stabilizes the enzyme (Fig. 6). Second, organic solutes inactivate the enzyme in shaken media (Fig. 6A, B) but not in still media (Fig. 6C). Similarly, other enzymes are inactivated by shaking in viscous media (12). Third, the decrease in PL production associated with lowered A_w when the organism was grown in still culture (Fig. 7) suggests that the results obtained in shake culture (Figs. 3 and 4) are not solely due to enzyme inactivation from shaking but also to an A_w effect.

These studies, however, do not explain whether the lowering of the A_w affected the synthesis or the excretion of PL. Previous studies have shown that A_w affects the intracellular levels of enzymes in *Pseudomonas fluorescens* (11) and membrane permeability in *Staphylococcus aureus* (5). Elucidation of the mechanism whereby A_w affects PL production demands further study.

Shaw (15) found the *Erwinia amylovora* failed to grow *in vitro* below 97% RH, both in liquid and on solid media. Pear fruits and shoots maintained in environments of different RH's failed to develop disease below 97% RH when inoculated with *E. amylovora*. Therefore the minimum RH at which growth occurred *in vitro* corresponded to the minimum RH in which disease developed. Studies on the infection of maize at different RH's by *E. chrysanthemi* are in progress.

Literature Cited

1. Alberghina, A., U. Mazzucchi, and P. Pupillo. 1973. On the effect of an endopolygalacturonate trans-eliminase on potato tissue. the influence of water potential. Phytopathol. Z. 78:204-213.
2. Bagley, S.T., and M.P. Starr. 1979. Characteristics of intracellular polygalacturonic acid trans-eliminase from *Klebsiella oxytoca, Yersinia enterolitica* and *Erwinia chrysanthemi.* Current Microbiol. 2:381-386.
3. Duniway, J.M., 1976. Water status and imbalance. Pp. 430-449 in R. Heitefuss and P.H. Williams, eds. Physiological Plant Pathology N. S. Vol. 4, Springer Verlag, Berlin.
4. Hoppe, P. E., and A. Kelman. 1969. Bacterial top and stalk rot disease of corn in Wisconsin. Plant Dis. Rep. 53:66-70.
5. Kanemasa, Y., T. Katayama, H. Hayashi, T. Takatsu, K. Tomochika and A. Okabe. 1976. The barrier role of cytoplasmic membrane in salt tolerance mechanism in *Staphylococcus aureus.* Zentralbl. Bakteriol Parasitenkd. Infektionskr. Hyg. Abt. 1 Suppl. 5:189-201.
6. Kelman, A., L. H. Person, and T. T. Hebert. 1957. A bacterial stalk rot of irrigated corn in North Carolina. Plant Dis. Reptr. 41:798-802.
7. Mildenhall, J. P., 1974. Bacterial stalk rot of maize in the Eastern Cape Province. Fort Hare Papers 6:27-34.
8. Mildenhall, J. P., B. A. Prior, and L. D. Trollope. 1981. Water relations of *Erwinia chrysanthemi:* growth and extracellular pectic acid lyase production. J. Gen. Microbiol. (in press).
9. Perombelon, M. C. M., and A. Kelman. 1980. Ecology of the soft rot Erwinias. Annu. Rev. Phytopathol. 18:361-387.
10. Prior, B.A., C. Casaleggio, and H. J. J. Van Vuuren. 1977. Psychometric determination of water activity in the high A_w range. J. Food Protect. 40:537-539.
11. Prior, B.A., and C. P. Kenyon. 1980. Water relations of glucose catabolizing enzymes in *Pseudomonas fluorescens.* J. Appl. Bacteriol. 48:211-222.
12. Reese, E. T. 1980. Inactivation of cellulase by shaking and its prevention by surfactants. J. Appl. Biochem. 2:36-39.
13. Robinson, R. A., and R. H. Stokes. 1955. Electrolyte solutions. Butterworth Scientific Publications. London.
14. Scott, W. J., 1953. Water relations of *Staphylococcus aureus* at 30°C. Aust. J. Biol. Sci. 6:549-564.
15. Shaw, L., 1935. Intercellular humidity in relation to fireblight susceptibility in apple and pear. Memoir 181. Cornell Univ. Agric. Expt. Sta. Ithaca, N.Y.

Relationship of Oxygen Status of Potato Tubers to Pectic Enzyme Maceration of Tissue

E. A. Maher, A. Kelman
University of Wisconsin
Madison, WI, U.S.A.

Abstract

Whole potato tubers were injected with sterile culture filtrates containing pectolytic enzymes from *Erwinia carotovora* (EC) and with viable suspensions of EC and incubated at low and ambient oxygen levels. With both types of injections, tuber tissue decayed readily under low oxygen conditions, but was resistant to decay under aerobic conditions. Culture filtrates contained pectate lyase and polygalacturonase; these enzymes were not inactivated aerobically *in vitro*. A host-mediated response affecting pectic enzyme substrates or activity rather than direct inhibition of bacterial growth may be involved in resistance of potato tubers to bacterial soft rot under aerobic conditions.

Introduction

Bacterial soft rot caused by *Erwinia carotovora* has been more severe when infected potato tubers were held under conditions of reduced oxygen (17, 19, 20, 22). Low oxygen conditions evolve in storage when potatoes are covered with a film of water as the result of condensation or in transit when freshly washed potatoes are not dried before they are packaged (5). The numbers of cells required to elicit a soft rot lesion in potato tubers increase from 10^5 to 10^9 cells as oxygen concentration increases from 5% to 20% oxygen (8). On the basis of these and related studies, tests for susceptibility of potatoes to bacterial soft rot are now completed under conditions of reduced oxygen (8, 13, 18).

The objective of this study was to examine the relationship of oxygen status to maceration of potato tuber tissues by pectolytic enzyme of *Erwinia carotovora*.

Materials and Methods

Bacterial Strains and Media

Erwinia carotovora subsp. *atroseptica* (Eca) (SR 8) and *Erwinia carotovora* subsp. *carotovora* (Ecc) (SR 16, SR 318, SR 319) were the strains used in this study.

Cultures were maintained in test tubes of sterile water and cultured on a casamino acid-peptone-glucose medium (CPG) (6). Cultures of Eca or Ecc that had been incubated at 22°C for 48 to 72 h were transferred from CPG plates to 500 ml of a sodium pectate broth. To maintain low oxygen levels in cultures, freshly autoclaved or freshly steamed and cooled pectate-based broth in rubber-stoppered flasks was sparged with nitrogen gas for several minutes. Aerobic flasks had cotton plugs. Flasks were incubated at 24°C on a rotary shaker for four days. After four days, cultures were centrifuged and standard procedures were followed to obtain partially purified enzyme preparations. Following centrifugation, ammonium sulphate precipitation, and dialysis, the partially purified culture filtrates were stored frozen.

Injection Technique

Potato tubers (cv Russet Burbank) were removed from storage at 4°C, immersed twice in 10% sodium hypochlorite for 20 minute periods, rinsed with sterile distilled water, and allowed to air dry. Sterile micropipets (Pipetman, Rainin Instrument Co., Woburn, MA, 01801) containing injection volumes of 0.1 to 1.0 ml were pushed into the potato and the tips were left in place at a depth of 7 to 10 mm. Injected potatoes were incubated anaerobically either in a 190 l drum that was flushed continuously with nitrogen or in anaerobe jars with catalyst (BBL, Cockeysville, MD, 21030) and 80% (vol/vol) nitrogen, 10% (vol/vol) hydrogen, and 10% (vol/vol) carbon dioxide at 20°C. In each test, one series was incubated under aerobic conditions. Prior to injection, culture filtrates were filter-sterilized. Most of the macerating activity was observed in the resuspended precipitate from a 90% ammonium sulfate fraction and this was the portion used routinely in injections.

Enzyme Assays

In assays for pectolytic enzyme activity, culture filtrates that had been heat-treated for 15 minutes at 100°C were used as controls. Pectolytic enzyme activity was determined using rotating spindle viscometry (23).

Polygalacturonic acid transeliminase (PATE) activity was measured by recording the increase in absorbance at 230 nm (25) using a Varian DB spectrophotometer. Hydrolytic activity (polygalacturonase) was measured by the viscometric technique described above and by testing levels of reducing sugars (21). Proteolytic enzymes were assayed using the technique outlined by Tseng and Mount (27). Protein concentrations in culture preparations, tuber extractions, and ammonium sulphate fractions were determined using the Lowry method (16).

Growth Curves

Growth curves were completed on CPG broth cultures under aerated and low oxygen conditions by measuring turbidity with a Klett-Summerson colorimeter.

Results

Growth and Pectic Enzyme Production Under Low Oxygen Conditions
In Vitro

Growth under conditions of reduced oxygen was inhibited in cultures of Eca and Ecc compared to growth measured under aerated culture

conditions. Both growth rate and yield were affected, as observed in the change of Klett units per hour and the final Klett reading at 22 hours (Table 1). Total specific activity of pectate lyase was less when produced under non-aerated than aerated conditions (Table 1).

Table 1. Growth and PATE activity of *Erwinia carotovora* subsp. *carotovora* grown in aerated and non-aerated broth cultures.

	Aerated	Non-aerated
Growth (Klett Units)[a]	222	55
PATE activity[b]		
(nmoles product/mg protein)	6930	826

[a]Final turbidity measurements made at 22 h on CPG broth cultures using a Klett-Summerson photoelectric colorimeter.
[b]PATE production determined by increase in absorbance at 230 nm in a culture filtrate from a pectate-based broth after 90% $AmSO_4$ saturation, resuspension, and dialysis.

In Vivo Tissue Maceration

When tubers were injected with either viable suspensions of Ecc or with a sterile culture filtrated and maintained under conditions of reduced oxygen, tissue was macerated. In contrast, under aerobic conditions, a dry, necrotic lesion similar to that caused by simple mechanical injury formed at the injection point with cells of Ecc or sterile culture filtrate. Injection of a heat treated culture filtrate caused no tissue maceration under anaerobic conditions (Table 2).

Table 2. Tissue maceration in potato tubers after 96 h incubation under aerobic and anaerobic conditions induced by cells and sterile culture filtrates of *Erwinia carotovora* subsp. *carotovora*.

Preparation Injected[a]	Average Weight of Tissue Macerated (gm)[b]	
	Aerobic	Anaerobic
Cell suspension of *E. carotovora*	0	0.42
Culture filtrate	0	0.39
Heat-treated culture filtrate[c]	0	0

[a]Injection volumes were 0.1 ml.
[b]Average wet weight of tissue macerated/injection site.
[c]Culture filtrates were heat-treated in a water bath at 100°C for 15 minutes.

Activity of Culture Filtrate *In Vitro*

Viscometric assays of pectolytic enzyme activity were completed at both pH 8.5 and pH 4.5 using culture filtrates that had been sparged with air or nitrogen for three hours. PATE and hydrolase activity were not altered significantly by either treatment.

Discussion

The use of whole potato tubers in assays for the macerating activity of pectolytic enzymes has not been reported previously. In general, macerating activity in enzyme preparations has been studied using tuber disks or slices (2, 12, 15). Since resistance to tissue maceration is oxygen-related when either viable EC cells or sterile pectic enzyme preparations are injected into whole tubers, the significance of assays using tissue slices in relation to *in vivo* conditions needs to be re-evaluated.

As a facultative anaerobe, *Erwinia carotovora* is able to grow when oxygen is limited, but our data and others (28) clearly demonstrate that optimal growth occurs *in vitro* under aerobic rather than anaerobic conditions. In contrast, *in vivo* growth and tissue maceration is greater under low than ambient oxygen levels (8). The high resistance of potato tubers to bacterial soft rot under ambient oxygen conditions has been attributed mainly to a host response resulting in formation of compounds that directly inhibit bacterial growth. The data obtained in these studies using whole tubers injected with sterile pectolytic culture filtrates indicate that resistance to tissue maceration under ambient oxygen conditions may involve a host-mediated response to extracellular enzymes produced by the bacterium rather than direct inhibition of bacterial growth.

The stability of pectolytic enzyme activity of the culture filtrate *in vitro* following sparging with air indicates that the loss of effectiveness of the filtrate under aerobic conditions *in vivo* is not attributable to exposure to oxygen.

Although the precise mechanism of cell death caused by pectolytic enzymes has not been determined (2, 3), maceration of plant tissues by filtrates with polygalacturonic acid trans-eliminase (PATE) activity from wild type and mutant strains indicate that this enzyme is the primary moiety involved in tissue degradation by *Erwinia chrysanthemi* (9). Both polygalacturonase and PATE were associated with tissue maceration by virulent strains of Ecc (4). The PATE enzyme has not been separated from hydrolytic activity in purified preparations from Ecc culture filtrates (24), although two distinct proteins may be present.

Rishitin is toxic to Ecc at concentrations that are formed in infected tissue under aerobic conditions (20); under low oxygen conditions production of the phytoalexin rishitin decreases. Although these observations can be correlated with the pattern of resistance under different oxygen levels, induction of a resistant response in whole tubers apparently does not require the presence of the bacterium. Furthermore, rishitin does not affect the activity of pectolytic enzymes (17).

Resistance to bacterial soft rot in ptotatoes under ambient air has been correlated also with the inhibition of bacterial growth by toxic quinones formed by the oxidation of phenolic compounds (15) and to increased

phenolic concentrations in tuber tissue (26). However, a direct inhibitory effect by a toxic compound on the bacterium may not be the primary basis for resistance under aerobic conditions if sterile extracellular pectolytic enzymes lose effectiveness under these same conditions.

Mechanisms for potato tuber resistance to soft rot under aerobic conditions also may include inactivation of pectic enzymes involved in tissue maceration (1). The *in vitro* polymerization of proteins by quinones or free radicals generated by oxidative enzymes from plant tissue may provide a mechanism for pectic enzyme inactivation under oxidative conditions (14).

Resistance to bacterial soft rot under aerobic conditions can be explained on the basis that the pectate substrates in the middle lamella are protected in some manner from pectic enzymes produced by the bacterium. The roles of suberization and wound cork formation have been investigated as barriers to infection (10, 11). Lignification of cell walls may also interfere with the action of pectic enzymes. Since these processes are blocked under low oxygen conditions (29), they may be involved in resistance to tissue maceration under aerobic conditions by making the pectate substrates less accessible to pectolytic enzymes.

Literature Cited

1. Albersheim, P., and A. J. Anderson. 1971. Proteins from plant cell walls inhibit polygalacturonase by plant pathogens. Proc. Nat. Acad. Sci. 68:1815-1819.
2. Basham, H. G., and D. F. Bateman. 1975. Killing of plant cells by pectic enzymes :the lack of direct injurious interaction between pectic enzymes or their soluble reaction products and plant cells. Phytopathology 65:141-153.
3. Basham, H. G., and D. F. Bateman. 1975. Relationship of cell death in plant tissue treated with a homogeneous endopectate lyase to cell wall degradation. Physiol. Pl. Path. 5:249-262.
4. Beraha, L., E. D. Garber and B. A. Billeter. 1974. Enzyme profiles and virulence in mutants of *Erwinia carotovora*. Phytopathol. Z. 81:15-22.
5. Burton, W., and M. J. Wigginton. 1970. The effect of a film of water upon the oxygen status of a potato tuber. Potato Res. 13:180-186.
6. Cuppels, D., and A. Kelman. 1974. Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. Phytopathology 64:468-475.
7. De Boer, S. H., R. J. Copeman, and H. Vruggink. 1979. Serogroup of *Erwinia carotovora* potato strains determined with diffusible somatic antigens. Phytopathology 69:316-319.
8. De Boer, S. H., and A. Kelman. 1978. Influence of oxygen concentration and storage factors on susceptibility of potato tubers to bacterial soft rot (*Erwinia carotovora*). Potato Res. 21:65-80.
9. Ferguson, M. W., and A. K. Chatterjee. 1981. Characterization of *Erwinia chrysanthemi* mutants deficient in synthesis of polygalacturonic acid transeliminase. (Abstr.). Phytopathology 71:216.
10. Fox, R. T., J. G. Manners and A. Myers. 1971. Ultra-structure of entry and spread of *Erwinia carotovora* var. *atroseptica* into potato tubers. Potato Res. 14:61-73.

11. Fox, R. T. V., J. G. Manners and A. Myers. Ultra-structure of tissue disintegration and host reactions in potato tubers infected by *Erwinia carotovora* var. *atroseptica*. Potato Research 15:130-145.

12. Hahn, W., 1969. Untersuchugen zur Faulnisabwehrreaktion der Kartoffelknolle gegenuber dem Erreger der Schwarzbeinigkeit und Knollennassfaule. *Pectobacterium carotovora* Jones (Waldee). Acta Phytopathol. Acad. Scient. Hung. 4:63-76.

13. Kelman, A., and E. A. Maher. 1979. Bacterial soft rot potential; A means of assessing potato tuber quality. Proc. 18th Ann. Washington State Potato Conf. 35-40.

14. Leatham, G. F., V. King and M. A. Stahmann. 1980. *In vitro* protein polymerization by quinones or free radicals generated by plant or fungal oxidative enzymes. Phytopathology 70:1134-1140.

15. Lovrekovich, L., H. Lovrekovich and M. A. Stahmann. 1967. Inhibition of phenol oxidation by *Erwinia carotovora* in potato tuber tissue and its significance in disease reaction. Phytopathology 57:737-742.

16. Lowry, O.H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.

17. Lund, B. M., 1979. Bacterial soft rot of potatoes. *In:* "Plant Pathogens." D. W. Lovelock and R. Davies, (Eds.). Soc. Appl. Bacteriol. Tech. Series 12 Academic Press, NY.

18. Lund, B. M., and A. Kelman. 1977. Determination of the potential for development of bacterial soft rot of potatoes. Am. Potato J. 54:211-225.

19. Lund, B. M., and G. M. Wyatt. 1972. The effect of oxygen and carbon dioxide concentrations on bacterial soft rot. I. King Edward potatoes inoculated with *Erwinia carotovora* var. *atroseptica*. Potato Res. 15:174-179.

20. Lyon, G.D., B. M. Lund, C. E. Bayliss, and G. M. Wyatt. 1975. Resistance of potato tubers to *Erwinia carotovora* and formation of rishitin and phytuberin in infected tissue. Physiol. Pl. Path. 6;43-50.

21. Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.

22. Perobelon, M.C.M., and R. Lowe. 1975. Studies on the initiation of bacterial soft rot in potato tubers. Potato Res. 18:64-82.

23. Sherwood, R. T., and A. Kelman. 1964. Measurement of pectinolytic and cellulolytic enzyme activity by rotating spindle viscometry. Phytopathology 54:110-112.

24. Stack, J. P., M. S. Mount, P. M. Berman and J. P. Hubbard. 1980. Pectic enzyme complex from *Erwinia carotovora:* a model for degradation and assimilation of host pectic fractions. Phytopathology 70:267-281.

25. Star, M.P., and G. Moran. 1962. Eliminative split of pectic substances by phytopathogenic soft rot bacteria. Science 135:920-921.

26. Tripathi, R. K., M. N. Verma, and R. P. Gupta. 1977. Interactions of potato tuber polyphenols and proteins with *Erwinia carotovora. In* "Cell Wall Biochemistry Related to Specificity in Host-Pathogen Interactions." B. Solheim and J. Raa (Eds.). Norway Universitetsfrolaget, Oslo, Norway.

27. Tseng, T. C., and M. S. Mount. 1974. Toxicity of endopolygalacturonate trans-eliminase, phosphatidase, and protease to potato and cucumber tissue. Phytopathology 64:229-236.

28. Wells, J. M., 1974. Growth of *Erwinia carotovora, E,. atroseptica,* and *Pseudomonas fluorescens* in low oxygen and high carbon dioxide atmospheres. Phytopathology 64:1012-1015.

29. Wigginton, M. J., 1974. Effects of temperature, oxygen tension, and relative humidity on the wound-healing process in the potato tuber. Potato Res. 17:200-214.

Regulation of Pectolytic Enzymes in Soft Rot *Erwinia*

A. K. Chatterjee, K. K. Thurn, D. J. Tyrell

Kansas State University
Manhattan, KS, U.S.A.

Abstract

In *Erwinia carotovora*, polygalacturonase (PG) was produced constitutively. On the other hand, polygalacturonic acid *trans*-eliminase (PATE) in *E. carotovora* and both PATE and PG in *E. chrysanthemi* were inducible. PATE production in E. carotovora stopped in the presence of chloramphenical and inducer(s), suggesting that the stimulation of the enzymatic activity resulted from *de novo* protein synthesis. In *E. carotovora*, the differential rates of PATE synthesis were the same with polygalacturonate (PGT) and saturated digalacturonate (SDG), but higher compared to the rate with unsaturated digalacturonate (UDG). On the other hand, in *E. chrysanthemi* the rate with SDG was much higher than that with PGT or UDG. In *E. carotovora* and *E. chrysanthemi*, the rate of PATE synthesis was markedly stimulated with cAMP and UDG and only moderately stimulated with PGT and cAMP. Such stimulation was not detected with SDG and the nucleotide. PATE synthesis in *E. chrysanthemi* occurred without an apparent lag in the presence of UDG or UDG + cAMP. However, in *E. carotovora* the lag period was abolished in the presence of UDG + cAMP, but not in the presence of UDG alone. Our results suggest that a common metabolic derived from either SDG or UDG induces PATE synthesis in these bacterial species.

Introduction

Most members of the "soft rot" group of the genus *Erwinia* produce enzymes, such as polygalacturonic acid *trans*-eliminase (PATE) and polygalacturonase (PG), which cause deploymerization of pectate (polygalacturonic acid). There is genetic and biochemical evidence for an essential role of PATE in tissue-maceration caused by *E. carotovora* and *E. chrysanthemi* (1, 2, 6, 7, 12, 18, 27). Comparative studies with pathogenic and nonpathogenic pectolytic bacteria (5, 31) have suggested that differences in the rates of synthesis and excretion of pectolytic enzymes might be critical in the elicitation of tissue-macerating diseases. Thus, in understanding the expression of virulence of soft rot bacteria, it is essential to consider genetic and physiological factors that control the synthesis of these enzymes as well as the mechanism(s) underlying transport of these proteins across the bacterial cell envelope.

Although the effects of certain substrates on PATE production in *E. carotovora* and *E. chrysanthemi* have been examined to some extent, the

knowledge of PATE regulation largely remains incomplete. For example, Tsuyumu (29) reported that in *E. carotovora* unsaturated digalacturonate (UDG), a catabolic product of polygalacturonate (PGT) (Fig. 1; 16), induced intracellular PATE synthesis earlier and at a higher differential rate than did PGT or galacturonate, and *c*AMP further stimulated the UDG effect.
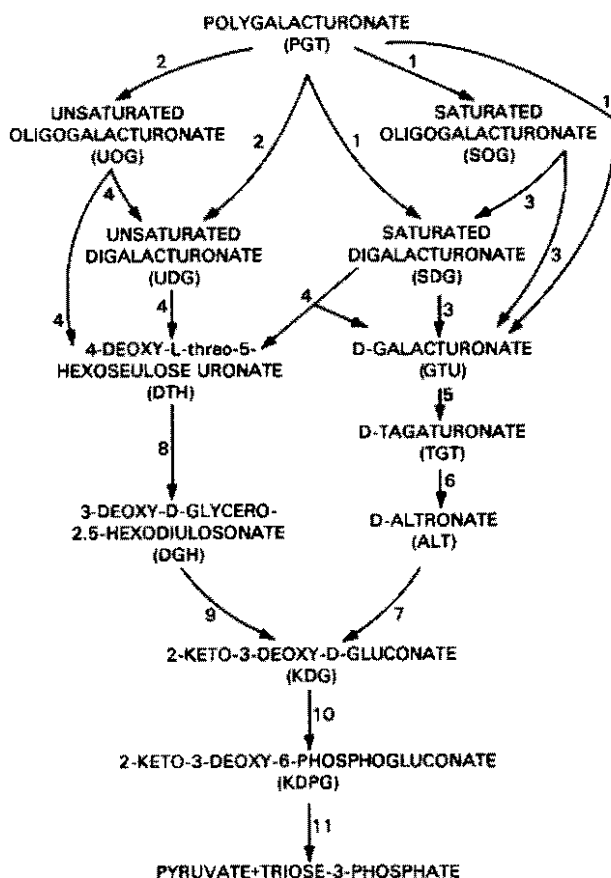
Fig. 1. Major pathways of polygalacturonate catabolism in bacteria. Enzymes for the catabolic steps are: (1) polygalacturonase; (2) polygalacturonic acid *trans*-eliminase (pectate lyase); (3) α-galacturonidase or oligogalacturonate hydrolase; (4) oligogalacturonate *trans*-eliminase; (5) D-glucuronate-ketol-isomerase; (6) D-altronate oxidoreductase; (7) D-altronate hydrolyase; (8) 4-deoxy-*L*-threo-5-hexoseulose uronic acid isomerase; (9) 2-keto-3-deoxy-D-gluconate (3-deoxy-D-glycero-2, 5-hexodiulosonic acid) dehydrogenase; (10) ketodeoxygluconate kinase; (11) ketodeoxyphosphogluconate aldolase. D-galacturonate (GTU) pathway consists of steps 5, 6, 7; 5-deoxy-*L*-threo-5-hexoseulose uronate (DTH) pathway follows the sequence 8, 9; 2-keto-3-deoxy-D-gluconate (KDG) pathway consists of steps 10, 11.

Unfortunately, the effect of saturated digalacturonate (SDG), a product of both PATE and PG activities (Fig. 1; 16), was not examined nor was the effect of these substrates on extracellular enzyme(s). Stack *et al.* (25) recently reported that most of the induced PATE activity was extracellular in a strain (EC14) of *E. carotovora*. Since the quantity of secreted PATE might vary between bacterial strains (5), in comparing inducer efficiency of substrates it is desirable to examine the effects of the total (i. e., both intra- and extracellular) enzymatic activity.

PATE synthesis in *E. carotovora* and *E. chrysanthemi* was glucose (catabolite) repressible (5, 17, 30). Cyclic adenosine monophosphate (*c*AMP) apparently relieved glucose repression and induced PATE synthesis in a *c*AMP -deficient mutant of *E. carotovora* (14, 19). In *E. chrysanthemi,* we (11, M. W. Ferguson and A. K. Chatterjee, unpublished data) and others (9) found that UDG or SDG, compared to PGT, was a more effective inducer of extracellular PATE and that *c*AMP potentiated the effects of UDG. While these findings collectively implicated *c*AMP in regulation of PATE production, the mechanism(s) underlying the effect remained unclear.

In contrast to PATE of these *Erwinia* spp., much less is known of polygalacturonase, the pectolytic enzyme that causes depolymerization by hydrolytic cleavage of the polymeric substrate (21). Consequently, the role of this enzyme in bacterial pathogenicity or in the catabolism of polyga- lacturonate largely remains unknown.

With the development of genetic systems in such soft rot pathogens as *E. carotovora* (23, A. K. Chatterjee and K. K. Thurn, In progress) and *E. chrysanthemi* (3, 4, 6; also see Chatterjee *et al.,* these proceedings,) it became feasible to examine the organization and expression of genes that specify pectolytic enzymes and the polygalacturonate catabolic pathway. Knowledge of metabolic pathways and their physiological regulation is helpful in designing genetic experiments, particularly in the isolation of mutants. As a prelude to genetic analysis of pectolytic enzymes and catabolism of polygalacturonate, we compared the effects of various substrates on the production of PATE and PG in *E. carotovora* and *E. chrysanthemi.* In this report we discuss our findings on the effects of polygalacturonate and its catabolic products, UDG and SDG, on pectolytic enzyme production, and the stimulation of enzyme synthesis by *c*AMP.

Materials and Methods

Wild-type strains of *E. carotovora* (EC, EC15) and *E. chrysanthemi* (EC16) were used. Cultural conditions were as described earlier (5).

Polygalacturonic acid *trans*-eliminase (PATE) was assayed using the procedure of Starr *et al.* (26) in a 0.6 ml reaction mixture. The rate of reaction at 30°C was measured using a Gilford recording spec- trophotometer. One unit of PATE activity is defined as the amount of enzyme that produced a change in absorbance of 1.0 at 235 nm.

Polygalacturonase (PG) was assayed according to Starr *et al.* (26), except that the reaction mixture (3 ml) contained 0.8% (w/v) polygalacturonic acid. One unit of PG activity is the amount of enzyme that liberated 1.0 nmole of aldehyde equivalent at pH 5.2 and 30°C.

Unsaturated digalacturonate (UDG) was prepared using an *E. chrysanthemi* (EC16) enzyme preparation (M. W. Ferguson and A. K. Chatterjee, unpublished data) and purified by the method of Davé *et al.* (10). Saturated digalacturonate (SDG) was produced using yeast endopolygalacturonase prepared according to the method of Phaff (24), except that the ammonium sulfate precipitation step was omitted. The products were precipitated as their strontium salts as described by Luh and Phaff (15) and purified SDG was obtained by a modified method of Nagel and Wilson (20) (Chatterjee *et al.*, manuscript in preparation). Column fractions were assayed using the arsenomolybdate assay (22) and the diuronides were identified using paper chromotography on Whatman #1 paper using the solvent system of Davé *et al.* (10).

Results and Discussion

Polygalacturonase (PG) production. The differential rates of PG synthesis in *E. carotovora* (EC) in cultures grown in the presence of glycerol and polygalacturonate (PGT) were 75 units/Klett unit and 55 units/Klett unit, respectively. The bacterium grew better with glycerol and produced a higher level of PG after 4 h incubation than with PGT or glucose. To determine if this feature was common to another wild-type strain of this bacterial species, the production of PG in strain EC15 was examined. Although the levels of PG varied to some extent depending upon bacterial strain tested, in each instance PG synthesis occurred in cells grown with glycerol, and the level of enzymatic activity was not stimulated further with polygalacturonate. These results indicated that PG was produced constitutively in *E. carotovora* strains EC and EC15. It is noteworthy that constitutive production of PG occurs in at least one other pectolytic microorganism (24).

In contrast to the strains of *E. carotovora*, *E. chrysanthemi* (EC16) grown with glycerol or glucose produced low amounts of PG activity and PGT stimulated the production of PG (Table 1). After 4 h the level was 2-fold higher than detected with glycerol. The amount of PG in induced cells of *E. chrysanthemi* was considerably lower than that detected with *E. carotovora* (Table 1). Consistent with the findings of others (8), we have noted that PG of *E. chrysanthemi* was of the exo-type whereas that of *E. carotovora* was predominantly of the endo-type.

Polygalacturonic acid *trans*-eliminase production. In *E. carotovora* (EC), the differential rate of PATE synthesis was higher with SDG and PGT than with UDG or glycerol (Table 2). Maximal rate of PATE synthesis in *E. carotovora* usually commenced after 90 to 180 min of growth, and the substrates (PGT, SDG, UDG, or glycerol) did not alter the lag period significantly (detailed data not presented). Our data do not support the finding of Tsuyumu (28, 29) that higher levels of PATE production commenced sooner with UDG than with PGT. Whether this apparent discrepancy resulted from strain differences or differences in the composition of growth medium remains to be determined. It should be noted, however, that we have determined the total PATE activity whereas Tsuyumu (1979) investigated substrate effects on intracellular activity.

Table 1. Effects of carbohydrates on the production of polygalacturonase (PG) and polygalacturonic acid trans-eliminase (PATE) in *Erwinia* spp.[a]

Bacterial Species	Activity (units/min/ml/100 Klett units) after 4 hr with					
	glucose		*glycerol*		*polygalacturonate*	
	PATE	PG	PATE	PG	PATE	PG
E. carotovora	0.35	1.1	1.8	2.3	3.6	1.4
E. chrysanthemi	2.7	< 0.02	2.7	0.03	25	0.07

[a]Bacteria were grown at 30°C in minimal salts casamino acids medium [6] in the presence of the desired carbohydrate (final concentration 0.1% W/V). After 4 h incubation, samples of cultures were removed, sonicated, and assayed for enzymatic activity. See Materials and Methods for the assay conditions and the definition of units of activity.

Thus, an alternative explanation might be a preferential stimulation of the intracellular PATE by UDG.

In *E. carotovora*, the addition of cAMP to PGT, SDG, or glycerol grown cultures did not appreciably alter the differential rate of PATE synthesis (Table 2) or the lag period, when compared to cultures grown in the absence of cAMP. In contrast, UDG used in conjunction with cAMP caused a two-fold higher rate of PATE synthesis (Table 2) and abolished the lag period. A similar response of *E. carotovora* with cAMP and UDG was noted by Tsuyumu (29). Our data also indicated that while the differential rate with SDG or PGT without cAMP was not significantly different from that of cells growing in the presence of cAMP, the total amount of PATE produced was consistently higher in the cAMP containing cultures. Therefore, we concluded that cAMP stimulated production of PATE with these substrates.

To determine if the stimulation of PATE synthesis in the presence of inducer(s) was due to *de novo* protein synthesis, chloramphenicol, an inhibitor of protein synthesis, was added to cultures of *E. carotovora* (EC) growing exponentially in the presence of SDG and oligogalacturonides. The growth and enzymatic activities were then measured at various time intervals. In the absence of the drug (Fig. 2) the culture grew and produced PATE at constant rate. The rate of secretion of the enzyme also remained constant up to 90 min incubation and then declined slightly. The synthesis of PATE stopped within 30 min of the addition of the drug, although the turbidity of the culture slowly increased up to 120 min, the duration of the experiment. In the chloramphenicol treated culture, secretion of PATE occurred, albeit at a slower rate, up to 30 min and then stopped. Judging from the mode of action of cloramphenicol (13), it is safe to conclude that the stimulatory effect of the substrates was exerted at the level of gene expression, i.e., stimulation of the formation of *pat* gene(s) product(s). Our data also suggest a partial uncoupling of PATE synthesis and excretion in the presence of chloramphenicol.

Table 2. Differential rates of polygalacturonic acid trans-eliminase synthesis in *Erwinia carotovora* (EC) and *E. chrysanthemi* (EC16) with various substrates in the presence or absence of cAMP[a].

| Substrate | cAMP | Differential rate with | |
		E. carotovora	*E. chrysanthemi*
PGT	−	108	147
	+	131	424
UDG	−	51	86
	+	103	1273
SDG	−	105	1217
	+	108	1231
Gly	−	79	48
	+	88	58

[a]Bacterial cultures in mid log phase of growth at 30°C in a minimal salts casamino acids medium (6) with glycerol were centrifuged, resuspended in 55 mM phosphate buffer, pH 7.2, inoculated to minimal salts casamino acids medium containing appropriate substrates (final concentration = 0.1º/o) to a Klett value of approximately 30, and incubation continued at 30°C. When cAMP was added, a final concentration of 5 mM was used. At desired time intervals (usually 30 to 60 min), culture turbidity was measured using a Klett Summerson colorimeter, and samples for enzymatic assays were removed and frozen. Enzymatic activity was measured after sonication of the thawed culture samples. To obtain differential rates, units of activity/min/ml were plotted against bacterial growth (Klett units) and the values were calculated from segments of the curves showing the maximal enzymatic activity. Differential rate = units of activity/ml ($\times 10^{-3}$) synthesized by bacterial population in a Klett unit. Klett unit = 5×10^{6} cell/ml.

PATE synthesis in *E. chrysanthemi* (EC16) was stimulated by SDG, UDG, and PGT in that higher rates of enzyme synthesis commenced sooner with these substrates than with glycerol (Fig. 3). SDG caused a dramatic increase in PATE synthesis as evidenced by a differential rate 8-fold higher than that of PGT grown cultures and by a shorter lag period. Although PATE synthesis in the presence of UDG commenced without an apparent lag, the rate of synthesis remained low throughout the duration of the experiment (Fig. 3). cAMP stimulated the effects of PGT and UDG in EC16 by stimulating the rate of PATE synthesis (Fig. 4; Table 2). The effect was most striking with UDG where cAMP caused a 15-fold higher rate of synthesis than noted with UDG in the absence of the nucleotide. The high differential rate of PATE synthesis with SDG was not stimulated further by the addition of cAMP (Table 2). We might note that this differential rate is similar to that of UDG + cAMP grown cultures. Moran *et al.* (16) have shown that

oligogalacturonide *trans*-eliminase (OGTE) of *E. carotovora* produced 4-deoxy-5-hexoseulose uronate (DTH; Fig. 1) from SDG or UDG. Recent findings (9) suggest that DTH is an apparent inducer of PATE. Our results are also consistent with the view that PATE synthesis is induced by a common metabolite derived from diuronides.

Investigations seeking clarification of the role of *c*AMP in the expression of *pat* are under way. It has been suggested that *c*AMP effect could have resulted from the relief of "self catabolite" repression caused by UDG or its catabolic products (9, 29). However, other possibilities such as enhanced inducer transport or interaction with *c*AMP of a regulator molecule (protein) must be considered in assessing the effect of the nucleotide in the regulation of PATE in *E. carotovora* and *E. chrysanthemi.*
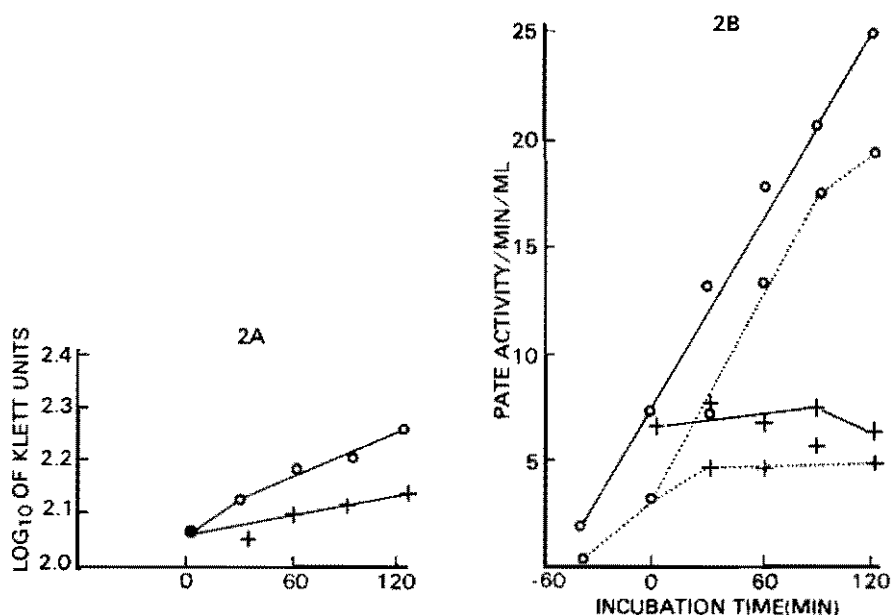
Fig. 2. Effect of chloramphenicol on growth and PATE synthesis in *E. carotovora* (EC). Bacterial cultures were treated as described in Table 2 (footnote a). Minimal salts casamino amino acids medium containing inducers (final concentration = 0.1% w/v) was inoculated to a Klett value of approximately 30 and incubation continued at 30°C. Aliquots of the culture were removed at 30 min intervals, frozen, sonicated and assayed for PATE (as described in the Materials and Methods). After induction had occurred (usually following 120 to 150 min incubation), the culture was divided into 2 parts, and chloramphenicol (final concentration = 185 ng ml⁻¹) was added to one culture at O time and the other culture served as a control. Samples of both cultures were removed at 30 min intervals and divided into 2 parts. One such sample was frozen, sonicated and assayed for PATE activity (total lysate). The other sample was centrifuged and the supernatant assayed for PATE activity. Fig. 2A illustrates the growth of EC in the absence (0) or presence (+) of chloramphenicol. Fig 2B illustrates the rate of PATE synthesis and the quantity of excreted PATE in the presence or absence of chloramphenicol.
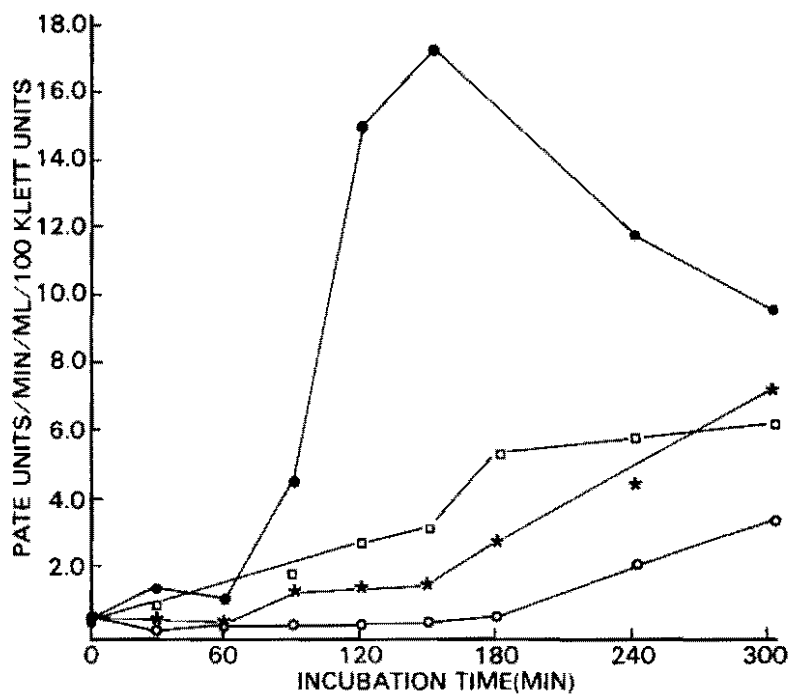
Fig. 3. PATE synthesis in *E. chrysanthemi* (EC16) in the presence of various substrates. Cultures were treated as described in Table 2 (footnote a).
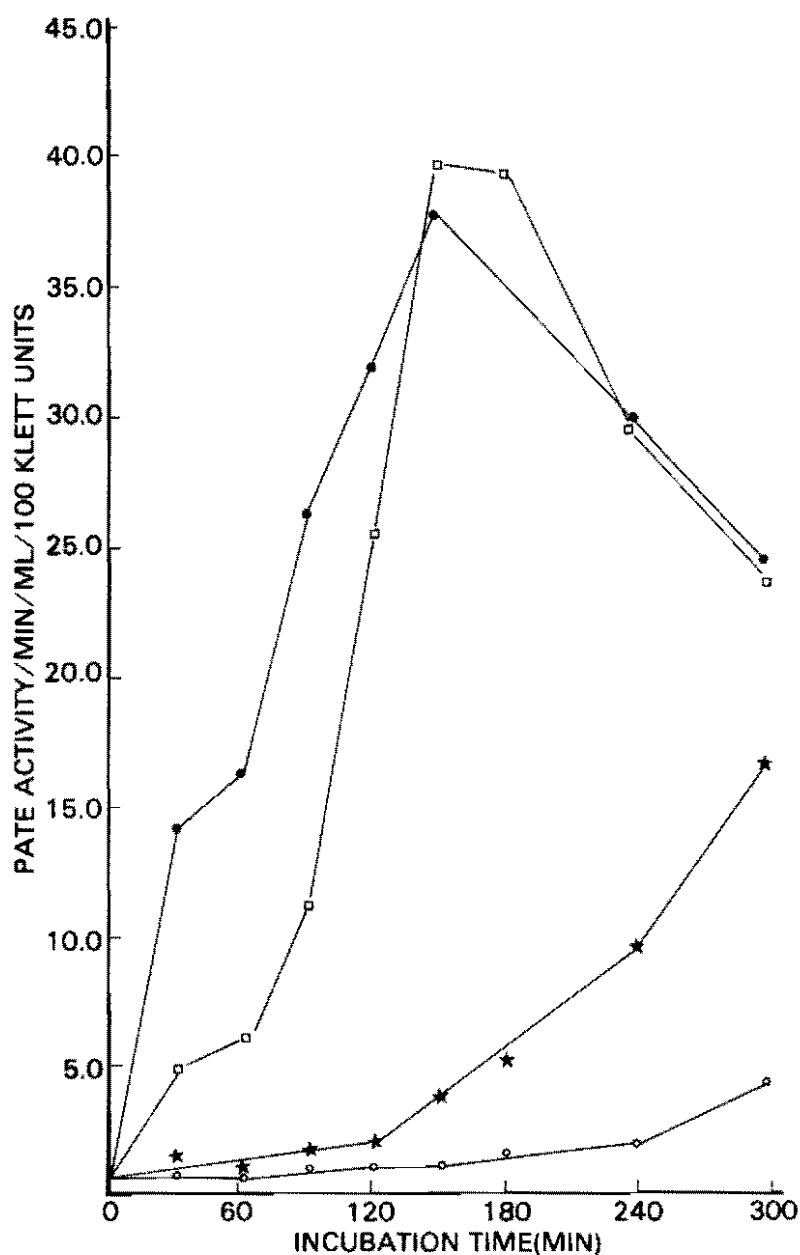
Fig. 4. Effect of various substrates and 5mM cAMP on the rate of PATE synthesis. Cultures of *E. chrysanthemi* (EC16) were treated as described in Table 2 (footnote a).

Literature Cited

1. Beraha, L., and E. D. Garber. 1971. Avirulence and extracellular enzymes of *Erwinia carotovora*. Phytopathol. Z. 70:335-344.
2. Basham, H. G., and D. F. Bateman. 1975. Relationship of cell death in plant tissue treated with a homogeneous endopectate lyase to cell wall degradation. Physiol. Plant Pathol. 5:249-262.
3. Chatterjee, A. K., 1980. Acceptance by *Erwinia* spp. of R plasmid R68.45 and its ability to mobilize the chromosome of *Erwinia chrysanthemi*. J. Bacteriol. 141:111-119.
4. Chatterjee, A. K., and M. A. Brown. 1980. Generalized transduction in the enterobacterial phytopathogen *Erwinia chrysanthemi*. J. Bacteriol. 143:1444-1449.
5. Chatterjee, A. K., G. E. Buchanan, M. K. Behrens, and M. P. Starr. 1979. Synthesis and excretion of polygalacturonic acid *trans*-eliminase in *Erwinia, Yersinia,* and *Klebsiella* species. Can. J. Microbiol. 25:94-102.
6. Chatterjee, A. K., and M. P. Starr. 1977. Donor strains of the soft-rot bacterium *Erwinia chrysanthemi* and conjugational transfer of the pectolytic capacity. J. Bacteriol. 132:862-869.
7. Chatterjee, A. K., and M. P. Starr. 1980. Genetics of *Erwinia* species. Annu. Rev. Microbiol. 34:645-676.
8. Collmer, A., and D. F. Bateman. 1979. An exo-poly-α-galacturonosidase from *Erwinia chrysanthemi*: Purification, characterization and aspects of regulation. Phytopathology 69:1025 (Abstr.)
9. Collmer, A., and D. F. Bateman. 1981. Impaired induction and self-catabolite repression of extracellular pectate lyase in *Erwinia chrysanthemi* mutants deficient in oligogalacturonide lyase. Proc. Nat. Acad. Sci. USA 78:3920-3924.
10. Davé, B. A., R. H. Vaughn, and J. B. Patel. 1976. Preparation, separation and degradation of oligouronides produced by polygalacturonic acid *trans*-eliminase of *Bacillus pumilus*. J. Chromatogr. 116:395-406.
11. Ferguson, M. W., and A. K. Chatterjee. 1981. Regulation of extracellular polygalacturonic acid *trans*-eliminase in *Erwinia chrysanthemi*. Phytopathology 71:216 (Abstr.)
12. Garibaldi, A., and D. F. Bateman. 1971. Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. Physiol. Plant Pathol. 1:25-42.
13. Garrod, L. P., and F. O'Grady. 1968. Antibiotic and Chemotherapy. 2nd. Ed. The Williams and Wilkins Co., Baltimore 123-125.
14. Hubbard, J. P., J. D. William, R. M. Niles, and M. S. Mount. 1978. The relation between glucose repression of endo-polygalacturonate *trans*-eliminase and adenosine 3'5'-cyclic monophosphate levels in *Erwinia carotovora*. Phytopathology 68:95-99.
15. Luh, B. S., and M. J. Phaff. 1954. End products of hydrolysis of pectin and pectic acid by yeast polygalacturonase (YPG). Arch. Biochem. Biophys. 51:102-112.
16. Moran, F., S. Nasuno, and M. P. Starr. 1968. Oligogalacturonide *trans*-eliminase of *Erwinia carotovora*. Archives of Biochem. and Biophys. 125:634-741.
17. Moran, F., and M. P. Starr. 1969. Metabolic regulation of polygalacturonic acid *trans*-eliminase in *Erwinia*. European J. Biochem. 11:291-295.
18. Mount, M. S., D. F. Bateman, and H. G. Basham. 1970. Induction of electrolyte loss, tissue maceration, and cellular death of potato tissue by an endopolygalacturonic *trans*-eliminase. Phytopathology 60:924-931.

19. Mount, M. S., P. M. Berman, R. P. Mortlack, and J. P. Hubbard. 1979. Regulation of endopolygalacturonate *trans*-eliminase in an adenosine 3'5'-cyclic monophosphate-deficient mutant of *Erwinia carotovora*. Phytopathology 69:117-120.

20. Nagel, C. W., and T. M. Wilson. 1969. The isolation of oligogalacturonic acids by column chromatography. J. Chromatog. 41:410-416.

21. Nasuno, S., and M. P. Starr. 1966. Polygalacturonase of *Erwinia carotovora*. J. Biol. Chem. 241:5298-5306.

22. Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. Biol. Chem. 153:375-380.

23. Pérombelon, M. C. M., and C. Boucher. 1968. Developing a mating system in *Erwinia carotovora*. Pages 47-52 in: Proc. of the IVth International Conference on Plant Pathogenic Bacteria. Angers, France.

24. Phaff, H. J., 1966. [109]α-1,4-polygalacturonide glycanohydrolase (endopolygalacturonase) from *Saccharomyces fragilis*. Enzymes of Complex Saccharide Utilization. P. 636-641. In: Colowick, S. and P. Kaplan (eds.) Methods in Enzymology: Vol. VIII Academic Press, New York.

25. Stack, J. P., M. S. Mount, P. M. Berman, and J. P. Hubbard. 1980. Pectic enzyme complex from *Erwinia carotovora*: A model for degradation and assimilation of host pectic fraction. Phytopathology 70:267-272.

26. Starr, M. P., A. K. Chatterjee, P. B. Starr, and G. E. Buchanan. 1977. Enzymatic degradation of polygalacturonic acid by *Yersinia* and *Klebsiella* species in relation to clinical laboratory procedures. J. Clinical Microbiol. 6:379-386.

27. Stephens, G. J., and R. K. S. Wood. 1975. Killing of protoplasts by soft rot bacteria. Physiol. Plant Pathol. 5:165-181.

28. Tsuyumu, S., 1977. Inducer of pectic acid lyase in *Erwinia carotovora*. Nature (London) 269:237-238.

29. Tsuyumu, S., 1979. "Self-catabolite repression" of pectate lyase in *Erwinia carotovora*. J. Bacteriol. 137:1035-1036.

30. Zucker, M., and L. Hankin. 1970. Regulation of pectate lyase synthesis in *Pseudomonas fluorescens* and *Erwinia carotovora*. J. Bacteriol. 104:13-18.

31. Zucker, M., L. Hankin, and D. Sands. 1972. Factors governing pectate lyase synthesis in soft rot and non-soft rot bacteria. Physiol. Plant Pathol. 2:59-67.

Virulence of *Pseudomonas solanacearum* in Relation to Extracellular Polyasaccharides

C. O. Ofuya, R. K. S. Wood

**Imperial College of Science and Technology
London, England**

Abstract

In isolate of low virulence was obtained from a highly virulent isolate of *Pseudomonas solanacearum*, after plating on tetrazolium chloride agar and selection from still cultures. Both isolates were grown on a basal medium supplemented with sucrose, a potato extract, or with one of various polysaccharides of types found in cell walls of higher plants. The virulent wild type isolate produced, in culture, enzymes which degraded the polysaccharides of types found in cell walls of higher plants. The virulent media containing particular substrates, with few exceptions, the activity of an enzyme was highest when the medium contained its substrate. The isolate of low virulence either did not produce these enzymes or produced much less. Loss of virulence was, therefore, closely related to loss in the capacity to produce a number of enzymes that degrade certain polysaccharides of types found in cell walls.

Introduction

Many plant pathogens produce in culture extracellular enzymes which degrade one or other of the polysaccharides of types found in the cell walls of higher plants (1, 2). In some of the diseases caused by these pathogens, the importance of one or more of the enzymes has been established (1, 2). But in vascular wilts caused by fungi and bacteria, the role of the enzymes is still uncertain.

Pseudomonas solanacearum is a well known and important vascular wilt pathogen of many crops. There is evidence that certain characteristic symptoms of the disease are caused by an extracellular polysaccharide slime which is readily and abundantly produced by virulent strains in culture (5). The pathogen also produces in culture a number of polysaccharidases which, if produced and active in infected plants, could account for some of the symptoms. A cellulasa has been studied in this way (6). Another approach in assessing the role of enzymes is to compare the virulence of different isolates of a vascular wilt pathogen with their capacity to produce one or more of the enzymes in cultures (4, 11, 12). Results from this type of work have been contradictory and inconclusive, partly because culture media and other conditions were probably not

263

always suitable for production of the enzymes *in vitro*, particularly in light of recent work on induction of synthesis of various polysaccharidases by certain vascular wilt fungi (3).

This paper summarizes work with isolates of *P. solanacearum* of high and low virulence to tomato grown in media and in conditions suitable for the production and accumulation of various polysaccharide degrading enzymes.

Materials and Methods

Bacteria

Isolate 115 of *P. solanacearum* was isolated originally from a wilting tomato plant in Ibadan (Nigeria). After it was re-isolated from bacterial ooze on an inoculated, infected tomato plant, isolate 115 of *P. solanacearum* was stored in sterile distilled water and, as needed, grown on a glycerol peptone agar (10). This isolate was highly virulent to tomato. An isolate 115-A, of much lower virulence, was produced after plating on tetrazolium chloride agar and selection from still cultures (7).

Production of Extracellular Polysaccharidases

Bacteria were grown in rotary shaken cultures at 28°C on E. medium containing 0.24% KH_2PO_4, 0.08% $NaHPO_4$, 0.02% $MgSO_4 \cdot 7H_2O$, 0.3% $(NH_)_2SO_4$, 0.1% yeast extract, 0.1% ferric citrate, 1.0% peptone, 1.0% main carbon source, in distilled water. The following carbon sources were used: sodium poly pectate (Sunkist Growers Inc.), citrus pectin (Sunkist Growers Inc.), dehydrated potato extract (Difco Clarified, desiccated water soluble extractives from potatoes), carboxymethyl cellulose (sodium salt, high viscosity, degree of substitution 0.7 - 0.8 BDH), purified wood cellulose (solka floc), xylan (larchwood, Sigma Chemical Company), arabinan, galactan (larchwood, Kock-Light Laboratories), and sucrose. Pectic substrates, arabinan, galactan, and xylan were washed in acidified 70% ethanol, then in 90% ethanol, until free from chloride, dried at 70°C and stored at room temperature. Media were autoclaved at 121°C for 10 min. Fifty ml in 250 ml flasks were inoculated with 1 ml of bacterial suspension from 24 h cultures in E-medium + sucrose. After incubation for 48 h, cultures were centrifuged at 10,000 x g for 10 min to remove bacterial cells and insoluble substrates. Culture filtrates were dialyzed against distilled water at 4°C overnight and were used immediately or after storage at -20°C which had little or no effect on enzyme activity.

Numbers of Bacteria

Total bacteria in cultures were estimated by the viable count technique described elsewhere (10).

Enzyme Assays

Enzymes which degrade sodium polypectate, carboxymethyl cellulose, arabinan, galactan, xylan, and wood cellulose, were assayed by measuring the release of reducing groups (9). Samples containing 1.0 ml of culture filtrate were added to 1 ml 0.5% solutions or suspensions of the substrate in 0.1 M citrate buffer, pH 5.0 to 5.5, or 0.1 M Tris-HC1 buffer, pH 8.5,

before incubation at 30°C for 0.5 h or 1 h. Zero-time reaction mixture or mixtures containing boiled enzyme solutions were used as controls. Standard curves for solutions of 10 - 500 ng glucose, arabinose, and xylose, were used to estimate reducing groups formed after enzymes had acted on substrates.

Virulence

Five 6-week old tomato plants, cv. Money-maker, were inoculated by root injury technique (13). After 7 days at 30°C, wilting was scored on a scale of 0 to 5, in which 0 represented no symptoms and 5 represented complete wilting and death of plants.

Results

Virulence

All plants inoculated with suspensions containing c. 4×10^6 viable cells ml-¹ of the virulent isolate 115 were almost completely wilted within 7 to 10 days with extensive degradation of stems and petioles. Symptoms were somewhat less pronounced after this period with inocula containing c. 3.5 $\times 10^5$ viable cells ml-¹ and still less pronounced when c. 2×10^4 viable cells ml-¹ were used. In contrast, inoculation with the avirulent isolate 115-A caused only very slight symptoms within 7 days at c. 2.4×10^6 viable cells ml-¹ and no symptoms at lower concentrations.

With further incubation all plants inoculated with the virulent isolate became still more severely wilted or were killed. In contrast, symptoms developed little if at all in plants inoculated with isolate 115-A.

Polysaccharidases in Cultures

The activities of polysaccharidases in cell-free liquid from cultures of virulent and avirulent strains of *P. solanacearum* on media containing 1% substrate are summarized in Tables 1 and 2.

The virulent strain (115) produced a wide range of extracellular polysaccharidases which degraded polysaccharide other than wood cellulose (Table 1). Pectic enzymes were the most active: both polygalacturonase and pectate lyase were produced. Arabinanase in cultures on arabinan was of lower but comparable activity. Cellulase (Cx), galactanase and xylanase activities were considerably lower.

The activities of pectic and cellulolytic enzymes in filtrates from cultures containing potato extract were as high or higher than in cultures containing substrates for these enzymes. For arabinanase and xylanase, activities were about a half and activity of galactanase was very much less.

For cultures on media containing particular substrates, with few exceptions the activity of an enzyme was highest when the medium contained its substrata. A striking exception was xylanase, for which cultures on media containing pectin were about twice as active as were those on media containing xylan.

Growth of the bacterium was of the same order in most of the cultures but it was substantially lower in cultures on pectin, possibly because of the decrease in pH. Initial pH of E medium was c. 6.8. Growth was also lower in cultures containing wood cellulose for which the final pH was higher than in other cultures.

Table 1. Virulent *Pseudomonas solanacearum* isolate 115- Polysaccharidases and growth in culture.

Growth Medium	Final pH	Bacterial[d] Numbers	Enzyme Activities[a] (Units ml^{-1})						
			PG[e] (NaPP)[c]	PL (NaPP)	C_x (CMC)	C_1 (Wood cellulose)	AR (Arabinan)	GAL (Galactan)	XYLANASE (Xylan)
EM[b] + Sucrose	7.2	3.2 x 10^6	0.4	0.0	0.9	0.0	0.2	0.0	0.0
EM + NaPP^5	7.4	1.6 x 10^6	15.2	16.9	0.0	0.0	0.0	0.0	0.0
EM + Pectin	5.1	5 x 10^5	9.3	12.8	4.8	0.0	2.2	0.4	9.3
EM + Potato Extract	7.1	1.6 x 10^6	15.0	17.2	6.1	0.9	5.0	0.2	2.2
EM + CMC	6.8	1.3 x 10^6	3.0	0.0	3.4	0.0	0.0	0.0	0.0
EM + Wood cellulose	7.8	7.9 x 10^5	0.0	0.0	4.1	0.0	0.0	0.0	3.7
EM + Arabinan	7.3	2.0 x 10^8	0.0	0.0	0.0	0.0	12.7	0.1	0.6
EM + Galactan	7.5	1.2 x 10^6	0.0	0.0	0.0	0.0	0.9	2.1	0.2
EM + Xylan	7.1	2.0 x 10^6	2.0	0.0	0.0	0.0	0.0	0.0	5.9

[a]Enzyme activities as units ml^{-1} were means for two determinations. Units = ug product min^{-1} ml^{-1} in the assay mixture. [b]EM — E-medium (Materials and Methods). [c]Substrates for assay. [d]Viable cells ml^{-1} [e]NaPP sodium polypectate, CMC carboxymethyl-cellulose, PG polygalacturonase, PL Pectate lyase, C_x Cellulase C_1 Cellulase, AR Arabinanase, GAL Galactanase. Initial pH of media was c. 6.8.

Table 2. Avirulent *Pseudomonas solanacearum* Isolate 115-A. Polysaccharidases and growth in culture.

Growth Medium	Final pH	Bacterial[d] Numbers	PG[e] (NaPP)[c]	PL (NaPP)	C_x (CMC)	C_1 (Wood cellulose)	AR (Arabinan)	GAL (Galactan)	XYLANASE (Xylan)
			Enzyme Activities[a] (Units ml^{-1})						
EM[b] + Sucrose	7.2	2.5×10^8	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EM + NaPP[5]	6.4	1.6×10^6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EM + Pectin	4.8	1.6×10^5	0.0	0.0	1.9	0.0	0.9	0.0	3.7
EM + Potato Extract	7.4	2.5×10^5	0.0	0.0	1.9	0.0	0.0	0.0	2.7
EM + CMC	6.8	1.3×10^6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EM + Wood cellulose	7.8	5×10^5	0.0	0.0	3.3	0.0	0.0	0.0	0.0
EM + Arabinan	6.2	2.0×10^8	0.0	0.0	0.0	0.0	4.2	0.0	0.0
EM + Galactan	7.6	2.5×10^6	0.0	0.0	0.0	0.0	0.0	0.0	0.0
EM + Xylan	7.1	2.5×10^6	0.0	0.0	0.0	0.0	0.0	0.0	0.0

[a]Enzyme activities as units ml^{-1} were means for two determinations. Units = ug product min^{-1} ml^{-1} in the assay mixture. [b]EM — E-medium (Materials and Methods). [c]Substrates for assay. [d]Viable cells ml^{-1} [e]NaPP sodium polypectate, CMC carboxymethylcellulose, PG polygalacturonase, PL Pectate lyase, C_x Cellulase C_1 Cellulase, AR Arabinanase, GAL Galactanase. Initial pH of media was c. 6.8.

In contrast, under similar conditions, the avirulent isolate either did not produce these extracellular enzymes or produced much less. None of the culture filtrates had pectic enzymes, C_1, or galactanase activity. Activities of C_x, arabinanase and xylanase were, with a few exceptions much less than in corresponding cultures of the virulent isolate.

Growth of the avirulent isolate differed from that of the virulent isolate in most cultures on media containing the different substrates. It was substantially less in media containing pectin, possibly because of the decrease in pH.

Discussion

The original highly virulent isolate of *Pseudomonas solanacearum* and the isolate of very low virulence (avirulent) to tomato plants obtained from it grew at different rates in cultures of a basal medium supplemented with sucrose, a potato extract, or with one of a group of polysaccharides of types found in cell walls of higher plants. Enzymes that degrade each of the polysaccharides, except a wood cellulose, accumulated in cultures of the virulent isolate in a pattern largely determined, although with a number of exceptions, by the presence in the culture of the substrate of the enzyme. In striking contrast, cultures of the avirulent isolate did not contain enzymes that degrade pectic polysaccharides, a soluble cellulose, or galactan, and much less of enzymes that degrade arabinan and xylan. Because of high activity in cultures of the virulent isolate, the differences for polygalacturonase and pectate lyase were, perhaps, especially pronounced. This type of evidence has been used to support claims that pectic enzymes and possibly other polysaccharidases, are important in causing certain symptoms of vascular wilts. Thus pathogenic species have been distinguished from non-pathogenic species of *Verticillium* by their ability to produce pectic enzymes (4) Also, mutants of *Fusarium oxysporum* f. sp. *lycopersici,* which did not produce polygalacturonase in culture, were less virulent to tomato plants than the parent isolate which produced this enzyme freely (8).

Similar type of evidence has been used to argue that peptic enzymes are not important in vascular wilts. Thus mutants of *V. dahliae* which were much less virulent than the parent strain nevertheless produced pectic enzymes in culture (11).

This study provides a good experimental basis for virulence because the results show a direct correlation between the production of extracellular enzymes or increased synthesis of these enzymes and virulence of *P. solanacearum.* But the evidence is by no means conclusive because the type of low virulence isolate used in this study was experimentally produced. Thus, conclusions may differ from those of a study of wild type strains.

Arguments for or against a role for pectic, and possibly, other enzymes in pathogenesis face the difficulty that isolates differ in other ways besides their capacity to produce these enzymes. Usually it will be difficult to establish the pectic role one way or the other, except by genetic manipulation. A later paper will describe attempts to do so.

Literature Cited

1. Albersheim, P., T. M. Jones, and P. D. English. 1969. Biochemistry of the cell wall in relation to infective processes. Annual Review of Phytopathology 7:171-194.
2. Bateman, D.F., and R. L. Millar. 1966. Pectic enzymes in tissue degradation. Annual Review of Phytopathology 4:119-146.
3. Cooper, R. M., and R. K. S. Wood. 1975. Regulation of synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. lycopersici. Physiological Plant Pathology 5. pp. 135-156.
4. Dimond, A. E., 1970. Biophysics and biochemistry on the vascular wilt syndrome. Annual Review of Phytopathology 8:302-322.
5. Husain, A., and A. Kelman. 1958. Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. Phytopathology 48 Pp. 155-165.
6. Kelman, A., and E. B. Cowling. 1965. Cellulase of *Pseudomonas solanacearum* in relation to pathogenesis. Phytopathology 55. pp. 148-155.
7. Kelman, A., and J. Hruschka. 1973. The role of motility and aerotaxis in the selective increase of avirulent bacteria in still cultures of *Pseudomonas solanacearum*. Journal of General Microbiology 76. pp. 177-188.
8. McDonnel, K., 1958. Absence of pectolytic enzymes in pathogenic strain of *Fusarium oxysporum* f. *lycopersici*. Nature 182. Pp. 1025-1026.
9. Marais, J. P., J. L. Dewit, and G. V. Quickie. 1966. A critical examination of the Nelson Somogyi method for the determination of reducing sugar. Analytical Biochemistry 15, pp. 373-381.
10. Ofuya, C. O., and R. K. S. Wood. 1979. Pathogenicity and biochemical characteristics of isolates of *Pseudomonas solanacearum* in Nigeria. 4th Int. Conf. Plant Path. Bact. Angers (France): 835-840.
11. Puhalla, J. E., and C. R. Howell. 1975. Significance of endo-polygalacturonase activity to symptom expression of *Verticillum* wilt in cotton, assessed by the use of mutants of *Verticillum dahliae* Kleb, Physiological Plant Pathology, 7:147-152.
12. Talboys, P. W., and L. V. Busch. 1970. Pectic enzymes produced by *Verticillium* species. Transactions of the British Micological Society. 55(3):367-381.
13. Winstead, N. N., and A. Kelman. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42:628-634.

Cell Wall Degrading Polysaccharidases and Extrachromosomal DNA in Wilts Caused by *Pseudomonas solanacearum*

C. O. Ofuya, R. K. S. Wood

Imperial College of Science and Technology
London, England

Abstract

Naturally occurring, low virulence isolates of *Pseudomonas solanacearum* did not produce polysaccharidases that degraded tomato cell walls *in vitro.* Such enzymes were produced by virulent isolates; those degrading pectic polysaccharides were particularly active. Avirulent isolates obtained from virulent isolates did not produce cell wall degrading polysaccharidases. When extrachromosomal DNA from cells of a virulent isolate was incubated with cells of an avirulent isolate, isolates were obtained which produced the cell wall degrading enzymes. Virulence to tomato plants was also restored but not to the level of that of the virulent wild type isolate. The extrachromosomal DNA was resolved into three peaks in 5 to 30% (w/v) alkaline sucrose gradients. The results suggest that virulent isolates of *Pseudomonas solanacearum* contain extrachromosomal DNA which codes for synthesis of a range of cell wall degrading enzymes and this synthesis is l closely related to virulence.

Introduction

Previous research with *Pseudomonas solanacearum* has indicated virulence and ability to kill young succulent tomato plants rapidly is an unstable character because isolates appearing spontaneously and frequently from cultures of a virulent isolate have shown far less virulence. The virulent isolate, when grown culture with each of a number of polymers of types found in cell walls of higher plants, produced enzymes which degraded these polymers, with the exception of wood cellulose, *in vitro.* The avirulent isolate derived from the virulent isolate produced little if any of these enzymes under similar conditions (15). This was taken as evidence that virulence, as the ability to cause severe symptoms to develop rapidly in inoculated tomato plants, depended at least in part, on the production by the pathogen of one or more of these polysaccharide degrading enzymes.

This paper describes further work on the relation between synthesis of these enzymes and virulence, especially as these may be controlled by DNA associated with extrachromosomal elements of the pathogen.

Materials and Methods

Origin and Growth of Bacteria

All strains of *Pseudomonas solanacearum* except one were obtained from diseased tomato plants in Nigeria GM1 1000 was supplied by Dr. C. Boucher, INRA, Station Pathologie Végétale, Versailles, France. Bacteria were grown routinely on a glycerol peptone agar (14). Cultures from which DNA was to be extracted were grown as follows. One ml water containing c. 10^5 bacteria from agar cultures of virulent isolate 1 15 were placed in 250 ml flasks containing 50 ml of P-medium containing K_2HPO_4, 0.05%; $MgSO_4.7H_2O$, 0.025%; yeast extract, 0.1%; peptone, 0.5%; sucrose, 1.0% in glass distilled water. Cultures were grown for 24 h on a rotary shaker at 28°C. The contents of one flask were then transferred to 500 ml P-medium in a 1 l flask and incubated at 28°C for 48 h. Cells were harvested by centrifugation at 10,000 x g for 10 min in pre-weighed ethanol sterilized centrifuge tubes. The pellets were washed and suspended in saline-EDTA (0.15 M NaC1, 0.01 M EDTA pH 8.0) which was then kept at - 20°C. Within 24 h of freezing, cells were thawed at room temperature and then refrozen at -20°C for a further 24 h to predispose to lysis with sodium lauryl sulphate.

Isolation of DNA from Bacteria

The procedure was based on the techniques of Hirt (8) as modified by Guerry *et al.* (7). Bacterial cells obtained as described above were lysed at 37°C in 1% sodium lauryl sulphate containing 400 ng ml^{-1} protease (type V, Sigma Chemical Company, U.S.A.). When lysis was complete after c. 40 min, flasks were cooled and NaCl was added to a final concentration of 1 M to enhance separation of DNA from RNA and polysaccharides. (DNA is soluble in high salt concentration.)

Lysates were stored overnight at 4°C and then centrifuged at 17,000 x g for 30 min at 4°C. This precipiated chromosomal DNA, leaving any lower molecular weight extrachromosomal DNA in the supernatant, which was then removed and mixed with an equal volume of saline-EDTA pH 8.0 to lower the salt concentration.￣

The supernatant fluid was treated with 2 volumes of cold 95% ethanol and kept at -20°C overnight to precipitate the extrachromosomal DNA. The mixture was centrifuged at 4°C, 8,000 x g for 3 min and the precipitate was dissolved in 5 ml saline citrate (0.015 M trisodium citrate pH 7.0, 0.15 M NaCl.) The ethanol precipitated DNA was purified by Marmur's procedures (11). The precipitate was dissolved in c. 5 ml of dilute saline citrate (0.15 M NaCl, 0.0015 M trisodium citrate) by gently shaking or pipetting. Ten ml of concentrated saline citrate (1.5 M NaCl, 0.15 M trisodium citrate pH 7.0) was added to maintain the ionic strength of the dissolved DNA, which was deproteinized by shaking with an equal volume of chloroform-isoamyl

alcohol (24:1) for 10 min and centrifuged. The supernatant was removed with a 10 ml wide bore syringe.

The supernatant obtained after a series of deproteinization was precipitated with cold 95% ethanol and dispersed in saline citrate as described above. One ml of acetate - EDTA (3.0 M sodium acetate, 0.001 M EDTA pH 7.) was added to the solution, to give a suitable ionic environment in the selective precipitation of DNA from RNA, by adding, slowly, 0.54 volumes of isopropanol. The gel which formed was centrifuged at 8,000 x g for 3 min at 4°C.

The final precipitate was washed free of acetate and salt by gently stirring in progressively increasing 70 to 90% portions of cold ethanol. It was then dissolved in saline citrate pH 7.0.

Alkaline Sucrose Gradients

DNA is denatured at high pH. Sedimentation through alkali is often used to distinguish the various conformations of extra-chromosomal DNA. This technique was therefore used as an additional detection procedure for extrachromosomal DNA.

Samples of 0.5 ml of supernatant from lysates were layered onto 5 to 30% (w/v) sucrose gradient solutions containing 0.3 M NaOH and 0.001 M EDTA pH 12.5. Gradients were prepared by a gradient mixer and tubes were placed in SW 27 rotor, certrifuged at 86,400 x g for 24 h at 4°C in a Beckman L2-65B ultracentrifuge.

Gradients were analyzed and 1.3 ml fractions were collected from the tubes using an Inco gradient analyser. The gradients were stabilised against convection by using 50% sucrose during elution. Two and one-half ml of saline citrate (0.15 M NaCl, 0.0015 M trisodium citrate) was added to each fraction and absorbance at 260 nm was measured.

Fractions under peaks were pooled and concentrated by dialysis against polyethylene glycol 6000 in dialysis tubing pretreated before use by boiling successively in 0.1 EDTA and 0.1 M sodium bicarbonate and thoroughly rinsing in water to reduce pore size and remove plasticiser. After overnight dialysis the solution containing DNA was stored under toluene at -20°C.

Incorporation of Extrachromosomal DNA

This involved the preparation of competent cultures of recipient cells (Isolate 115-A), and the transfer of the DNA from isolate 115.

Competent Cultures of Isolate 115-A

Twenty ml of P-medium in a 250 ml flask was inoculated with cell suspensions of a single colony of isolate 115-A from a 24 h agar culture. This was incubated at 28°C for 24 h. The culture was then placed in a 50 ml fresh medium and incubated for 3 h and diluted again with an equal volume of starvation medium (S-medium), to predispose the cells for absorption of the DNA. S-medium contained 1% triptone, 0.1% yeast extract, and 1% glucose in glass distilled water.

The cultures were shaken for 1.5 h. Then 1.5 ml of 15% glycerol were added per 9 ml of culture before dispensing in 0.9 ml aliquots in 5 ml bottles and quickly freezing at -20°C. Cells were used within 4 days of storage.

Transfer of DNA

An aliquot (0.4 ml) of DNA solution c. 40 ng was added to 0.9 ml competent culture c. 4.8×10^4 viable bacteria and incubated at 28°C for 45 min. Four tenths ml of sterile saline citrate replaced the DNA solution in the controls. Also, 0.4 ml DNA solution was treated for 15 min with 0.4 ml DNase solution (RNase free, 400 ng ml^{-1} in 0.2 M M_gSO_4) before incubation with 0.9 ml competent culture.

Characteristics of Bacteria in Relation to Virulence

These were colony shape and color on tetrazolium chloride agar (10), rate of degradation of isolated tomato cell walls, and severity of symptoms in inoculated tomato plants.

Isolation of Tomato Cell-Walls

Cell walls were prepared from 6-week old tomato plants, cv. Money-Maker. Plants were destarched by placing them in the dark for 20 h. They were then cut immediately below the cotyledons and laminae of leaves were removed; stems and petioles were then plunged into liquid nitrogen and ground to fine power (6). The powder was mixed at high speed in 10 vol. (v/w) of 0.1 m potassium phosphate buffer, pH 7.0. The suspension was centrifuged at 10,000 x g for 15 min. The pellet was resuspended in fresh buffer and the procedure repeated twice. The insoluble residue was washed in distilled water then mixed at high speed in 10 vol. chloroform-methanol (1:1,v/v). Insoluble material was collected on sintered glass, washed three times in the chloroform-methanol mixture and finally in several changes of acetone. All extractions were done in an ice-bath. The finely divided walls were handled aseptically after the acetone extraction. They were dried and then stored.

Enzyme Production and Assay

Bacterial cultures were grown on a rotary shaker for 48 h on E-medium (15) containing 0.2% isolated tomato cell walls or 1% of a number of polymeric substrates. At the end of incubation, cultures were centrifuged at 10,000 x g for 10 min to remove bacterial cells and insoluble substrate. Culture filtrates were dialysed against distilled water at 4°C overnight and were used immediately for assays.

The activity of cells wall degrading polysaccharidases in cell-free fluids from cultures in the medium containing 0.2% cell walls was measured against 0.5% (w/v) suspension of susceptible tomato cell walls in 0.1 M citrate buffer pH 5.0, and in 0.1 M Tris-HC1 pH 8.5. Assay mixtures contained 1 ml suspension of substrate and 1 ml culture filtrate at 30°C. After 0.5 h and 1 h, the reaction mixture was centrifuged at 8,000 x g for 5 min and then supernatant was analysed for reducing groups (12).

Polygalacturonase, pectase lyase, cellulase(C$_x$), arabinase, galactanase, and xylanase were assayed by following the release of reducing groups from solutions or suspensions of the following: 0.5% sodium polypectate (pH 5, and 8.5), carboxymethyl cellulose, arabinan, galactan and xylan (12).

Protease was assayed on congocoll in 0.1 M Tris-HCl buffer pH 8.5 at 37°C for 0.5 h (13). Assay mixtures contained 1 ml culture filtrate, 5 mg

congocoll, and 1 ml 0.1 M Tris-HC1 buffer pH 8.5. After incubation, tubes were removed and 4 ml of water was added to the mixture, and undergraded substrate was removed by filtration. Concentration of the released dye was measured at 495 nm.

Virulence

Virulence was determined by a root injury technique on 6-week old tomato plants, cv. Money-Maker (14). Lateral roots of plants in pots were cut with a scalpel along one side of the plant and 10 ml suspensions of inoculum containing $c.$ 2.4 x 10^6 cells ml-[1] were poured over the severed roots bordering the slit which was then closed with displaced soil. Disease was measured on a 0 to 5 scale where 0 = no symptoms; and 5 = complete wilting and death.

Results

Extraction of Extrachromosomal DNA from Virulent
Pseudomonas solanacearum

Extrachromosomal DNA was extracted as described in Materials and Methods. The presence of deoxyribose in the supernatant of lysate of virulent cells (isolate 115) was detected by the Dische reaction using diphenylamine reagent (5). A blue color specific for deoxyribose developed in the sample which was obtained from the supernatant of lysate of virulent cells. About 500 ng ml-[1] of extrachromosomal DNA was extracted from $c.$ 2.52 g, wet weight, of virulent bacteria cells. The absorption ratios of the extracted DNA at 260/230 nm and 260/280 nm were 1.7 and 1.2, respectively. The results of the sedimentation of the DNA in the supernatant of lysates of virulent isolate 1 15 through alkaline sucrose gradients are shown in Fig. 1. Three peaks, a, b, c, were obtained, which may correspond to three forms of the extrachromosomal DNA which differed in their sedimentation rates. Peaks a and b, occurring in the slowly sedimenting fraction of the gradients, represent linear fragments and open circles, respectively. Peak c, occurring in the radpidly sedimenting fraction of the gradients represent covalent circles, which are $c.$ 2.7% of the total DNA.

Growth on Tetrazolium Chloride Agar

Before transfer of the extrachromosomal DNA, competent cultures of the avirulent isolate (115-A) produced colonies which, when isolated on the tetrazolium chloride agar, were butyrous with red centers (mean diameter $c.$ 3.0 mm). After the extrachromosomal DNA isolated from the parent virulent isolate (115) was incubated and plated on tetrazolium chloride agar, $c.$ 1.2% of the colonies which developed were pink, round, and small. Their mean diameter was $c.$ 1.4 mm. The colonies differed from those of isolate 115, 115-A in size, appearance, and growth rate and they showed a much reduced growth rate on the tetrazolium chloride agar after 2 or 3 generations. Cultures of the avirulent isolate (115-A) treated for 15 min with sterile saline citrate (0.015 M trisodium citrate pH 7.0, 0.15 M NaCl) or with a mixture of DNase and extrachromosomal DNA solution, produced on
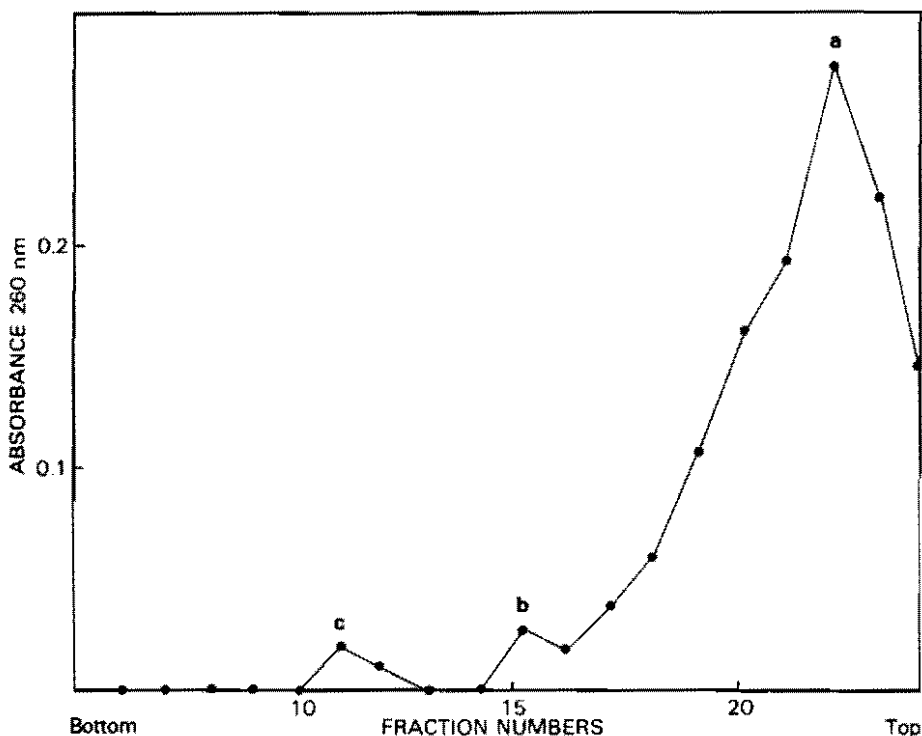
Fig. 1. Elution profile of 5-30% (w/v) alkaline sucrose gradients of supernatant from lysates of virulent *Pseudomonas solanacearum* (Isolate 115). Cells of virulent isolate 115 were lysed at 37°C in 1% sodium lauryl sulphate containing 400 ng ml⁻¹ protease. After lysis, NaCl was added to a final concentration of 1M. Lysates were stored at 4°C, centrifuged at 17,000 x g for 30 min at 4°C. Samples of 0.5 ml of supernatant were centrifuged at 86,400 x g through 5 to 30T (w/v) alkaline sucrose gradients. After 24 h, the gradients were analyzed. Three peaks, a, b, and c, were obtained which correspond to three forms of the DNA, which differed in their dissemination rates. The a may represent linear fragments; b, open circles; and c, covalent circles.

tetrazolium chloride agar, colonies of unchanged appearance with characteristic red centers.

Enzyme Activity of Culture Filtrates

Results for the parent wild type virulent isolate (115), isolate derived from it, and for a number of other wild type isolates are shown in Table 1. Cell wall degrading polysaccharidases had little or no activity at pH 8.5 so, data shown are for activity at pH 5.0. In contrast, protease was inactive at pH 5.0 and data shown are for activity at pH 8.5.

As assessed by release of reducing groups, tomato cell walls were degraded by filtrates from cultures of wild type virulent isolate 115 and GM1 1000 but not by those from cultures of wild type isolates 82 and 93 of low virulence. Filtrates from cultures of isolate 115-A, of low virulence

Table 1. Characteristics of virulent and avirulent isolates of *Pseudomonas solanacearum*.

Isolates	Origin	Virulence	Disease[a] Index	Enzyme Activities (Units ml - 1)[b,e]							
				PTCW	Protease	PG	PL	C_x	AR	GAL	WYL
115[c]	Wild type	High	4.5	1.8	0.5	3.3	6.1	0.6	1.5	1.5	0.0
115-A	Derived from 115	Very low	0.5	0.0	0.0	0.0	0.3	0.0	0.7	0.4	0.0
115-B	115-A + DNA(E)	Intermediate	2.0	3.3	1.2	1.1	7.3	2.3	1.6	1.0	0.6
115-B[d]	115-A + DNA(E)	Intermediate	2.0	—	0.6	6.2	8.3	2.3	4.8	2.2	0.6
115-C	115-A + DNA(E) + DNase	Very low	0.0	0.0	0.6	0.0	0.0	0.8	1.3	0.2	0.0
GM1 1000	Wild type	High	3.2	1.3	0.3	3.7	4.6	2.5	1.2	0.6	0.3
82	Wild type	Low	1.0	0.0	0.7	0.7	3.8	1.1	0.0	0.0	0.3
93	Wild type	Low	1.0	0.0	0.1	1.9	3.3	1.2	0.0	0.0	1.4

[a] Disease index for tomato plants 0 to 5, 0 = no symptoms, 5 = plants completely wilted. [b]Enzyme activities were means for two determinations: (1) for enzymes acting on cell walls and on substrates other than protein, enzyme activity = ng equivalent glucose min^{-1} ml^{-1}, and (2) One unit of protease activity = increase in absorbance per unit time. [c]Isolates grown in tomato cell wall media except [d]115-B, which was grown in media containing enzyme substrates/gelatin.

[e] PTCW = polysaccharidases on tomato cell wall, PG = polygalacturonase, PL = pectate lyase, C_x = cellulase, AR = arabinanase, GAL = galactanase, XYL = xylanase. DNA(E) = Extrachromosomal DNA, DNase = Deoxyribo-nuclease.

obtained from isolate 115, also did not degrade cell walls. But filtrates from cultures of isolate 115-B, i.e. isolate 115-A treated with extrachromosomal DNA from isolate 115, did degrade cell walls. Treatment of the DN with DNase nullified this effect.

For the DNA treatment, similar results were obtained for polygalacturonase, pectate lyase, arabinanase and galactanase. Results for protease and cellulase (Cx) were less clear because treatment of isolate 115-A with extrachromosomal DNA enhanced activity beyond that of the parent isolate 115 and treatment of the extrachromosomal DNA with DNase gave isolates about as active as the parent isolate. Also, results for xylanase were anomalous in that only isolate 115-A treated with extrachromosomal DNA produced active culture filtrates. Again, however, treatment of the DNA with DNase nullified this effect.

After isolate 115-A had been treated with extrachromosomal DNA and then grown in media containing one or other of a number of polysaccharidases that degrade polymers of types found in cell walls of higher than in cultures of the original 115 on tomato cell walls. This may be due to a disproportionate number of bacterial cells in the two cultures.

In comparing wild type isolates of high and low virulence, the differences in enzyme activities were striking for enzymes collectively degrading cell walls, and for arabinanase and galactanase. Differences were somewhat less striking for polygalacturonase, still less so for pectate lyase. Differences for protease, cellulase (C_x), and xylanase did not conform to virulence.

Virulence of Isolates

Isolate 115-A, obtained from virulent isolate 115 was of very low virulence to tomato plants and caused no more than mild symptoms, some two weeks after inoculation when most plants inoculated with isolate 115 were dead. Treatment of isolate 115-A with extra chromosomal DNA from isolate 115 gave 115-B of intermediate virulence which caused pronounced symptoms in plants but did not kill them within two weeks as usually happened when the parent isolate 115 was used. Extrachromosomal DNA treated with DNase did not restore virulence to isolate 115-A.

Discussion

Many plant pathogens produce, *in vitro* and *in vivo,* one or more of the polysaccharidases that degrade polymers of types found in cell walls of higher plants (1, 2, 3, 4). But other than in soft rots evidence that these enzymes are important in pathogenesis either is lacking or is inconclusive, especially in the vascular wilts for which direct evidence is difficult to obtain. In these diseases, arguments for or against an important role depend largely on indirect evidence of the type presented elsewhere (15) for *Pseudomonas solanacearum* and tomato plants in which virulence of different isolates was found to be reasonably well related to production *in vitro* of certain polysaccharidases, especially those acting on pectic substrates.

The data given above show that an isolate of very low virulence obtained from a highly virulent isolate became moderately virulent after it had been

treated with extrachromosomal DNA, obtained from wild type virulent isolate and that this did not occur if the DNA were first treated with DNase. Treatment with extrachromosomal DNA but not with DNase-treated extrachromosomal DNA also restored the characteristic ability of the virulent wild type strain to produce in culture enzymes that degrade tomato cell walls and, more specifically, certain polysaccharidases (especially polygalacturonase, arabinanase, and galactanase) but not others and also not protease. Because of the heterogeneity of the extrachromosomal DNA and because of probable fragmentation during extraction and purification, only those cells which received the functional portions of the DNA produced these enzymes.

The production of certain enzymes by the avirulent isolate derived from the wild type virulent isolate may be due to the development of an alternative system of production which was activated upon loss of the extrachromosomal DNA which may control the synthesis of these enzymes.

We believe that the work reported in this paper goes some way toward establishing that factors for virulence of *P. solanacearum* are carried on the DNA associated with extrachromosomal elements which may be lost from cells which lose virulence in culture, and that the factors for virulence include one or more of the enzymes that degrade polysaccharides in cell walls.

Literature Cited

1. Anderson, A. J., 1978. Extracellular enzymes produced by *Collectotrichum lindemuthianum* and *Helminthesporium* maydis during growth on isolated bean and corn cell walls. Phytopathology 68, 1585-1589.
2. Bateman, D. F., H. D. Van Etten, P. D. English, D. J. Nevins and P. Albersheim. 1969. Susceptibility to enzymatic degradation of cell walls from bean plants resistant and susceptible to *Rhizoctonia solani* Kuhn. Plant Physiology 44, 641-648.
3. Chatterjee, A. K., and M. P. Starr. Genetics of pectolytic enzyme production in *Erwinia chrysanthemi* Proc. 4th Int. Conf. Plant Path. Bact. Angers, France. 89-94.
4. Cooper, R. M., and R. K. S. Wood. 1973. Induction of synthesis of extracellular cell wall degrading enzymes in vascular fungi. Nature 246, 309-311.
5. Dische, Z., 1955. Color reactions of nuclei acid components. In the Nucleic Acid Vol. 1. Chargaff, E., and Davidson, J. N. (eds). Academic Press, New York. 285-305.
6. English, P. D., J. B. Jurale and P. Albersheim. 1971. Host pathogen interactions II. Parameters affecting polysaccharide degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. Plant physiology 47, 1-6.
7. Guerry, P., D. J. Le Blanc and S. Falkow. 1973. General method for the isolation of plasmid deoxyribonucleic acid. Journal of bacteriology 116 (2) 1064-1066.
8. Hirt, B., 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. Journal of Molecular Biology 26, 365-369.

9. Keegstra, K., K. W. Talmadge, W. D. Bauer, and P. Albersheim. 1973. The structure of plant cell walls III. A model of the walls of suspension cultured sycamore cells based on interconnection of the macromolecular components. Plant Physiology 51, 188-197.

10. Kelman, A., 1954. The relationship of pathogenicity in *Pseudomonas* Phytopathology 44, 693-695.

11. Marmur, J., 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. Journal of Molecular Biology 3, 208-218.

12. Marais, J. P., J. L. Dewit, and G. V. Quickie. 1966. A critical examination of the Nelson-Somogyi method for the determination of reducing sugars. Analytical Biochemistry 15, 373-381.

13. Nelson, W.L., E. I. Ciaccio, and G. P. Hess. 1961. A rapid method for the quantitative assay of proteolytic enzymes. Analytical Biochemistry 2, 39-44.

14. Ofuya, C. O., and R. K. S. Wood. Pathogenicity and biochemical characteristics of isolates of *Pseudomonas solanacearum* in Nigeria. Proc. 4th Int. Conf. Plant Path. Bact. Angers, France, 835-840.

15. Ofuya, C. O., and R. K. S. Wood. 1981. Virulence of *Pseudomonas solanacearum* in relation to extracellular polysaccharidases. 5th Int. Conf. Plant Path. Bact. Abstract. CIAT, Cali, Colombia.

Changes in Enzymic Activity During Pathogenesis of Vascular Wilt Caused by *Pseudomonas solanacearum*

C. O. Ofuya, R. K. S. Wood

Imperial College of Science and Technology
London, United Kingdom

Abstract

Extracts of tomato plants infected by isolates of *Pseudomonas solanacearum* contain extracellular polysaccharidases. Extracts of low enzyme activity were obtained from plants which were infected by low virulence isolates whereas plants infected by a highly virulent isolate contained extracts of high enzyme activity.

Both polygalacturonase and pectate lyase were produced *in vivo*. The activity of pectic enzymes was particularly high during the early stages of infection. The first disease symptoms of epinasty and drooping of leaves appeared when polygalacturonase had reached its maximum activity. As the infection progressed to an advanced state activity of polygalacturonase decreased. Increased synthesis of pectate lyase was detected in the early stages of infection but decreased significantly when all plants had wilted. Activity of β-1,4 endoglucanase was very low at the early stages of infection but increased later at advanced stage of wilting. The results suggest a close association between the development of symptoms, synthesis, and activity of extracellular polysaccharidases.

Introduction

The pathogenesis of wilts caused by *Pseudomonas solanacearum* has been studied extensively (4, 7, 8, 9). However, despite the many approaches that have been used to study the development of the disease, the ways in which symptoms of the disease are produced after the pathogen is established in vascular tissues is not fully understood. Little is known about the first stages of infection during which bacteria become established in xylem elements. One suggestion is, that in vascular wilts caused by bacteria and fungi, certain substances such as slime, enzymes, and growth regulators secreted by the pathogen in the tracheal fluids may damage the plant one way or the other (6, 14).

P. solanacearum produces one or more extracellular polysaccharidases in infected plants (7, 8). But the role of these enzymes in pathogenesis is not certain. A study of the enzymic responses of infected plants should help

clarify some of the subtle components involved in the development of disease in susceptible plants infected by virulent *Pseudomonas solanacearum*. This study describes work on cell wall degrading enzymes which are produced during pathogenesis of bacterial wilt of tomato.

Materials and Methods

Bacterial Cultures

Three isolates of *P. solanacearum* were used in this study. Isolates 98 and 115 were originally isolated in 1977 from wilting sweet pepper and tomato plants. Isolate 1049 was obtained from the National Collection of Plant Pathogenic Bacteria, Harpenden, England. The isolates were routinely grown in glycerol peptone agar and maintained in 10 ml sterile distilled water in 25 ml bottles (12).

Plants

Seeds were first treated in a 5% solution of sodium hypochlorite for 5 min, washed several times in sterile distilled water, then planted in Fisons Lexington compost. Seedlings were grown at 26 to 28°C in a 12 h photoperiod. When well developed cotyledons had appeared (*c.* 10 to 15 days), seedlings were transplanted to individual pots (7 x 8 cm diameter) containing fresh compost. Seven days after transplanting, they were fed at intervals with a liquid manure. Plants were used six weeks after sowing when they were about 14 cm high, with six expanded leaves.

Inoculation of Plants

Cell suspensions prepared from growth on tetrazolium chloride agar were usually introduced into the plants by the stem puncture technique. A drop of inoculum was placed in the axil of the second or third leaf from the top. A sterile needle was carefully inserted through the drop into the stem without piercing the opposite side.

Inoculum levels of *c.* 10^6 bacterial cells ml^{-1} were used. The tetrazolium chloride agar contained 0.5% glycerol, 1% peptone, 0.1% casamino acids, 0.05% 2,3,5-triphenyl tetrazolium chloride, 1.8% agar.

Disease Scale

Inoculated plants were kept in a growth cabinet at *c.* 30°C. Five plants per isolate were usually inoculated. Wilting of plants was rated at intervals after inoculation on a 0 to 5 scale for plants with six expanded leaves. Zero = no symptom, 5 = plant and terminal shoot completely wilted.

Bacterial Population in Infected Plants

Bacteria numbers were estimated on the top 3 cm of stem. About 0.8 g of stem tissues were cut from the stem under aseptic conditions and placed in 5 ml sterile distilled water for 5 to 10 min. The presence of bacteria was indicated by the turbidity of the water. Bacteria numbers were determined by the viable plate technique on tetrazolium chloride agar.

Extraction of Enzymes From Infected Tissue

Shoots of infected plants were cut at soil level, washed with the leaves and epidermal layers removed, then stored at -20°C. Frozen tissue was

ground in an ice cooled mortar and was taken up in cold 0.1 M phosphate buffer pH 6.0, containing 0.2 M NaCl, 1 mM dithiothreitol, and 5% insoluble polyvinypolypyrrolidone (PVP), in the proportion of 1 g tissue per 4 ml buffer. NaCl was used to deabsorb protein (3), and PVP and dithiothreitol were used to adsorb phenols and prevent oxidation by phenol oxidases (1).

Extraction was at 1°C in a sorvall omnimixer, for 2 min at lowest speed, followed by 10 min at high speed, and 5 min at low speed. The extract was filtered through a layer of cheese cloth and was then spun down at 20,000 x g for 15 min. The extract was dialysed and then used immediately for enzyme assay or kept at -20°C. Extracts were also prepared from healthy uninoculated tomato plants by a similar procedure.

Determination of Enzyme Activity

Polygalacturonase (PG) and *Pectate lyase* (PL). The activities of polygalacturonase and pectate lyase were determined by the thiobarbituric acid technique as used by Ayers, Papavizas, and Diem (2). Ten ml of reaction mixture contained the following, 8 ml of 1% polygalacturonic acid (PGA) or sodium polypectate (NaPP); 1 ml enzyme (extract), 0.7 ml of 0.1 M citrate buffer, pH 5.0 or 0.7 ml 0.1 M Tris-HCl, pH 8.5; and 0.3 ml of 0.1 M $CaCl_2.2H_2O$, incubated at 30°C. After 1 or 4 h, enzyme action was stopped by removing tubes from incubation and adding 0.6 ml of 9% $ZnSO_4$ and 0.6 ml 0.5 N NaOH, successively. The mixture was shaken vigorously in capped centrifuge tubes, and then centrifuged at 16,000 x g for 15 min.

Five ml of clarified reaction mixture was added to tubes containing 3 ml of 0.04 M TBA(thiobarbituric acid), 1.5 ml of 1 N HCl, and 0.5 ml of distilled water. The tubes were placed in a boiling water bath for 0.5 h and cooled; absorbance of the solutions was determined at 515 nm to detect PG activity and 550 nm for products of lyase activity. Zero = time reaction mixtures containing active enzyme were used as controls. Enzyme activity is expressed as ng product min^{-1}ml^{-1} based on calibration curves for unsaturated digalacturonic acid and galacturonic acid.

Pectinmethylesterase (PME)

The activity of pectinmethylesterase was determined by the continuous titration technique of Kertesz, 1937 (10) by measuring the methoxyl released during the reaction period. One ml enzyme was added to 5 ml of 1% washed pectin solution, previously adjusted to pH 7.0. The mixture was incubated at 30°C and at intervals the mixture was adjusted to pH 7.0 with 0.1N NaOH. Reaction mixture containing boiled enzyme was used as controls. The alkali was added at the rate required to keep the mixture at pH 7.0. (Usually for about 5 to 7 min). The total volume of base used during 1 h reaction period was equivalent to PME activity, since it neutralized carboxyl groups freed on hydrolysis of ester linkages in pectin. Enzyme activity is expressed as units ml^{-1}, one unit COO-h$^{-1}\equiv$ mg methoxyl released per h under the conditions of the assay.

β -1,4 endoglucanase (Cellulase C_x)

The activity of cellulase (C_x) was determined by viscometry. Ten ml of reaction mixture contained 8 ml of 1% carboxymethylcellulose, 1 ml of buffer (0.1 M citrate buffer pH 5.0 - 5.5), and 1 ml of enzyme (extract) in size

200 viscometer. Incubation was at 30°C. Enzyme activity is expressed as relative viscometric units (RVU), which is equivalent to $1000/t_{50}$ where t_{50} is the time (min) for 50% reduction in viscosity of the substrate.

Results

Extracellular Polysaccharidases in Infected Tomato Plants

In preliminary experiments, six-week tomato plants were inoculated with 1×10^8 cell suspensions of isolates 98 (low virulence), 1049 (moderately virulent) and 115 (highly virulent), by stem puncture described in Materials and Methods. One week after inoculation, plants were harvested and extracts from stem tissues were assayed for pectic enzyme activity (Table 1).

Table 1. The effect of extract from tomato plants infected by *Pseudomonas solanacearum* on viscosity of 1°/o sodium polypectate.

Isolate	Virulence	Enzyme activity (RVU)[a]	
		pH 5.5	pH 8.5
98	Low	2.0	0.0
1049	Moderate	5.0	0.2
115	High	76.9	22.2
Healthy plants	—	0.0	0.0

[a] Enzyme activity was assayed by viscometry. Ten ml of assay mixture contained 8 ml substrate, 1 ml enzyme, 0.7 ml buffer, and 0.3 ml of 0.1 M $CaCl_2.2H_2O$. RVU Relative viscometric units = $1000/t_{50}$. t_{50} is the time required for 50°/o reduction in the viscosity of 1°/o NaPP. Values are means for two determinations.

The results show that extract from infected tomato plants contained pectic enzymes, which were more active at pH 5 than at pH 8.5. Enzyme activity was correlated with virulence, because activity was highest in extracts from severely wilted plants infected by isolate 115, and lowest in plants infected by isolate 98, which caused very mild symptoms. Extracts from healthy plants were inactive. The pH of plants infected by the highly virulent isolate 115 (water extract) was *c.* 6.4.

Changes in Enzyme Activities During Pathogenesis

To show the relationship between wilt symptoms and the activities of extracellular polysaccharidases, tomato plants were inoculated with isolate 115 and then harvested at intervals during the course of pathogenesis. The results are shown in Fig. 1.

The first evidence of infection was the detection of pectic enzymes activity, which was particularly high during the early stages of infection. By the second day, the first disease symptoms of spinasty and drooping of
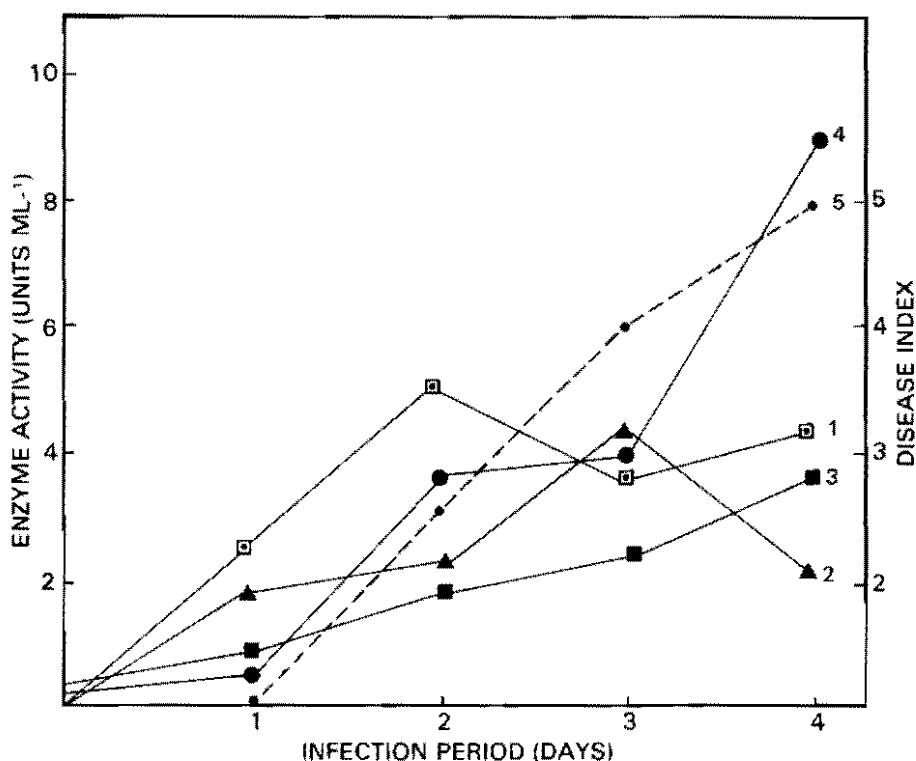
Fig. 1. Activities of extracellular polysaccharidases during infection of tomato plants by *Pseudomonas solanacearum,* Isolate 115. (1) Polygalacturonase (TAB assay). (2) Pectate lyase (TBA assay). (3) Pectinmethylesterase (continuous titration test). (4) Cellulase C (Viscometry). (5) Disease index (0-5 scale). Units of enzyme activity; PG = ng galacturonic acid min-¹ml-¹; PL = ng unsaturated galacturonic acid min-¹ml-¹; Cx = relative viscometric units (100/t50), t50 = time required for 50% reduction in the viscosity of 1% NaPP; Pectinmethylesterase - equivalent to COO-released h-¹.

leaves appeared. Polygalacturonase reached its maximum activity. As the infection progressed to an advanced state (i. e by the fourth day), activity of PG decreased.

Pectate lyase was also detected in infected plants. Its activity increased gradually as the infection progressed, reaching its maximum on the third day when most of the disease symptoms had appeared, but decreased significantly when all plants had wilted. Decreased enzyme activity at advanced wilting may be due to the effects of inhibitors such as phenols which are known to increase during infection. Decrease in the activity of PG is probably due to enzyme inhibitors, too. Pectinmethylesterase PME, increased steadily as symptoms developed.

Cellulase C_x activity was very low at the early stages of infection, much lower than activity of pectic enzymes. High activity appeared later at an advanced stage of wilting (i. e. by the fourth day after infection).

The development of disease symptoms correlated with growth of bacteria (Fig. 2). Bacterial numbers per top 3 cm of stem (i.e 0.8 g of stem tissues) increased sharply at first, reaching a maximum on the third day and then decreased slightly when the plants had fully wilted.
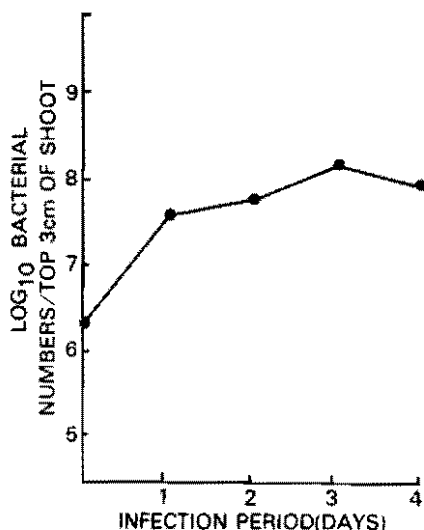
Fig. 2. Growth of virulent *Pseudomonas solanacearum* in tomato plants, cv. Moneymaker.

Discussion

Extracellular polysaccharidases were detected in tomato plants following infection by *Pseudomonas solanacearum*. Both PG and PL were produced. They were highly active during the initial stages of infection, but appeared to be less active as infection advanced to the terminal death stage. This may indicate a possible inhibition as suggested by Deverall and Wood, 1961 (5), who studied the relationship between phenolase of bean (*Vicia faba*) and pectic enzymes of *Botrytis faba* and *B. cinerea*. Phenols and phenol oxidases are said to accumulate as infection progresses, and these substances may inactivate the enzymes.

Pectinmethylesterase was present in infected tissue but in relatively small amounts. Cellulase (C_x) reached its maximum activity at the advanced stages of infection. The appearance and activity of these enzymes in infected plants may suggest a role for them in the disease process. Cellulase had been studied in this connection (9).

The advance of disease symptoms was closely related to growth of bacteria and synthesis of extracellular polysaccharidases. A rapid increase in disease symptoms occurred at a time corresponding to a rise of enzyme synthesis. This suggests an association between bacterial metabolism in plant tissues and severity of disease. A similar association between symptoms of *Fusarium* wilt and quantity of fungus and enzyme activity in tomato stems has been reported (11).

The early production and high activity of the pectic enzymes secreted by the pathogen during pathogenesis would influence tissue susceptibility or the rate of tissue disintegration. A direct consequence of the enzymic activity of the pathogen is the modification of the cell wall of the vascular tissues. This activity may release certain sugars which serve as inducers of other wall degrading enzymes such as cellulase C_x, which appeared later in pathogenesis. The establishment and growth of the pathogen in the tissue are highly influenced by these biochemical events. Talmadge et al., 1973 (15) have suggested that endopectic hydrolases and lyases increase the susceptibility of cell walls to non-uronide degradation. Recent ultrastructural studies (13, 16) have shown that breakdown of walls of vessels occurs during pathogenesis of wilts caused by P. solanacearum. It is suggested that this phenomenon is associated with the activities of pectic enzymes and possibly other polysaccharidases secreted during pathogenesis.

Literature Cited

1. Anderson, A. J., 1978. Extraction of enzymes and ultracellular organelles from plant tissue. Phytochemistry 7, 1973-1988.
2. Ayers, W. A., G. C. Papavizas and A. E. Diem. 1966. Polygalacturonate transeliminase and polygalacturonase production by Rhizoctonia solani. Phytopathology 56, 1006-1011.
3. Blackhurst, F. M., and R. K. S. Wood. 1963. Verticillum wilt of tomatoes: Further experiments on the role of pectic and cellulolytic enzymes. Annals of Applied Biology 52, 89-96.
4. Buddenhagen, I. W., and A. Kelman. 1964. Biological and Physiological aspects of bacterial wilt caused by Pseudomonas solanacearum. Annual Review of Phytopathology 2, 203-230.
5. Deverall, B. J., and R. K. S. Wood. 1961. Chocolate spot of beans (Vicia faba L.) interactions between phenolase of host and pectic enzymes of the pathogen. Annals of Applied Biology 49, 473-487.
6. Diamond, A. E., 1970. Biophysics and biochemistry of the vascular wilt syndrome. Annual Review of Pathology 8, 301-322.
7. Husain, A., and A. Kelman. 1958a. Relation of slime production to mechanism of wilting and pathogenicity of Pseudomonas solanacearum. Phytopathology 48, 155-165.
8. Husain, A., and A. Kelman. 1958b. The role of pectic and cellulolytic enzymes in pathogenesis by Pseudomonas solanacearum. Phytopathology 48, 377-386.
9. Kelman, A., and E. B. Cowling. 1965. Cellulase of Pseudomonas solanacearum in relation to pathogenesis. Phytopathology 55, 148-155.
10. Kertesz, Z. I., 1937. Pectic enzymes: the determination of pectinmethyoxylase activity. J. Biol. Chem. 121, 589-597.
11. Matta, A., and A. E. Dimond. 1963. Symptoms of Fusarium wilt in relation to quantity of fungus and enzyme activity in tomato stems. Phytopathology 53, 574-578.
12. Ofuya, C.O., and R. K. S. Wood. 1978. Pathogenicity and biochemical characteristics of isolates of Pseudomonas solanacearum in Nigeria. Proc. 4th Int. Conf. Plant Path. Bact. Angers, France, 835-840.

13. Schmit, J., 1978. Microscopic study of early stages of infection by *Pseudomonas solanacearum* E. F. S. on '*in vitro*' grown tomato seedlings. Proc. 4 th Int. Conf. Plant Path. Bact. Angers, France. 841-856.
14. Talboys, P. W., 1975. Simulation of the symptoms of hop *Verticillium wilt* by model toxins . Proc. Int. Symp. on Path. Wilting of Plants. Madras (India). 231-242.
15. Talmadge, K. W., K. Keegstra, W. D. Bauer and P. Albersheim. 1973. The structure of plant cell walls I. The macromolecular components of the walls of suspension cultured sycamore cells with a detailed analysis of the pectic polysaccharides. Plant Physiology 51, 158-173.
16. Wallis, F. M., and S. J. Truter. 1978. Histopathology of tomato plants infected with *Pseudomonas solanacearum* with emphasis on ultrastructure. Physiological Plant Pathology 13, 307-317.

Phaseolus Vulgaris - Pseudomonas syringae **pv.** phaseolicola System:

Role of Host Tissue Age and Pathogenic Toxin in Establishment of Pathogen in Host

T. Oguchi , B. V. Gantotti, G. K. H. Ho, Suresh Patil

University of Hawaii
Honolulu, Hawaii, U.S.A.

Abstract

Pseudomonas syringae **pv.** *phaseolicola,* the casual agent of the halo blight of beans produces an extracellular non-specific toxin in culture and in inoculated plants. Both toxigenic and non-toxigenic strains of the pathogen multiply in inoculated tissues of young plants of susceptible cultivars and induce watersoaking, but only toxigenic strains induce systemic chlorosis and stunting, indicating that the toxin is a virulence factor and is not required for pathogenicity of the bacterium in such tissues. However, in previous studies which have been extended in this report, toxin has been shown to specifically suppress hypersensitive resistance to pv. *phaseolicola* in "genetically" resistant bean cultivars, suggesting a role for the toxin in the pathogenicity of the bacterium. Additional data support the role of the toxin in pathogenic establishment. Tissues of a susceptible cultivar Red Kidney (RK), which are susceptible to certain of both toxigenic and nontoxigenic strains of pv. *phaseolicola* gradually become resistant to them as they mature and eventually react to the pathogen in a manner similar to that of tissues of a resistant cultivar, GN Nebraska # 27. In mature tissues, bacterial proliferation is severely restricted, and instead of watersoaking, an HR-like response is produced. Further, as in the reaction of "genetically" resistant tissues, toxin content of inoculated mature tissues is drastically reduced as compared to young tissues of the same susceptible cultivar. Thus the manifestations of the resistance reaction of mature susceptible tissues are similar to those of tissues of "genetically" resistant cultivars.

It is proposed that the reason why nontoxigenic strains of pv. *phaseolicola* grow in young RK tissues is that the specific resistance of RK to the pathogen is not expressed in such tissues, and bacteria grow passively. It appears that when toxigenic strains are used to inoculate young RK tissues, the toxin produced during the passive growth prevents subsequent expression of resistance as the tissues mature. A model to explain the role of the toxin in pathogenic establishment in the host is presented.

Introduction

When susceptible cultivars of bean (*Phaseolus vulgaris* L.) are infected with *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of the halo blight of beans, the affected tissues show watersoaking, stunting, systemic chlorosis, and accumulation of ornithine (4, 5, 11, 19). The stunting, systemic chlorosis, and ornithine accumulation are caused by an extracellular toxin(s) produced by the pathogen (4, 11, 18, 19). The toxin(s), which is referred to as phaseotoxin (8) or phaseolotoxin (12), is a potent and specific inhibitor of ornithine carbamoyltransferase (OCT) of bean (20). The first step in the induction of chlorosis in bean leaves treated with the toxin(s) appears to be the inhibition of OCT by the toxin(s) (14).

Other studies indicate that in addition to its role as a specific inhibitor of OCT, the toxin(s) may be involved in the establishment of the pathogen in the host. When plants of resistant cultivars are inoculated with pv *phaseolicola*, they respond with the production of hypersensitivity (HR). Bacterial growth is sharply curtailed and no toxin(s) is detected in tissues of such plants (1). However, when plants of the same resistant cultivars are treated with the toxin(s) prior to inoculation with the pathogen, the HR is suppressed, bacterial multiplication increased, and typical susceptible symptoms are observed (2). Furthermore, preliminary studies (3), showed that the toxin(s) suppresses HR induced by only pv. *phaseolicola*, but not by several other phytobacteria. These data indicate that in hypersensitively responding plants, the production of toxin(s) may be suppressed, and normal expression of the resistance mechanism occurs in such plants. That large quantities of toxin(s) are detected (1) in inoculated susceptible tissues lends further support to the idea that the toxin(s) may help the bacteria to establish themselves in the host.

One way to test this hypothesis would be to determine if non-toxigenic mutants and naturally occurring nontoxigenic strains of the pathogen are able to establish themselves in bean tissues. An ultraviolet light-induced mutant of a toxigenic strain (G50) was isolated. When it is inoculated in young primary leaves of a susceptible cultivar (Red Kidney, RK), it multiplies as well as its toxigenic parent and produces watersoaking, but no systemic chlorosis (13), indicating that unlike in resistant bean tissue, in susceptible tissues toxin(s) is not necessary for pathogenic establishment.

However, we often observed that unless primary leaves of RK seedlings are inoculated soon after unfolding, typical symptoms are not observed. Indeed, it later developed (10) that if fully expanded mature (14 days) primary leaves were inoculated with either toxigenic or non-toxigenic pv. *phaseolicola* strains, the leaf tissues would produce an HR-like response. This means that even in susceptible cultivars, resistance expression can occur. If the manifestations of the mature tissue resistance of susceptible cultivars are qualitatively the same as those of the "genetically" resistant tissues, it would give further credence to the proposed role of the toxin(s) in pathogenic establishment in bean tissues.

We report here studies on mature tissue resistance of susceptible RK plants to pv. *phaseolicola*. Also presented are further results on the specificity of HR suppression of the toxin(s) of pv. *phaseolicola*. Based on these results we have constructed a model to explain the role of the toxin(s) in pathogenic establishment.

Materials and Methods

Bacterial Isolate

Pseudomonas syringae pv. *phaseolicola* isolates used in these studies were, HB-36, G50 (toxigenic); HB-20, G50 TOX-(nontoxigenic). Other phytobacteria used were *P. syringae* pv. *tabaci* (ICPB PT5), *P. syringae* pv. *tomato* (ICPB PT 111) and *P. syringae* pv. *lachrymans*. The bacterial isolates were maintained as glycerol stocks at -20°C and revived by transferring them on to plates of yeast extract, dextrose, and calcium carbonate agar (YDC). Growing of inoculum, which was adjusted to 10^7 cells/ml, inoculation of bean primary leaves, and determination of bacterial populations in ininoculated leaves have been described elsewhere (1, 2, 3).

Bean Cultivars

Bean cultivars used in these studies were Red Kidney (RK), Resistant Cherokee Wax (RCW), which are susceptible to pv. *phaseolicola,* and GN Nebraska #27 (GN) and PI 150514 (PI), which are resistant. Red Mexican UI-3 (RM) was used as a differential cultivar which is resistant to race 1 of pv. *phaseolicola* but susceptible to race 2. Plant culture and growth conditions have been described previously (1, 3).

Toxin Preparation, its Determination, Enzyme Assays, and Toxin Treatment of Plants

Toxin was isolated from primary leaves of RK inoculated with pv. *phaseolicola.* Watersoaked leaves from inoculated plants were ground in 70% methanol. The slurry was filtered through Whatman no. 1 filter and the methanol extract evaporated to a small volume. This was filtered through a UM-2 (amicon) membrane. The concentrated ultrafiltrates were Chromatographed on a 1.5 x 102 cm. column of Sephadex LH-20 developed with deionized water. Toxin concentration of inoculated tissues was determined as before (1).

The ornithine carbamoyltransferase (OCT) assay has been described previously (7, 20). The amount of OCT in healthy bean leaf tissues was determined by grinding 1 g of primary leaves in 1.2 ml of HEPES (pH 7.0), centrifuged, and the supernatent dialyzed against the same buffer. Bean plants of the reistant cultivars were treated with the toxin as described before (3). The toxin concentration was 4000 n/ml. Metabolic inhibitors, cyclohexamide and blasticidin S (kindly supplied by Professor Nishimura, Tottori University, Japan), were added to the inocula to achieve final concentrations of 0.5 ng/ml and 10 ng/ml, respectively, when the effect of these inhibitors on bacterial multiplication in GN leaves was studied.

Results and Discussion

Specificity of Phaseotoxin-induced Suppression of Hypersensitive Response

Both the no-toxin control plants and plants treated with inactivated toxin produced HR when inoculated with pv. *phaseolicola* (HB-36) and three other phytobacteria, pv. *tabaci,* pv. *tomato* and pv. *lachrymans.* Bacterial populations increased in all cases from 10^4-10^5/1.13 cm^2 by about two orders of magnitude within 24 h. In toxin treated plants only pv.

phaseolicola multiplied to a higher level (four orders of magnitude in 24 h); other bacteria multiplied to roughly the same extent as in untreated plants. At 48 h after inoculation, pv. *phaseolicola* population in toxin treated tissues was over 100-fold greater than in those treated with inactivated toxin or in no-toxin controls. Only in toxin-treated plants inoculated with pv. *phaseolicola* did HR fail to appear; instead, typical watersoaking was noted; other combinations showed HR. These results confirm and extend the previous findings (3).

Nonspecific Suppression of HR Resistance to Phytobacteria by Translation Inhibitors

Both blasticidin S and cycloheximide blocked the HR of GN plants when inoculated with pv. *phaseolicola* (HB-36). The inhibitors also neutralized the HR of GN against three other phytobacteria, pv. *tabaci*, pv. *tomato*, and pv. *lachrymans*. Twenty-four hours after inoculation, populations of all four bacteria increased from 10^4-10^5 cells/1.13 cm^2 leaf area to 10^8-10^9 cells/1.13 cm^2 leaf area. Concomitantly, visible HR of inoculated tissues was suppressed by both inhibitors in all four cases, and leaves developed watersoaking.

The above results clearly distinguish between nonspecific suppression of HR in beans to phytobacteria and the specific suppressions caused by toxin against pv. *phaseolicola*. Cycloheximide, a potent inhibitor of protein synthesis in plants, but not in bacteria, has been shown to suppress HR induced by bacterial pathogens (15). Recently, blasticidin S, which also inhibits protein synthesis at the translational level, was used to block HR in soybeans induced by *Pseudomonas* spp. (6). It is not known how the toxin affects the specific neutralization of the host defense mechanism. Nevertheless, the data indicate that in addition to its known role, as an inducer of chlorosis, the toxin plays a role on another level, i.e. the suppression of host resistance. In contrast to the specific effect of toxin in beans, in tobacco the toxin was reported to suppress visible HR against several phytobacteria (17). However, induction of susceptibility was apparently not observed.

Effect of Tissue Age of Bean Cultivars on pv. *phaseolicola* Bean Interaction

Primary leaves of 5 to 6 day old bean cultivar RK showed typical watersoaking symptoms 48 to 72 h after inoculation with pv. *phaseolicola* (HB-36). On the other hand, primary leaves of 14-day old RK, when inoculated, produced no watersoaking. Instead, an HR-like reaction (silvering of the lower leaf surface) was observed within 24 to 36 h. We conducted a detailed time course study in which symptomatology, bacterial growth, and toxin levels in primary leaves of RK beans of different ages inoculated with pv. *phaseolicola* were compared. When bacterial growth in 6-day old plants was compared to that in 14-day old plants, it was seen that in spite of the larger initial bacterial numbers in the 14-day old plants (due to thicker leaves), both the rate of multiplication and final level of the bacterial population were lower (roughly 100 fold less) in older plants than in younger ones. The toxin level of the two groups of inoculated leaves also showed the same relationship; there was roughly 100 fold less toxin present in older tissues, compared to younger ones.

Symptoms in 7 and 8-day old plants were mixed; both watersoaking and silvering were seen. Bacterial growth in these tissues was about the same as in 5 to 6 day old plants and toxin level was somewhat less. In primary leaves of plants 11 to 15 days old, there was no watersoaking (only silvering), lower population of bacteria as compared to 6-day old leaves, and drastically reduced levels of toxin.

One explanation for the reduced levels of toxin in older inoculated tissues could be that such tissues contain larger amounts of OCT than younger tissues. It is known that the toxin binds OCT covalently (7). However, we found that as the primary leaves mature, there is progressive decrease in the amount of OCT/mg protein in these tissues. Thus in older tissues either the toxin is degraded more rapidly than younger tissues, or its synthesis is suppressed.

In inoculated tissues, toxin induces accumulation of ornithine (12). We determined ornithine levels in the first trifoliate leaves of young and old inoculated plants. The data on ornithine accumulation was consistent with toxin content of leaves. That is, in young tissues where large amounts of toxin accumulated, their first trifoliate leaves also accumulated large amounts of ornithine. In older tissues the reverse was true. We determined whether or not the development of mature tissue resistance to pv. *phaseolicola* seen in RK was a general phenomenon that applied to various bean cultivars and various strains of the pathogen. Two additional cultivars, one "genetically" resistant (PI) and one susceptible (RCW) were used. Also, a differential cultivar (RM) which distinguishes between race 1 and race 2 of the pathogen was used.

When plants were 5 to 6 days old, all cultivars showed a susceptible response to appropriate strains and a resistant response when 12 to 14 days old. Further our work has shown that plants of even the "genetically" resistant GN cultivar respond in a susceptible manner if inoculated with the pathogen within 24 h after unfolding. These results suggest that in the bean, resistance or susceptibility to pv. *phaseolicola* is a function of tissue age. Although differences in individual cultivars exist with respect to the period of time (after unfolding of primary leaves) for which they are devoid of resistance, on the basis of these studies it can be concluded that eventually tissues of all cultivars react to pv. *phaseolicola* in a hypersensitive-like manner.

The results of studies on the suppression of HR·in GN tissues by the toxin(s) show that in this cultivar toxin(s) can neutralize its resistance specifically to pv. *phaseolicola*, indicating that the tissues of this cultivar possess more than one type of resistance mechanism; one for the pathogen, and one or more for other phytobacteria. The fact that nontoxigenic mutants of the pathogen or naturally occurring nontoxigenic strains are able to grow in young tissues of RK, a susceptible cultivar, may indicate that in this cultivar toxin(s) is not necessary for pathogenic establishment.

An explanation as to why the toxin(s) seems to be involved in bacterial establishment in the resistant but not the susceptible cultivars may be that whereas tissues of resistant cultivars are able to express specific resistance to pv. *phaseolicola*, tissues of susceptible cultivar cannot express this resistance and bacteria grow passively in such tissues. That

tissues of susceptible cultivars develop the ability to react to pv. *phaseolicola* in a manner similar to tissues of GN gives strong support to this contention. Among the manifestations of mature tissue resistance is the drastic reduction in the amount of toxin(s) present in inoculated tissues and reduced bacterial multiplication. Thus it appears that toxin(s) is implicated in pathogenic establishment.

Although the behavior of all bean cultivars studied conforms to the above observation there are what appear to be, at least at first glance, some exceptions. Prominent among them is the behavior of the cultivar RM with respect to pathogenic strains HB-33 and HB-36. Both of these strains produce toxin in culture, and cause systemic chlorosis in young RK plants. When inoculated in young RM tissues only HB-36 produces watersoaking and systemic chlorosis; HB-33 causes HR. Since mature tissues of RM are resistant to both of these strains it appears that only HB-36 produces enough toxin to neutralize RM's defense mechanisms. It has been shown that when RM plants inoculated with HB-33 are incubated at a lower temperature (16°C) than used in these studies, typical susceptible symptoms, including systemic chlorosis, are produced by the plants (9). Thus, it appears that the major factor in pathogenic establishment is production and maintenance of high enough levels of toxin(s) to suppress expression of host resistance mechanisms.

Based on our studies we propose the following generalized model to explain the possible basis of pathogenic establishment in beans. When tissues of bean cultivars are inoculated with pv. *phaseolicola* strains when they are relatively young, they allow the pathogen to grow passively because such tissues cannot express their specific resistance to the pathogen. During the passive growth phase, toxigenic strains of the pathogen produce the toxin(s) which prevents the subsequent expression of resistance as the tissues mature. Nontoxigenic strains also grow passively but cannot suppress subsequent expression of resistance. The final outcome of a specific host-pathogen combination depends upon the dynamic relationship between the ability of the host to express resistance and the ability of the pathogen to maintain toxin(s) concentration above the critical level for suppression of resistance of host tissues in that particular combination.

Literature Cited

1. Gnanamanickam, S. S., and S. S. Patil. 1976. Bacterial growth, toxin production, and levels of ornithine carbamoyltransferase in resistant and susceptible cultivars of bean inoculated with *Pseudomonas phaseolicola.* Phytopathology 66:290-294.
2. Gnanamanickam, S. S., and S. S. Patil. 1977. Phaseotoxin suppresses bacterially induced hypersensitive reaction and phytoalexin synthesis in bean cultivars. Physiol. Plant Pathol. 10:169-179.
3. Gnanamanickam, S. S., and S. S. Patil. 1977. Specificity of phaseotoxin induced suppression of resistance in bean to phytobacteria. Proc. Amer. Phytopath. Soc. 4:163 (Abstr.).
4. Hoitink, H. A. J., R. L. Pelletier, and J. G. Coulson. 1966. Toxemia of halo blight of bean. Phytopathology 56:1062-1065.

5. Jensen, J. H., and J. E. Livingston. 1944. Variation in symptoms produced by isolates of *Phytomonas medicaginis* var *phaseolicola*. Phytopathology 34:471-480.

6. Keen, N. T., T. Ersek, M. Long, B. Bruegger, and M. Holliday. 1981. Inhibition of the hypersensitive reaction of soybean leaves to incompatible *Pseudomonas* spp. by blasticidin S, streptomycin or elevated temperature. Physiol. Plant Pathol. 18:325-337.

7. Kwok, O. C. H., H. Ako, and S. S. Patil. 1979. Inactivation of bean ornithine carbamoyltransferase by Phaseotoxin: Effect of phosphate. Biochem. Biophys. Res. Comm. 89:1361-1368.

8. Mitchell, R. E., 1976. Isolation and structure of a chlorosis-inducing toxin of *Pseudomonas phaseolicola*. Phytochemistry 15:1941-1947.

9. O'Brien, F. L., and R. K. S. Wood. The hypersensitive reaction and other responses of bean leaves to bacteria. Ann. Bot. 40:479-491.

10. Oguchi, T., and S. S. Patil. 1979. Mature tissue resistance in bean to *Pseudomonas phaseolicola*. Proc. IX Intl. Congress of Plant Protection. (abstr. 641).

11. Patel, P. N., and J. C. Walker. 1963. Changes in free amino acids and amide content of resistant and susceptible beans after infection with the halo blight organism. Phytopathology 53:522-528.

12. Patil, S. S., 1974. Toxins produced by phytopathogenic bacteria. Annu. Rev. Phytopathol. 12:259-279.

13. Patil, S. S., A. C. Hayward, And R. Emmons. 1974. An ultraviolet-induced nontoxigenic mutant of *Pseudomonas phaseolicola* of altered pathogenicity. Phytopathology 64:590-595.

14. Patil, S. S., L. Q. Tam, and W. S. Sakai. 1972. Mode of action of the toxin from *Pseudomonas phaseolicola*. I. Toxin specificity, chlorosis, and ornithine accumulation. Plant Physiol. 49:803-807.

15. Pinkas, Y., and A. Novacky. 1971. The differentiation between bacterial hypersensitive reaction and pathogenesis by the use of cycloheximide. Phytopathology 61:906-907 (Abstr.).

16. Roebuck, P., R. Sexton, and J. W. Mansfield. 1978. Ultrastructural observations on the development of the hypersensitive reaction in leaves of *Phaseolus vulgaris* cv. Red Mexican inoculated with *Pseudomonas phaseolicola*(race 1). Physiol. Plant Pathol. 12:151-157.

17. Rose, E., and K. Rudolph. 1976. Einfluss des *Pseudomonas phaseolicola*. Toxins auf die Hypersensitivitatsreaktion von Tabak gegenuber *Pseudomonas lachrymans* und anderen Pseudomonaden. Phytopathol. Z. 87:241-254.

18. Rudolph, K., and M. A. Stahmann. 1966. The accumulation of L-ornithine in halo-blight infected bean plants (*Phaseolus vulgaris* L.) induced by the toxin of the pathogen *Pseudomonas phaseolicola* (Burkh.). Phytopathol. Z. 57:29-46.

19. Skoog, H. A., 1952. Studies on host-parasite relations of bean varieties resistant and susceptible to *Pseudomonas phaseolicola* and toxin production by the parasite. Phytopathology 42:475 (Abstr.).

20. Tam, L. Q., and S. S. Patil. 1972. Mode of action of the toxin from *Pseudomonas phaseolicola*. II. Mechanism of inhibition of bean ornithine carbamoyl-transferase. Plant Physiol. 49:808-812.

Crown-gall Tumor Stimulation or Inhibition: Correlation with Strand Separation

L. Le Goff, M. Beljanski

Laboratoire de Pharmacodynamie
Faculté des Sciences Biologiques et Pharmaceutiques
Chatenay-Malabry, France

Abstract

The effects of the carcinogen dimethylbenz (a)anthracene, of antimitotic drugs (cyclophosphamide and daunorubicin), of the plant hormone (auxin IAA) and the antibiotic mitomycin C were investigated *in vitro* on cancer and healthy DNA from pea seedlings, inoculated and not, with oncogenic *Agrobacterium tumefaciens*. These substances stimulate *in vitro* both synthesis and strand separation of crown-gall DNA as well as oncogenic *A. tumefaciens* DNA, while they have little effect on normal plant DNA as in the case with *E. coli* and non-oncogenic *A. tumefaciens* DNA. This correlates with the substance-enhancing-power on *in vivo* crown-gall cell multiplication. Growth-stimulatory or inhibitory-effects are antagonized by the tumorless action of *E. coli* small size RNA-fragments. Plant ribonuclease is under control of all these compounds and the RNA-fragments compensate for increased or decreased ribonuclease activity induced by cyclophosphamide, daunorubicin, dimethylbenz(a) anthracene or auxin. There appears to be a correlation between ribonuclease activity and crown-gall cell development.

Introduction

A relatively close relationship exists between the carcinogenic processes in the animal and vegetable kingdoms. Chemical carcinogens induce mammalian (1) and plant (3, 15) tumors and when used at low doses they stimulate cancer cell multiplication in both cases (2, 24). Chemotherapeutic agents (cyclophosphamide (CP), daunorubicin) inhibit human cancers (16) but their carcinogenic potency established *in vitro* (5) and *in vivo* in mammals (26), has been extended to plants. We have shown (24) that once the tumor process was started the *in vivo* multiplication of pea cancer cells induced by *A. tumefaciens* B $_6$ could be either substantially accelerated or inhibited by CP, daunorubicin and DMBA [(IAA has exhibited a similar action (13)]. The effects obtained are dose-dependent. Carcinogens, antimitotics, which behave as carcinogens, and steroids preferentially stimulate mammalian cancer cell *in vitro* DNA synthesis and induce the strand separation of the DNA (12). We show here that CP,

daunorubicin, DMBA, mitomycin C and IAA also stimulate crown-gall cell and *A. tumefaciens in vitro* DNA synthesis through a destabilisation of these DNA but have little effect on DNA from healthy plant cells.

In addition, a correlation exists between CP, daunorubicin, DMBA, and IAA effects and RNase activity in plant tumor cells. Nucleases may produce a large range of biologically active RNA-fragments; primers for DNA replication (9), tumor-inducing RNA (active exclusively in the presence of IAA) (8, 10, 22), tumor-inhibiting RNA (whose effect may be overcome by IAA) (23), or RNA-fragments used here. These last have no direct effect on animal and plant tumor inhibition or evolution but are capable of binding to some sites on DNA which thereafter are no longer accessible to other substances (11).

The influence of endogenous or exogenous substances on RNase activity may lead to the liberation of ribooligomers in the cell which are either purin-rich, in which case they generally activate protein synthesis (19), or pyrimidin-rich, in which case they inhibit it (19). Furthermore, we show here that each of the drugs used (carcinogen, antimitotic, hormone, and RNA) acts on the plant cell RNase activity and that there appears to be a correlation between RNase activity and plant tumor development.

Materials and Methods

Reagents

Pancreatic RNase 4 x crystalized: I. C. N. Pharmaceuticals Inc., Cleveland, USA. Deoxyribonucleoside-5'-triphosphates (d-XTP) ^{12}C and ^{3}H-lithium salt (sp. act. 24-27 Ci/mmol): Schwarz Bioresearch, USA. Indolacetic acid (IAA): Prolabo, France. Mitomycin C: Sigma Co., St. Louis, USA. Daunorubicin- Rhône-Poulenc, France. Cyclophosphamide (CP): Lab. Lucien, France. 9, 10-dimethyl-1,2-benzanthracene (DMBA):N.B.C., Cleveland, USA. Phenol: Backer Chemicals, Holland. 8-hydroxychinolin: Merck, France.

Plant Material

Two-day-old etiolated decapitated epicotyls of *Pisum sativum* L. cv Annonay were used as already described (20). The oncogenic agent was *Agrobacterium tumefaciens* B_6 (about 10^8 cells per wound), aerobically grown overnight at 28°C (20). IAA, DMBA, daunorubicin, CP, and RNA-fragments tested here were dissolved in a buffered physiological saline solution, pH 7.0 and filtered through millipore prior to use.

In some instances, the wounds were infected (time 0) with a mixture of bacteria-drugs and in other experiments the drugs were introduced into bacteria-preinfected wounds (time 24 or 48 h) after gentle tissue scarification. The sterile saline solution was applied to control plants. The treated seedlings (30 plants) continued to grow in darkness for 12 days, then the fresh weight of the excised tumors was determined, and mean weights were compared by the Student's t-test. The tumors were used to prepare cancerous DNA and crude extracts for RNase activity detection. In

yet other experiments, the drugs were applied immediately to the cut subapical region and the segments were cut after 1 or 2 days and used for the preparation of crude extracts.

Isolation of DNA

Crown-gall cells from peas were placed in a pH 8 Lerman buffer (25) and DNA was extracted with water-saturated phenol containing 0.2% 8-hydroxychinolin as described previously (28). Light-grown pea shoots (about 2 weeks old) were used for healthy DNA extraction. DNA from *A. tumefaciens* was prepared in the same way except that lysis was performed in sterile distilled water. The integrity of DNA was controlled before use (12). The hyperchromic effect on DNA in the presence of the different substances was determined by UV absorbance in Tris-buffer 0.01 M pH 7.65 as described for mammalian DNA (12).

Isolation of DNA-dependent DNA Polymerase

The preparation of a partly purified enzyme from *E. coli* and incubation conditions for DNA synthesis have been described (12). All experiments were performed in the absence or presence of compounds to be tested.

Sources and Isolation of ³H-labelled RNA and RNA-fragments

Bacteria *E. coli* T3000 aerobically grown at 37°C in a synthetic medium (6) supplemented with ³H-adenine (500 nc/1) and ³H-guanine (500 nc/1) were harvested during the exponental growth phase. Ribosomal RNA was isolated from washed cells as described (6). RNA-fragments were obtained by mild degradation of *E. coli* ribosomal RNA (r-RNA) using pancreatic RNase (11). Purin-rich RNA-fragments (about 25-50 nucleotides) are devoid of DNA. Their analysis and characteristics have been described (11).

Plant Ribonuclease

Healthy and cancerous tissues, drug treated or not (from 50 plants), were homogenized in a cold mortar with sterile distilled water. The homogenate was twice centrifuged (5000 g for 10 min) and the dialysed supernatant (crude extract) was used as the enzyme source. Proteins were determined by Folin's method. Five to 10 ng of proteins were added to the assay solution (final vol. 0.1 ml) containing 100 ng of ³H-r-RNA (15000-20000 CPM). After 10 min of incubation at 36°C the reaction was stopped by the addition of 5% TCA solution and the acid-precipitable product was filtered on a GF/C glass filter. It was then washed (TCA 5% dried, and its radioactivity was measured in a Prias Liquid spectrometer.

Results

Crown-gall and Healthy Cell DNA *in Vitro* Synthesis

Fast development of crown-gall tumors implies an accelerated replication of DNA from these cells compared to that of healthy cells. DNA replication and cell division are interdependent. Using template DNA purified from the healthy or tumorous cells of pea seedlings and DNA-dependent DNA polymerase, we compared template activities of both

during DNA *in vitro* synthesis. Tumorous DNA exhibits a higher template activity compared with that of DNA from normal plant cells (Fig. 1, see origin of curves). From this first observation it appeared that cancer DNA contained a large number of single-stranded DNA regions required for DNA-dependent DNA polymerase activity. DNA synthesis, which requires all four d-XTP, is strongly inhibited in the presence of DNase (Table 1).

To parallel numerous observations on human cancer therapy and because of their effects on mammalian DNA synthesis, we investigated the *in vitro* synthesis of DNA from crown-gall and healthy pea cells in the presence of CP, daunorubicin, DMBA, mitomycin C or IAA. At low concentrations every one of these substances substantially stimulated the synthesis of tumorous DNA while they slightly enhance normal cell DNA synthesis (Fig. 1). High concentrations were inhibitory. It thus appears that crown-gall cell DNA is very susceptible to the extremely diversified molecules used. It is remarkable that *A. tumefaciens* DNA *in vitro* synthesis is greatly stimulated by the above compounds while that of DNA from *E. coli* or the non-oncogenic *A. tumefaciens* B_6-Trl (7) is not (Fig. 1). In this
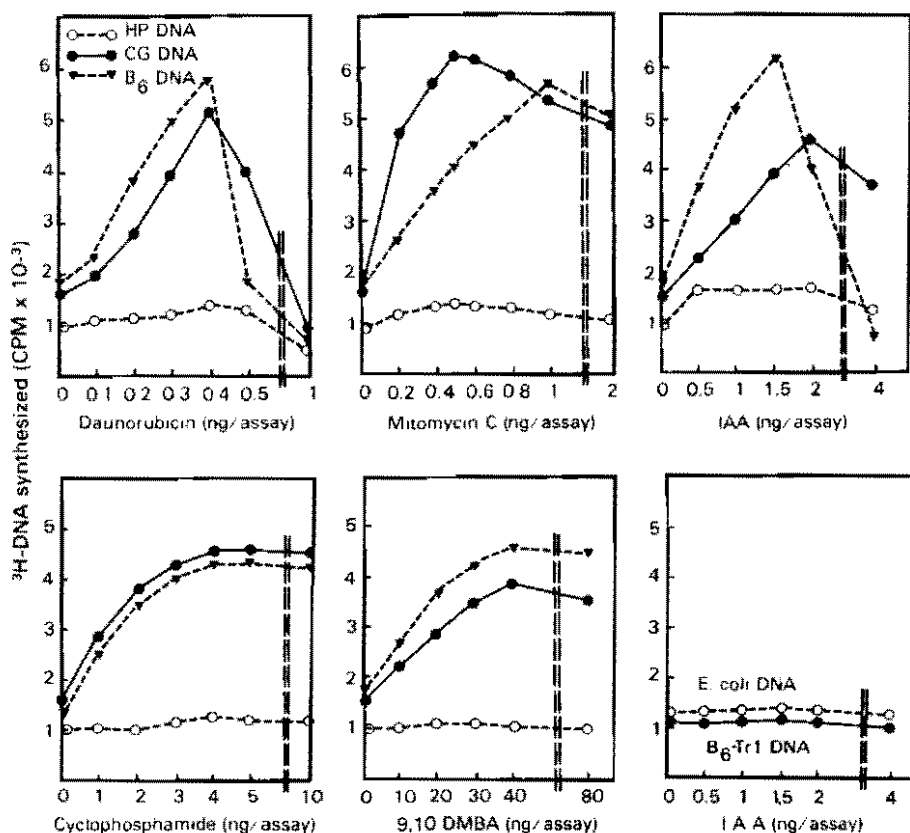
Fig. 1. Effects of various compounds on cancer and normal *in vitro* DNA synthesis of healthy pea cells DNA (HP DNA), crown gall DNA (CG DNA), and *A. tumefaciens* (B_6 DNA).

Table 1. Effect of DNase on crown-gall DNA *in vitro* synthesis.

Incubation medium (11)	^{3}H-TTP incorporated into DAN (CPM)	Inhibition %
Complete (0.5 ng of DNA)	6420	—
+ DNase 0.1 ng	643	90
+ DNase 1 ng	274	95
− d-ATP, d-CTP, d-GTP	447	93
heated enzyme at 100° for 5 min.	163	97

respect, crown-gall cell and *A. tumefaciens* DNA behave as do DNA purified from different mammalian cancerous tissues, i. e. carcinogens and several antimitotic drugs exhibit a considerable stimulatory effect on cancer DNA *in vitro* synthesis and a slight effect on that of DNA from healthy tissues (5).

Crown-gall Cells *in vivo* Multiplication

The particular *in vitro* stimulation of crown-gall DNA synthesis by the various substances used should be correlated by an *in vivo* acceleration of cancer cell multiplication. Thus, a range of concentrations of CP, daunorubicin or DMBA were introduced into preinfected wounds 24 or 48 h following bacterial infection (only a few hours are required for the transformation of normal pea cells into tumor cells). It is interesting that at low concentrations, these substances act as tumor-stimulants and increase tumor weight while at high concentrations they have a strong tumor-inhibiting action but do not disturb normal plant growth (Fig. 2). When drugs and bacteria are applied together on wounds (time 0), the same low concentrations have no effect on tumor development, whereas, high concentrations have a drastic inhibitory effect (Fig. 2). The non action of low concentrations at time 0 must be the consequence of the dilution or elimination of these substances unretained by healthy cells during the first hour following wounding. These results correlate the stimulation of *in vitro* crown-gall DNA synthesis and the absence of stimulation on DNA synthesis from healthy cells.

In Vitro DNA Strand Separation

The substantial and selective stimulation of *in vitro* crown-gall DNA synthesis and the significant increase of pea tumor cell multiplication under the influence of each of these compounds suggested that cancer DNA was "relaxed" compared to normal DNA and many undergo further destabilization in the presence of these substances. To verify this hypothesis, we studied the hyperchromic effect of these substances on healthy and cancer DNA. It is known that UV absorbance (260 nm) increases when DNA strands are separated. Maximum hyperchromicity obtained from both types of DNA on incubation with 0.1 M KOH ranges
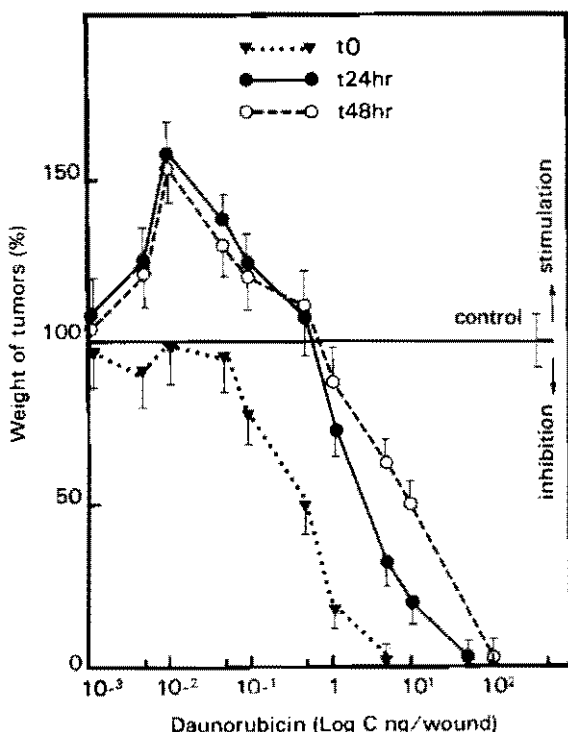
Fig. 2. Effect of daunorubicin on crown-gall tumor development. Drug was applied to the wounds either mixed with B_6 bacteria (to) or separately 24 hours (t24) or 48 hours (t48) after bacterial infection. Each point represents mean tumor weight value for 30 plants and vertical bars indicate ± SE of the mean. Similar responses were obtained with CP and DMBA.

between 35 and 42%, which confirms that both DNA are well polymerized. Now, Fig. 3 shows that in the presence of each of the substances used, UV absorbance increases considerably for crown-gall DNA but not at all or only slightly for control cells DNA. There are optimal concentrations for DNA strand separation which vary according to the drug used (Fig. 3). They also locally separate the strands of DNA from *A. tumefaciens* B_6 but not from *E. coli* or non-oncogenic B_6-Trl. It is important to note that plant hormone IAA, daunorubicin, CP, and mitomycin C behave as carcinogens on cancer cell DNA but act very poorly on normal cell DNA. We should recall that tumorogenesis requires IAA for tumor induction with RNA (8, 10, 22). This hormone exhibits a small but detectable hyperchromic effect on plant DNA. DNA strand separation may explain both the stimulation of *in vitro* DNA synthesis and the *in vivo* acceleration of cancer cell multiplication.
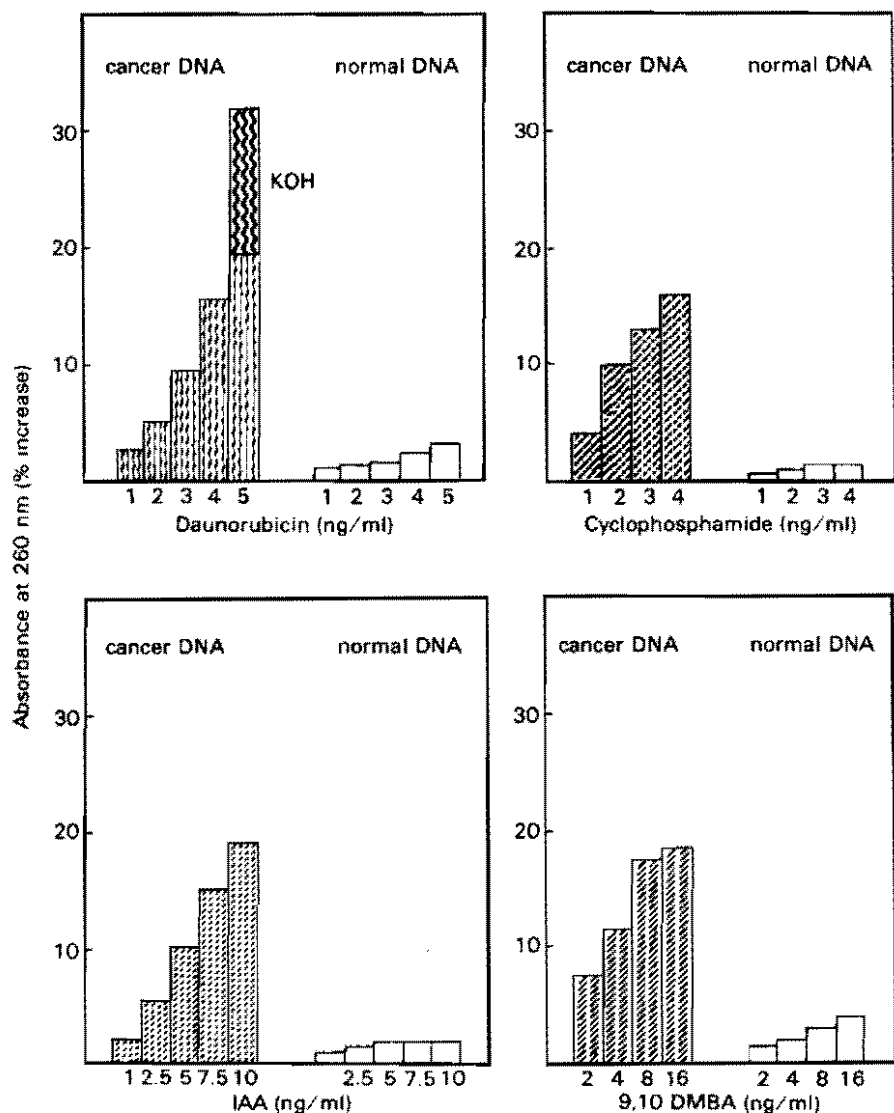
Fig. 3. Effects of drugs on crown-gall tumor and healthy pea cells DNA strand separation. UV absorbance (260 nm) of DNA was measured in the absence or presence of each compound tested at the indicated concentrations (see text).

Ribonuclease Activity in Healthy Crown-Gall Drug Treated Cells

It is well known that *in vitro* replication of DNA by DNA-dependent DNA polymerase requires the presence of RNA primers (oligoribonucleotides) whose origin may be multiple (9, 27). RNase may provide these primers and we have shown that non-oncogenic long chain RNA may be degraded into

more or less purin-rich RNA-fragments, some of which inhibit preformed cancer cells (23). Others initiate DNA *in vitro* replication with a relatively high specificity towards DNA (4, 11). Plant cancer cells can be induced with RNA only in the presence of IAA (10). Thus, one should expect that, depending upon the nature of the cell and the speed of its division, RNA primers would differ and RNase activity would be modified. We therefore attempted to establish a possible correlation between *in vivo* increase or decrease in weight of tumors and RNase activity in plant and tumor extracts according to various treatments.

The crude pea extracts for the RNase activity detection were prepared with 24 h following application of the different drugs on healthy wounds (normal tissues) or at the 12th day following bacterial infection of wounds which have been post-treated 24 or 48 h after B_6 infection (tumorous tissues). Enzyme activity was measured by observing the degradation of ^3H-labelled r-RNA. Results are expressed as TCA-precipitated RNA (Table 2). Two important observations may be made: 1) CP, daunorubicin and DMBA after a 24 or 48 h aging period in the healthy wounded cells, decrease RNase activity, with low and high drugs doses. 2) Small doses of drugs present in the crown-gall cells lead to a considerable increase in RNase activity, while high doses greatly depress this activity.

An increase of RNase activity corresponds to an increase of tumor weight; both appear in the presence of small concentrations of drugs (Table 2). High doses decrease the tumor weight as well as RNase activity. Small

Table 2.   RNase activity in the extracts from healthy pea cells and crown-gall tumors, both untreated or treated with different drugs.

Per cent of degraded radioactive RNA			
Extracts from healthy plants		Extracts from tumor tissues	
Extract, no drug	32	Extract, no drug	42
'' , CP 0.1 ng	32	'' , CP 0.1 ng	55
'' , CP 1 ng	10	'' , CP 1 ng	70
'' , CP 10 ng	8	'' , CP 10 ng	21
'' , CP 50 ng	1	'' , CP 50 ng	1
'' , daunorub. 0.005 ng	30	'' , daunorub. 0.005 ng	76
'' , daunorub. 1 ng	20	'' , daunorub. 1 ng	16
'' , daunorub. 2 ng	14	'' , daunorub. 2 ng	8
'' , daunorub. 5 ng	8	'' , daunorub. 5 ng	11
'' , DMBA 0.02 ng	33	'' , DMBA 0.02 ng	49
'' , DMBA 0.2 ng	32	'' , DMBA 0.2 ng	63
'' , DMBA 1 ng	30	'' , DMBA 1 ng	35
'' , DMBA 10 ng	28	'' , DMBA 10 ng	28
No extract	100	No extract	100

doses of RNA-fragments introduced into infected wounds treated with small doses of drugs suppress the increase in tumor weight (Fig. 4) and decrease the enhanced RNase activity (Table 3). In the presence of high doses of drugs, large doses of RNA-fragments increase the RNase activity and practically suppress the tumor inhibitory effect of drugs (Fig. 4 and 5). Over a limit in concentration of drugs, RNA-fragments cannot reverse the effects observed. Thus, the amount of exogenous RNA-fragments may well modulate RNase activity in tumorous cells and thus bring the weight of tumors and RNase activity back to values found in untreated cancer cells. It should be stressed that RNA-fragments introduced into the wounds of decapitated epicotyls increase RNase activity (measured 24 h after administration), which is suppressed by low or large doses of drugs.

Table 3. RNase activity in the extract from crown-gall tumors treated with different drugs and RNA fragments.

Treatment	Per cent of degraded radioactive RNA
Extract without drug	42
Extract , CP 0.1 ng	55
" , CP 0.1 ng + RNA-fragts 0.005 ng	38
" , daunorub. 0.005 ng	76
" , daunorub. 0.005 ng + RNA-fragts 0.1 ng	40
" , DMBA 0.02 ng	63
" , DMBA 0.02 ng + RNA-fragts 0.4 ng	41
No extract	100

Conclusions

We used crown-gall tumors as a model to establish a correlation between *in vitro* DNA synthesis, DNA local strand separation, and multiplication of cancerous plant cells, with differential susceptibility to low and high doses of CP, daunorubicin, DMBA, or IAA. At low concentrations, these compounds (also mitomycin C) strongly stimulate crown-gall and *A. tumefaciens* B_6 DNA *in vitro* synthesis and DNA strand separation measured as a UV absorbence increase, and enhance tumorous cells development. Used at low or high concentrations, they have practically no effect on DNA from healthy pea cells, DNA from *E. coli* or from B_6-Tr-1 *A. tumefaciens* non-oncogenic strain. The action of IAA in these events is of particular interest. This plant hormone is required to increase the size of healthy cells (14) and is needed for tumor induction with tumor-inducing RNA (8, 10, 22). *In vitro*, it strongly stimulates the synthesis of crown-gall and B_6 DNA and induces DNA strand separation of both. In this
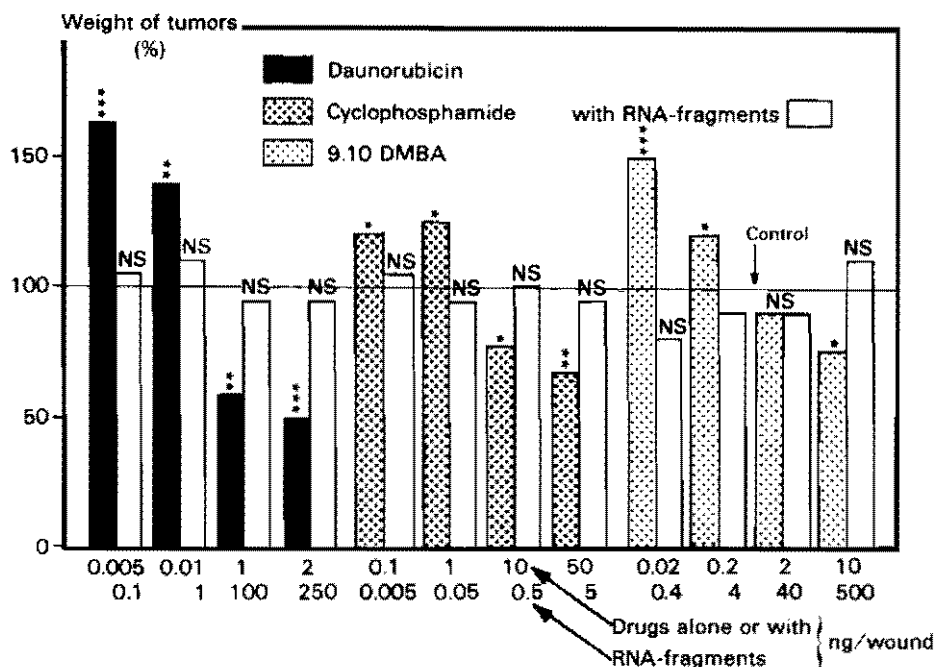
Fig. 4. Pea wound response following drugs or drugs + RNA-fragment application delayed by B$_6$ infection. The results based on 30 plants represent average of tumor weight expressed as percentage of control. NS; no significant difference. Significance of comparison to control (t-test), p values; 0.05*, 0.01**, 0.001***.
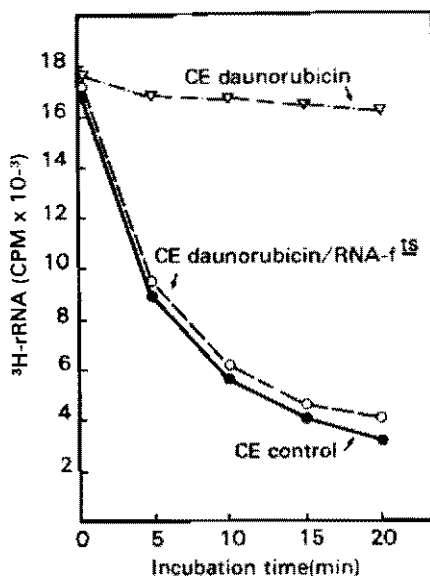
Fig. 5. RNase activity in the extracts from crown gall tumors treated with drug or drug + RNA fragments. The preinfected wounds were treated as described with distilled water (control), 2 ng daunorubicin or 2 ng daunorubicin + 100 ng RNA-fragments. Crude extracts (CE) were prepared (see text) and 10 ng proteins with 100 ng ^{3}H-r-RNA were used per assay. The results express the degraded radioactive r-RNA according to incubation time.

respect it has a detectable, although low, effect on DNA from healthy pea cells. Its contribution in tumor induction appears to be similar to that of carcinogenic compounds since its *in vitro* behavior is similar to theirs.

The interaction of hormones and ribonuclease was considered in plant cells (29). RNase activity is in our experiments of fundamental interest since this enzyme may provide RNA primers without which there is no possible DNA synthesis (9, 27) and, consequently, no cell division process. We have already demonstrated that, depending on the RNase, inert r-RNA may be used to provide either tumor-inducing RNA (8, 10, 22), tumor-necrosing RNA (23) or specific primers for this or that DNA template (4, 11). Thus, all modifications of RNase activity interfere with the endogeneous primers which this enzyme provides for cell multiplication.

It is worthwhile noting that a given cell DNA requires different RNA primers, according to its normal or cancerous origin (unpublished results). Here we saw that in healthy plant cells, low and high concentrations of these substances inhibit RNase activity while at low doses in crown-gall they strongly stimulate RNase activity. High doses are inhibitory and decrease tumor weight.

There appears to be a correlation between crown-gall tumor weight increment and the increased RNase activity of these cells. This correlation is observed only if small doses are introduced into wounded plants 24 h after infection with B_6. This indicated that these compounds act preferentially on tumorous cells whose formation requires at least 6 h following infection (18). Exogenously introduced RNA-fragments regulate RNase activity and suppress either the stimulating or inhibiting effects of the substances active in plant tumor development. It was reported that mitomycin C used at low concentration stimulated the multiplication of *A. tumefaciens* and consequently substantially increases tumor initiation (17). Moreover multiplication of *A. tumefaciens* inside tumorous cells does not appear to be essential for tumor weight increase (21). Here we have shown that mitomycin C at low doses induces crown-gall and B_6 DNA *in vitro* strand separation and strongly enhances DNA synthesis without affecting DNA from healthy pea cells. DNA local strand separation, as a part of the gene activation process in healthy cells and particularly in tumorous cells, provides single-stranded DNA regions required by DNA and RNA polymerases to accomplish their action necessary for cell growth and division.

Data presented here and other data obtained with mammalian cancer cells DNA (12) demonstrate the existence of a common denominator for cancer DNA i.e. that these DNA are destabilized and then become susceptible to the action of various compounds and may exhibit a high template activity in comparison to DNA from healthy cells.

Literature Cited

1. Arcos, J. C., and M. F. Argus. 1974. Chemical induction of cancer (Academic press, New York). Vol. II (A and B).
2. Bear, M. P., and F. H. Shneider. 1979. The effects of actinomycin D and cordycepin on neurite formation and acetyl-cholinesterase activity in mouse neuroblastoma cells. Expl. cell. Res. 123:301-309.

3. Bednar, T. W., and E. M. Linsmaier-Bednar. 1971. Induction of cytokinin-independent Tobacco tissues substituted fluorene. Proc. Nat. Acad. Sci. USA, 68:1178-1179.

4. Beljanski, M., 1975. ARN-amorceurs riches en nucleotides G et A indispensables á la réplication in vitro de 1'ADN des phage λX 174 et lambda (Φ) C. R. Acad. Sc. Paris, Sér. D. 280:1189-1192.

5. Beljanski, M., 1979. Oncotest: a DNA assay system for the screening of carcinogenic substances. IRCS Medical Science, 7:p. 476.

6. Beljanski, M., P. Bourgarel and M. Beljanski. 1970. Showdomycine et biosynthése D'ARN non complémentaires de 1'ADN. 118:253-276.

7. Beljanski, M., M. Beljanski, P. Manigualt and P. Bourgarel 1972. Transformation of *Agrobacterium tumefaciens* into a non-oncogenic species by an *Escherichia coli* RNA Proc. Nat. Acad. Sci. USA, 69:191-195.

8. Beljanski, M., M. I. Aaron-Da Cunha, M. Beljanski, P. Manigault and P. Bourgarel. 1974. Isolation of the tumor inducing RNA from oncogenic and non-oncogenic *Agrobaterium tumefaciens*. Proc. Nat. Acad. Sci. USA, 71:1585-1589.

9. Beljanski, M., M. Beljanski, M. Plawecki and P. Bourgarel. 1975. ARN-fragments, amorceurs nécessaires á la réplication in vitro des ADN. C. R. Acad. Sc. Paris, Sér. D., 280:363-366.

10. Beljanski, M., and M. I. Aaron-Da Cunha. 1976. Particular small size RNA and RNA-fragments from different origins as tumor inducing agents in *Datura stramonium*. Mol. Biol. Reports 2:497-506.

11. Beljanski, M., and M. Plawecki. 1979. Particulàr RNA-fragments as promoters of leukocyte and platelet formation in rabbits. Expl. Cell Biol 47:218-225.

12. Beljanski, M., P. Bourgarel and M. Beljanski. 1981. Correlation between in vitro DNA synthesis, DNA strand separation and in vivo multiplication of cancer cells. Expl. Cell Biol. 49:220-231.

13. El Khalifa, M. D., and J. A. Lippincott. 1968. The influence of plant-growth factors on the initiation and growth of crown-gall tumors on primary pinto bean leaves. J. Exp. Bot. 19:749-759.

14. Fan, D. F., and G. A. MacLachlan. 1967. Massive synthesis of ribonucleic acid and cellulase in the pea epicotyl in response to indolacetic acid, with and without concurrent cell division. Plant physiol. 42:1114-1122.

15. Garrigues, R., N. P. Buu-Hoï and A. Ramé. 1971. Production de tumeurs végétales par action de la N-méthyl-N-nitrose-aniline, composé cancérogéne chez l'animal. C. R. Acad. Sc. Paris, Sér. D., 273:1123-1125.

16. Gordon-Zubrod, C., 1972. Chemical control of cancer. Proc. Nat. Acad. Sci. USA. 69:1042-1047.

17. Heberlein, G. T., and J. A. Lippincott. 1967. Enhancement of *Agrobacterium tumefaciens* infectivity by mytomicin C. J. Bacteriol. 94:1470-1474.

18. Kurkdjian, A., and P. Manigault. 1969. Effet de la blessure sur 1'efficacité d'*Agrobacterium tumefaciens* au cours de l'induction des tumeurs de la plantule de Pois, *Pisum sativum* L., C. R. Acad. Sc. Paris, Sér. D, 268:2756-2757.

19. Lee-Huang, S., J. M. Sierra, R. Naranjo, W. Filipowitz and S. Ochoa. Eukaryotic oiligonucleotides affecting m-RNA translation. 1977. Arch. Biochem. Biophys. 180-276-287.

20. Le Goff, L., 1977. Participation á la tumorisation des cellules végétales d'acides ribonucléiques particuliers et action de ces ARN sur l'expression du pouvoir oncogene d'*Agrobacterium tumefaciens*. Thése de Doctorat d'Eat. Université P. et M. Curie. Paris.

21. Le Goff, L., 1980. Action in vitro d'ARN particuliers sur l'oncogenicité d'*Agrobacterium tumefaciens*. C. R. Acad. Sc. Paris, Sér. D., 290:283-286.

22. Le Goff, L., M. I. Aaron-Da Cunha and M. Beljanski. 1976. Un ARN extrait d'*Agrobacterium tumefaciens* souches oncogénes et non oncogénes, élément indispensable á l'induction des tumeurs chez *Datura stranomium*. Can. J. Microbiol. 22:694-701.

23. Le Goff, L., and M. Beljanski. 1979. Stimulation de l'induction-ou inhibition du développement des tumeurs de crown-gall par des ARN-fragments U_2 .Interférence de l'auxine. C. R. Acad. Sc. Paris, Sér D., 288:147-150.

24. Le Goff, L., and M. Beljanski. 1979. Cance/anti-cancer dual action drugs in crown-gall tumors. IRCS Medical Science. 7. p. 475.

25. Lerman, L. S., and L. J. Tolmach. 1957. Genetic transformation. I. Cellular incorporation of DNA accompanying transformation in *Pneumococcus*. Biochim. Biophys. Acta. 26:68-82.

26. Schmahl, O., and M. Habs. 1979. Carcinogenic action of low dose cyclophosphamide give orally to Sprague-dawley rats in a lifetime experiment. Int. J. Cancer 23:706-712.

27. Sugino, A., S. Hirose and R. Okasaki. 1972. RNA-linked nascent DNA fragments in *Escherichia coli*. Proc. Nat. Acad. Sci. USA. 69:1863-1867.

28. Thompson, W. F., and R. Cleland. 1971. Auxin and ribonucleic acid synthesis in pea stem tissue as studied by deoxyribonucleic acid-ribonucleic acid hybridization. Plant Physiol. 48:663-670.

29. Wilson, C. M., 1975. Plant nucleases. Ann. Rev. Plant Physiol. 26:187-208.

Factors Affecting Motility
of *Erwinia amylovora*

A. K. Raymundo , S. M. Ries

University of Illinois
Urbana, IL, U.S.A.

Abstract

Cultural and environmental factors influence the ability of *Erwinia amylovora* to move by flagellar motion. Synthesis of flagella, the organ of locomotion, is dependent on temperature with an optimum at 18-23°C. Bacterial movement is also dependent on pH with an optimum of between 6 and 8, and on the presence of a chelating agent in the motility medium. The energy for flagellar movement is derived from oxygen-dependent metabolism of an endogenous energy source, but motility will occur under anaerobic conditions if an energy source is provided which can be metabolized under anaerobic conditions. *Erwinia amylovora* is not motile when inside host tissue, but cells rapidly become motile when in contact with free water. Temperature, pH, and chemical factors also influence the directional movement (chemotaxis) of *Erwinia amylovora*. The bacterium is strongly attracted only to four carbon dicarboxylic organic acids such as malate, succinate, and oxaloacetate. Attraction to this entire class of compounds is uniformly inhibited by the presence of any member of the class suggesting a single chemoreceptor for all attractants.

Introduction

Most plant pathogenic bacteria are motile by means of flagella. It has been proposed that motility may be of survival value when nutrients become limiting (23, 9) and may increase the pathogen's infection potential by allowing movement toward sites of entry (16). Panopoulos and Schroth (16) demonstrated that motile strains of *Pseudomonas phaseolicola* were more infective than nonmotile strains.

Motility by bacteria may be a directed motion, and when it is in response to a chemical gradient, it is termed chemotaxis. Chemotaxis has been demonstrated in a variety of bacterial genera (10). Among plant pathogenic genera, chemotaxis studies have been limited to studies with *Xanthomonas oryzae* (8) and *Pseudomonas lachrymans* (6). Additional studies on chemotaxis in plant pathogenic bacteria are needed if generalizations concerning bacterial chemotaxis are to be made.

Erwinia amylovora (Burr.) Winslow *et al.*, a peritrichously flagellated bacterium and the causal agent of fire blight of rosaceous plants could serve as a model system to examine the role of motility and chemotaxis among plant pathogenic bacteria. Such studies may eventually aid in elucidation of the role of chemotaxis in plant pathogenesis.

This report summarizes our findings concerning the factors which affect movement and chemotaxis of *E. amylovora* (18, 19).

Materials and Methods

Bacterial Strains and Growth Conditions

The bacterial strains of *E. amylovora* used were a single cell isolate obtained from an infected apple shoot (A_1), and an American Type Culture Collection isolate #19382. *E. amylovora* was grown on modified Emerson medium (MEM) (20) and maintained in sterile water stock cultures.

Factors Affecting Motility

Effect of growth temperature, pH, chelating agent, energy source, and oxygen on motility were studied through microscopic examination and motility assay.

Motility was observed directly under a phase contrast microscope at 256 x magnification by focusing on the bottom of a drop of the bacterial suspension.

Motility assays were carried out by Adler's technique (1). In this method, a capillary tube containing a test chemical is inserted into a bacterial suspension. Bacteria swim to and accumulate in the capillary if they are attracted to the chemical. In the absence of a test chemical in the capillary, this technique becomes a motility assay (1). Bacteria move randomly but the number of bacteria in the capillary at the end of the assay period is a function of the rate of movement. The more vigorously motile the cells are, the more bacteria enter into the capillary.

Slight modifications were made with Adler's technique (1). Glass petri dishes were used instead of microscope slides. All materials used including one nl micropipets (Drummond Scientific Co., Bromall, Pennsylvania) were rinsed thoroughly with glass distilled water and sterilized. An assay chamber was formed by laying a U-shaped capillary tube with sealed ends between a glass petri dish and cover slip. The chamber was filled with 0.2 ml of bacterial suspension in motility medium into which a one nl capillary tube containing motility medium and sealed at one end was inserted.

Bacteria used in the assay were prepared from MEM broth. Cells from MEM plates were transferred to MEM broth and incubated overnight at 23°C, unless mentioned otherwise, on a shaker bath at 120-140 oscillations/min. These cultures were used to inoculate fresh broth and allowed to grow to an optical density of 0.2 to 0.4 at 660 mn. Cultures were harvested at 3400 g in a refrigerated centrifuge at 23 to 26°C and resuspended in motility medium to a final concentration of 4×10^7 cells/ml. Motility medium consisted of $10^{-3}M$ or $10^{-4}M$ ethylenedia-minetetraacetic acid (EDTA) in $10^{-2}M$ phosphate buffer.

The effect of growth temperature on motility was studied by growing cells at temperatures of 18 to 33°C. The cells were prepared as above, examined under the microscope and assayed for motility. For the effect of pH, *E. amylovora* was grown on MEM, adjusted to different pH values, and motility of resulting cells was determined by microscopic examination. The effect of pH on motility itself was determined by assays in motility medium with varying pH values using potassium phosphate buffer. The effects of EDTA (a chelating agent) and of different energy sources on motility were studied by adding either EDTA at 10^{-1}-$10^{-6}M$ concentrations or energy

sources at differing concentrations to the motility medium. The effect of oxygen was determined by examining motility of cells in a drop on a slide with the cover slip sealed with paraffin or nail polish or unsealed.

Assays were done at 23°C for 45 minutes. At the end of the incubation period, the capillaries were removed and the exteriors rinsed with water. The sealed ends were broken and the contents squirted into MEM broth. Appropriate dilutions were made, plated onto MEM, and resulting colonies counted after 24 to 48 h. All assays were replicated three times and number of bacteria per capillary was based on duplicate plate counts. The average percent standard deviation was 21% based on replicate determinations of the different treatments in the experiments.

Factors Affecting Chemotaxis

The modified Adler's capillary technique described above was also used to assess the effects of different factors affecting chemotaxis. A capillary tube containing a test chemical was inserted into a bacterial suspension. Bacteria accumulate in the capillary if they are attracted to the chemical. Temperature effects were studied by performing chemotaxis assays on slide warmers adjusted to the assay temperature and placed in a refrigerated room. The effects of pH, incubation time, $MgCl_2$, chemotaxis medium and bacterial concentration on chemotaxis were also investigated by varying each parameter.

Apple nectar extract served as the natural chemoattractant. Nectar extracts were obtained from flowers at full bloom of cultivars Jonathan and Golden Delicious at the University of Illinois apple orchard. Glass distilled water (10 nl) was deposited on flower nectaries exposed by manually removing other flower parts for 10 to 15 sec and then removed with an Eppendorf pipet. The nectar extracts were filter sterilized and either lyophilized or stored at -20°C. One ml of the extract was obtained from 130 flowers; it weighed 3.1 mg after freeze drying.

Fractionation of nectar extract into basic, neutral and amino acid, and organic acid fractions was performed with an anionic resin (Dowex 2-X8-chloride form, 74-38 nm) and a cationic exchanger (Dowex 50W-X8-hydrogen form, 74-38 nm). A vial of the freeze dried nectar extract containing 3.1 mg was rehydrated with 5 ml of glass distilled water and added to 5 ml of Dowex-X8 (wet volume) mixed, filtered, and the resin washed with 0.1 N HCl and refiltered. To the latter filtrate, Dowex 50W-X8 was added, and the same process was repeated as above. All filtrates were evaporated to dryness, dissolved in 10 ml of the chemotaxis medium (equivalent to a 10^{-1} dilution of the crude nectar extract) consisting of 10^{-3}M ethylenediaminetetraacetic acid (EDTA), 10^{-3}M mannitol, 10^{-2}M $MgCl_2$ and 10^{-2}M potassium phosphate buffer at pH 7. The pH was adjusted to 7 with KOH and the solutions assayed by the capillary technique.

The capillary tube assay was used to test various amino acids, sugars, and organic acid to determine if they were chemoattractants. The different test chemicals at 10^{-1}-10^{-7}M, depending upon solubility, were dissolved in the chemotaxis medium described previously. The solutions were adjusted to pH 7 with KOH as necessary. Assays were run for 30 minutes using a bacterial population of 8×10^6 cells/ml suspended in the chemotaxis

medium. In assays of sugar compounds, the bacteria were grown on Modified Miller Schroth Medium (MMS) (13) with a 10^{-2}M concentration of the sugar being assayed. Cysteine solutions were prepared just before assay.

All sugars used were D-form and all amino acids L-form (Sigma Chemical Co., St. Louis, MO.). All organic acids were reagent grade, obtained from various commercial sources.

Terms commonly used to describe chemotaxis responses are adopted (1, 11). A concentration response curve is a plot of responses vs. logarithm of concentration in the capillary. Peak concentration is defined as the concentration that causes the greatest accumulation of bacteria in a series of dilutions. Peak response is that number of bacteria that accumulate in the capillary containing the peak concentration. Threshold is the concentration which gives a detectable increase over the blank or control value (response to zero concentration of the compound) plus the standard deviation for replicate determination for the value. The threshold may be extrapolated from a concentration response curve on a double logarithmic plot. The blank value is the bacterial accumulation in the absence of an attractant. Relative response is the ratio of the number of bacteria per capillary to that of blank value (14). Any chemical with a relative response less than five at peak concentration is considered a weak attractant in this study. Threshold values of weak and non-attractants are not computed.

Results

Factors Affecting Motility

Effect of Temperature. Examination of cultures grown at temperatures of 18 to 23°C revealed more vigorously motile cells than those grown at 30°C or higher. Temperature effects on motility were confirmed by Adler's motility assay (Fig. 1). There were no significant differences between responses of cells grown at 18, 20, and 23°C but cells grown at those temperatures were significantly more motile than cells grown at 27, 30, or 33°C (P = .01). At 33°C, less than 1% of the cells were motile and those that were motile were sluggish. Cells were more vigorously motile when grown below 23°C, but growth was not as good as when bacteria were cultured at higher temperatures (Fig. 1).

When cells grown at 23°C were transferred to fresh broth with one set incubated at 23 and the other at 33°C, only cells incubated at 23°C were vigorously motile after 24 h When weakly motile cells (grown at 33°C) were the source of inoculum, cultures incubated at 23°C became fully motile after five generations (generation time of *E. amylovora* on MEM at 23°C is 81 minutes). Cells incubated at 33°C remained at a very low level of motility. When washed cells grown at 23 and 33°C were maintained in motility medium and incubated at either 23 or 33°C, 23°C grown cells remained motile for about 2 H while cells grown at 33°C, regardless of temperature of incubation, remained weakly motile. Cells grown at temperatures of 23°C had abundant flagella as determined by electron microscopy (19) while cells from cultures grown at 33°C had very few or no flagella.
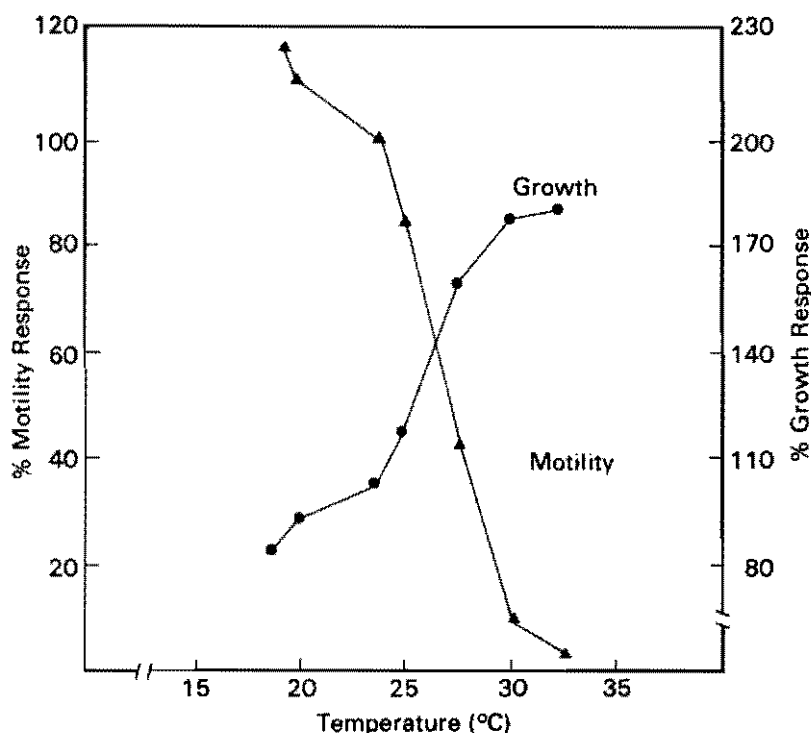
Fig. 1. Effect of incubation temperature on motility and on growth of *Erwinia amylovora*. The data are a composition of several experiments in which responses are adjusted to 100% with respect to the results obtained at 23°C. Motility assays were performed at 23°C for 45 min using 4×10^8 cells/ml suspended in 10^{-4}M EDTA and 10^{-2}M potassium phosphate buffer at pH 7. Growth responses were estimated by dilution plating. $FLSD_{05}$ = 14.4% for motility and 32.28% for growth.

Effect of Chelating Agents. The addition of EDTA to the motility medium enhances motility of *E. amylovora* as determined by Adler's method (Fig. 2). Maximum stimulation by EDTA was observed at 10^{-3}M and inhibition at 10^{-2}M or higher concentrations. Motility of cells in phosphate buffer plus 10^{-4}M EDTA was more vigorous than that of cells in buffer or distilled water alone. Addition of other reported chelating agents (1), including 1% peptone, 10^{-3}M L-glutamine, 10^{-3}M L-arginine or a drop of MEM broth enhanced motility.

Effect of pH. Active motility was observed through microscopic examination of cells cultured on MEM at pH values of 6 to 8, while pH values of 5 and 9 caused reduced motility and growth. The effect of pH on motility itself was assayed by adjusting the pH of motility medium to different pH values. Motility as reflected in numbers of cells per capillary was unaffected within the pH range of 6 to 9 (Fig. 2).
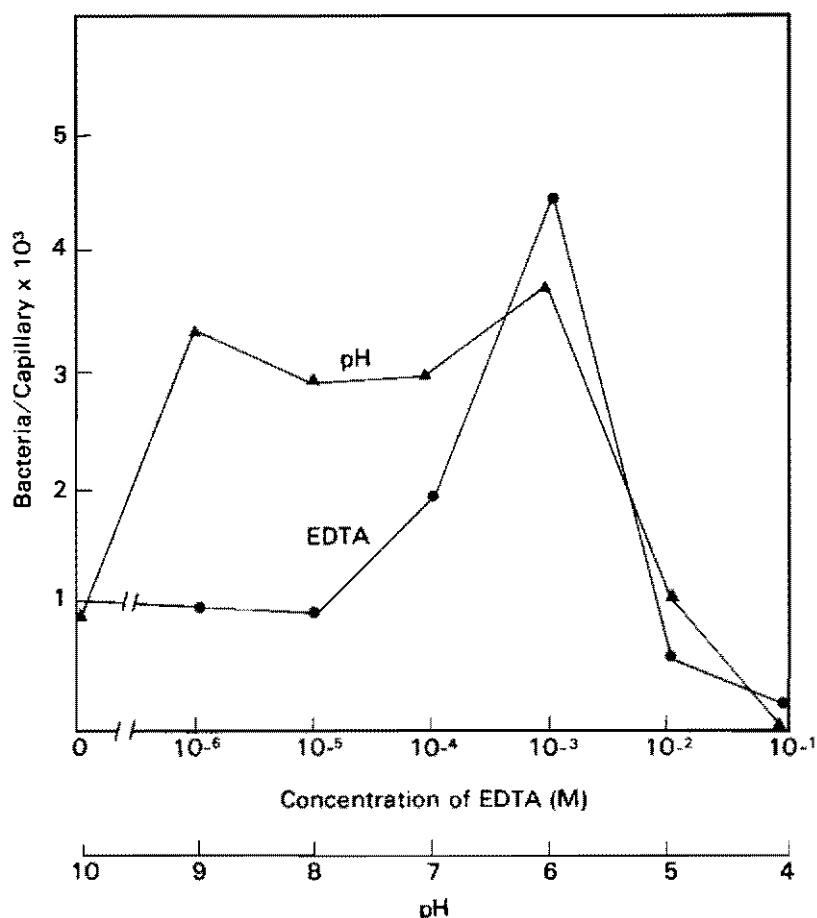
Fig. 2. Effect of EDTA concentration and pH on motility of *Erwinia amylovora*. Assays were performed at 23 C for 45 min using 4×10^7 cells per ml suspended in $10^{-2}M$ potassium phosphate buffer for EDTA experiments and $10^{-2}M$ potassium phosphate buffer plus $10^{-3}M$ EDTD for pH experiments. FLSD $_{05}$ = 525.03 for EDTA; 748.96 for pH.

Effect of Energy Sources. Motility was enhanced when certain energy sources were added to the motility medium as judged by microscopic examination. In this experiment the Miller and Schroth medium (13) minus the selective agents was used as the growth medium. Mannitol and glucose at $10^{-3}M$ gave the best stimulation as compared to cells in motility medium without an outside energy source. Glutamine, asparagine, galactose, fructose, and sucrose at $10^{-3}M$ also enhanced motility but not as well as mannitol and glucose. Glycerol at 0.1 or 0.05% or sodium lactate at $10^{-3}M$ and $10^{-4}M$ or inositol at $10^{-3}M$ did not stimulate motility.

Effect of Oxygen. Motility of cells (10^7 cells/ml) washed with and suspended in motility medium ceased after 30 to 45 min in a drop on a slide with the cover slip sealed. When the seal was broken, good motility was restored. Cells in the unsealed controls were motile for up to 2 to 3 hrs. With an added energy source, mannitol, in the medium, motility was maintained for 1 1/2 to 2 h and 8 h in the sealed and unsealed slides, respectively.

Factors Affecting Chemotaxis

Chemotaxis Medium Composition. Both an energy source and EDTA are essential for chemotaxis of *E. amylovora* toward nectar extract. The use of chemotaxis medium consisting of 10^{-3}M $(NH_4)_2SO_4$, 10^{-5}M $MGCl_2$, 10^{-3}M mannitol, 10^{-3}M EDTA, and 10^{-2}M potassium phosphate buffer at pH 7 resulted in an accumulation of 6400 bacteria/capillary. The removal of EDTA or mannitol but not $(NH_4)_2SO_4$ and $mgCl_2$ from the chemotaxis medium resulted in statistically fewer bacteria/capillary. Increasing concentration of $MgCl_2$ to 10^{-2}M increased the accumulation to 30,000 bacteria/capillary (Fig. 3). Based on these results, the chemotaxis medium used in our studies consisted of 10^{-2}M potassium phosphate buffer (pH 7), 10^{-3}M EDTA, 10^{-3}M mannitol, and 10^{-2}M $MgCl_2$.

Effect of Incubation Period. Accumulation of bacteria in the capillary attracted by 10^{-1} dilution of nectar extract reached a maximum after 30 minutes (Fig. 4). The responses obtained after 30 minutes did not vary significantly ($P = 0.05$) from responses at 45, 60, 75, and 90 minutes.
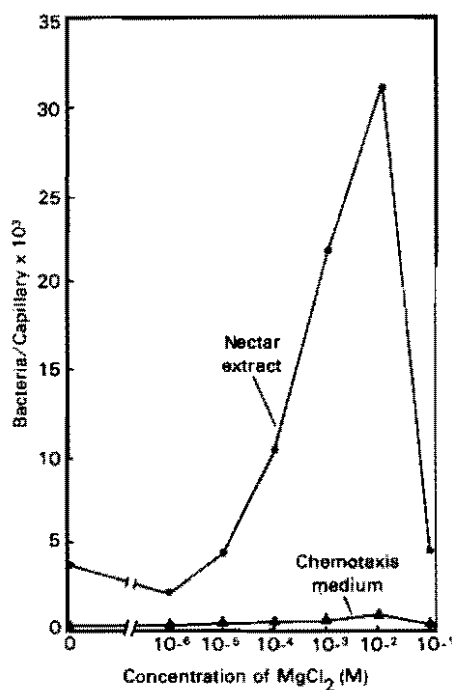
Fig. 3. Effect of $MgCl_2$ on taxis toward nectar extract. Assay run for 45 min at 23°C. Capillaries contained 10^{-1} dilution of nectar extract or chemotaxis medium alone. When magnesium chloride at 10^{-1} or 10^{-2}M is inside the capillary, accumulations were 270 and 420 bacteria/capillary, respectively.

Accumulation in the absence of an attractant increased linearly with time to about 830 bacteria in the capillary after 90 min of incubation. Therefore, an incubation period of 30 min was adopted.

Effect of pH. Chemotaxis towards 10^{-1} dilution of nectar extract was unaffected by pH within a range of 6 to 8. Values of pH 4 and 10 caused an almost complete inhibition of chemotaxis. Responses at pH 5 and 9 were also significantly ($P = 0.05$) lower than those at pH 6 to 8.

Effect of temperature. Chemotaxis toward 10^{-1} dilution of nectar extract was insensitive to incubation temperature within the range of 20 to 28°C (Fig. 5). Responses at 18 and 30°C were significantly ($P = 0.05$) lower than those at 20 to 28°C, and lowest at 4 and 33°C. The assay temperature used in subsequent experiments was 23°C.
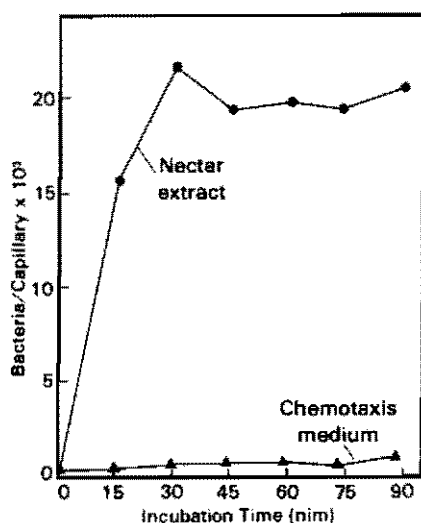
Fig. 4. Rate of accumulation of *Erwinia amylovora* in capillaries containing 10^{-1} dilution of nectar extract or chemotaxis medium alone. Assays were run for 30 min at 23°C.
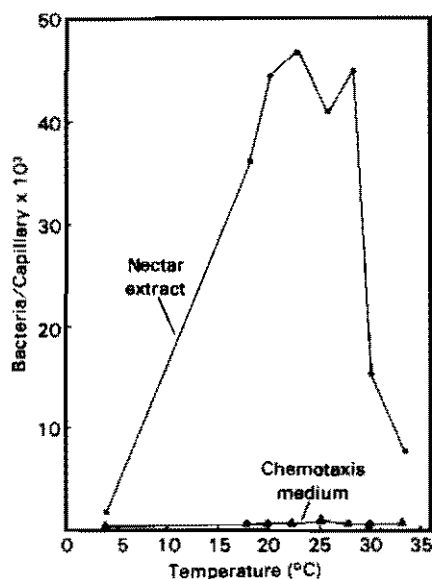
Fig. 5. Effect of temperature on taxis toward nectar extract. Capillaries contained 10^{-1} dilution nectar extract or chemotaxis medium alone. Assays were run for 30 min.

Effect of Bacterial Concentration. The accumulation of bacteria within the capillary containing 10^{-1} dilution of nectar extract increased linearly with increasing bacterial concentration outside the capillary up to about 4×10^7 cells/ml. At higher cell concentrations no additional accumulation occurred and a plateau was reached. Accumulation in the absence of an attractant increased linearly with increasing bacterial concentration up to 7.9×10^7 cells/ml, the highest concentration tested. The bacterial population used in all other experiments was 8×10^6 cells/ml.

Chemotactic Response Toward Different Compounds

Taxis toward Nectar Extract and its Components. The responses of *E. amylovora* to nectar extracts of Jonathan and Golden Delicious apple cultivars were identical. Peak concentration occurred at the undiluted form of the nectar extracts and the threshold was $<1 \times 10^{-6}$ dilution.

Chemotaxis toward the different Dowex fractions of nectar extract of Golden Delicious varied (Table 1). The organic acid fraction was the best attractant among the different fractions but was not as good an attractant as the unfractionated nectar extract. Response to the neutral and basic fraction and the amino acid fraction was significantly ($P = 0.05$) lower than the organic acid fraction and not significantly different from the control. No fractionation was performed on the Jonathan nectar extract.

Table 1. Response of *Erwinia amylovora* to the different fractions of nectar extract of apple cultivar Golden Delicious.

Fraction[a]	Bacteria/capillary[b]
No attractant	175w[c]
Neutral and basic	945w
Amino acid[d]	1,385w
Organic acid[d]	16,915x
Unfractionated nectar extract	25,025y

[a]Fractionated by ion exchange chromatography.
[b]Assay run at 23°C for 30 min with 8×10^6 cells/ml outside the capillary.
[c]Means followed by the same letters are not significantly different at 5% level of probability according to Fisher's least significant difference test.
[d]Adjusted to pH 7 with KOH. Accumulation in capillary containing 1 M KCl was 240 thus values reported are due to the acids themselves.

Taxis Toward Amino Acids. Among the amino acids giving positive significant ($P = 0.05$) response, only aspartate induced high response with an accumulation of 13,520 bacteria at peak concentration compared with 210 to 1,475 bacteria for the others. The relative responses at peak concentrations were 80.5 for aspartate and 1.5 to 4.8 for the other amino acids. The threshold value for aspartate is less than 10^{-7}M, the lowest concentration tested (Fig. 6). No significant responses ($P = 0.05$) were obtained with alanine, asparagine, L-carbamyl aspartate, glutamine, glycine, hydroxyproline, isoleucine, and lysine.

Taxis Toward Organic Acids. *Erwinia amylovora* demonstrated positive chemotaxis toward 8 of 15 organic acids tested (Table 2). Galacturonate and tartarate are weak attractants with peak relative responses of only 3.4 and 2.3, respectively. The peak relative response of maleate was 5.1, a much lower value than those of the other organic acid attractants. It has also a low threshold value of 8×10^{-3}M (Table 2 and Fig. 6). Fumarate has high peak and relative responses but a low threshold of 2×10^{-3}M.

Table 2. Comparison of responses of *Erwinia amylovora* to certain organic acids.

| Organic Acid[a] | Peak Response | | | |
	Concentration (M)	Calculated number of bacteria per capillary[b]	Relative[c] response	Threshold
DL–Tartarate	10^{-3}	575	2.3	
D–Galacturonate	10^{-1}	708	3.4	
Maleate	10^{-1}	2230	5.1	8×10^{-3}
DL–Malate	10^{-3}	25693	161.6	8×10^{-7}
Fumarate	10^{-1}	24915	56.4	2×10^{-3}
Succinate	10^{-1}	33295	334.0	2×10^{-7}
Malonate	10^{-1}	34164	214.5	3×10^{-5}
Oxaloacetate	10^{-1}	20720	57.0	1×10^{-7}

[a]Organic acids which showed significant responses.
[b]Blank values (no attractant in the capillary) were subtrated. Assays were run for 30 min at 23°C with 8×10^6 cells/ ml outside the capillary.
[c]Ratio of number of bacteria/capillary of peak response to that of blank value.

Oxaloacetate, succinate, malate, and malonate are good attractants by all criteria. All the attractants had peak concentrations of 10^{-1}M except malate and tartarate at 10^{-3}M (Table 2). However, reduced motility of *E. amylovora* was observed in the presence of 10^{-1}M concentrations of the above compounds.

Concentrations of citrate, isocitrate, oxalate, lactate, pyruvate, α-ketoglutarate and cis-aconitate at 10^{-1}M to 10^{-7}M concentration did not induce significant (P = 0.05) positive responses of *E. amylovora*. Induction of chemotaxis toward citrate was attempted by adding 10^{-2}M citrate to the growth medium. However, poorly motile cells resulted. At 10^{-3}M, cells were motile but not as vigorous as cells grown in the absence of citrate. No additional assays were done on citrate.

Taxis Toward Sugars. Sucrose, glucose, and fructose, which are present in nectar extract, did not elicit chemotactic response from *E. amylovora.* The same was true with galactose, mannitol, sorbitol, ribose, lactose, and raffinose. In this set of assays, glutamine previously established to a non-attractant (unpublished data), was used as energy source.
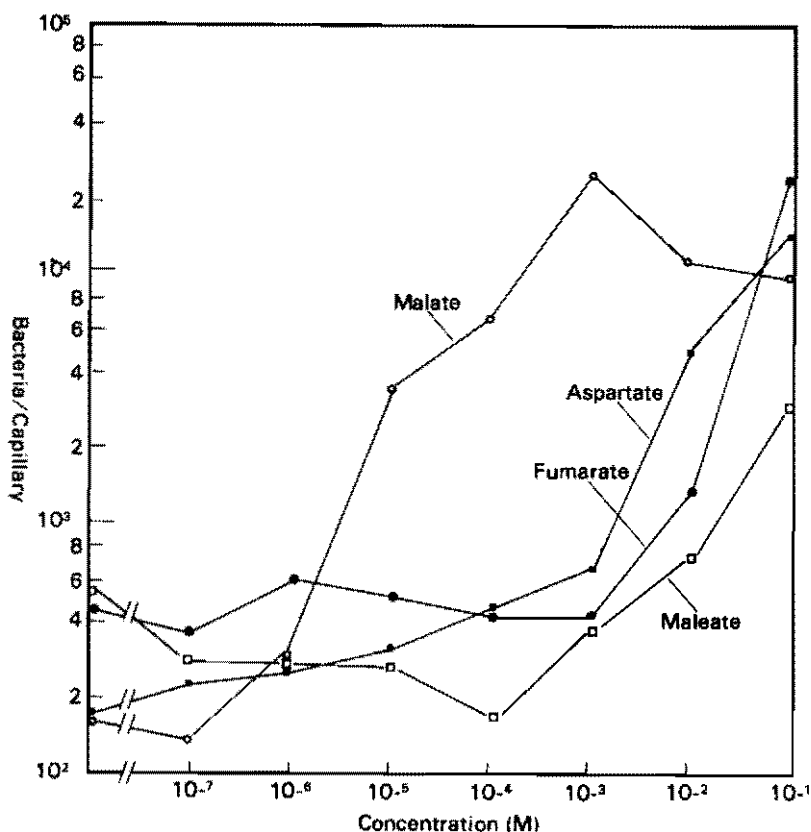
Fig. 6. Concentration response curves for aspartate, fumarate, malate, and maleate. The response at threshold (accumulation value in the absence of attractant plus standard deviation) was 30 for fumarate, 67 for malate, and 141 for maleate based on linear portions of the curves.

Inhibition of Chemotaxis by Other Attractants

Malate, an attractant, when present at $10^{-3}M$ (peak concentration) in the capillaries containing other attractants and in the cell suspension, inhibited chemotaxis toward all the other attractants tested (Table 3).

Comparison of Chemotaxis Between Two Strains of *E. amylovora*

The pattern of responses of our strain used in this study and ATTC strain #19382 to two sugars, three organic acids, and two amino acids was similar (Table 4). Neither strain responded to asparagine, fructose, glucose, or α-ketoglutarate. Strong taxis was observed in both strains toward aspartate, malate, nectar extract, and succinate.

Table 3. Inhibition of *Erwinia amylovora* chemotaxis by malate[a].

| Attractant[b] | Bacteria/capillary[c] | | o/o Inhibition |
	No malate	Malate present	
Fumarate	32100	2765	91
Malate	31000	0	100
Succinate	33800	2495	93
Maleate	3600	0	100
Oxaloacetate	28300	1650	94
Malonate	23150	1050	95
Galacturonate	1090	0	100
Aspartate	20525	1196	94
Nectar extract	17000	360	98

[a]Malate at 10^{-3} M present both in the capillaries and in bacterial suspension.
[b]Tested at 10^{-1} M except malate at 10^{-3} M and nectar extract at 10^{-1} dilution.
[c]Assays were run for 30 min at 23°C. A background accumulation of 420 was subtracted from each value.

Table 4. Response of two isolates of *Erwinia amylovora* to a variety of compounds.

| Compound[a] | Bacteria/capillary | |
	ATTC isolate no. 19328	A_1[b]
D—Fructose	175	185
D—Glucose	360	180
L—Asparagine	210	260
L—Aspartate	24700	26183
Succinate	38566	32250
—Ketoglutarate	140	130
DL—Malate	33450	31400
Nectar extract	16917	19600
No attractant	160	255

[a]Tested at 10^{-1} M concentrations except malate at 10^{-3} M and nectar extract at 10^{-1} dilution. pH adjusted to pH 7 when necessary.
[b]Isolated from University of Illinois apple orchard and used in all other experiments.

Discussion

Erwinia amylovora is peritrichously flagellated (5) and the synthesis of its flagella is dependent upon growth temperature. At the optimum temperature for motility (23°C or lower), *E. amylovora* produces abundant flagella (19). This temperature is not optimal for growth which in this study is 30°C. The temperature cited as minimum for occurrence of blossom blight is 18°C (17). This is of interest since motility is optimal at 18°C for *E. amylovora*. It is conceivable that vigorous motility can enhance the infection process, but motility is not an absolute necessity for either entry (16) or for pathogenesis becauses non motile *E. amylovora* cells cultured at 33°C are still pathogenic.

Environmental conditions and cultural factors influence motility of bacteria. Motility of *E. amylovora* is enhanced by EDTA, possibly because of its chelating ability (4). Motility of *E. amylovora* can occur without an outside energy source although a chelating agent is needed to demonstrate this. In *E. coli* this was shown to be due to an endogenous energy source (4). Motility of both *E. amylovora* and *E. coli* (4), however, is stimulated by exogenous energy sources.

Oxygen is required for motility of *E. amylovora* unless an energy source like mannitol, which is utilized anaerobically, is present. In *E. coli,* oxygen is required for utilization of endogenous energy sources (4). However, motility occurred under anaerobic conditions in the presence of serine, an energy source which the bacteria can metabolize in the absence of oxygen. A similar result was obtained with *Pseudomonas viscosa* (22) which utilized arginine as energy source for motility in the absence of oxygen.

The optimum pH for motility varies with the bacterium studied (4, 7). The effect of pH on flagella synthesis may not parallel the effect on motility itself. Flagella synthesis of *E. amylovora* is inhibited at pH 9 as shown by the poorly motile cells resulting from cultures grown on medium at pH 9. However, motility is unaffected at pH 9 since motility response was identical to that at pH 7.

Erwinia amylovora also exhibits chemotaxis. Medium components, including the energy source, the presence of chelating agent, and magnesium chloride, and environmental factors including temperature and pH, influence its response. *Erwinia amylovora* is attracted strongly to aspartate, and to several Kreb's cycle organic acids but to none of the sugars tested. This pattern of responses is different from other bacteria (3, 12, 15, 24).

Erwinia amylovora attractants studies appear to be detected by only one chemoreceptor site. This site appears highly specific. The substitutions of a hydroxyl group for a hydrogen on C_3 of malate, changes malate, a strong attractant, to tartarate, a weak attractant. The substitution of an amide group for a carboxyl group changes aspartate, a strong attractant to asparagine, a non-attractant. The addition of a carbon atom between carboxyl groups, i.e., aspartate to glutamate, also changes a strong attractant to a weak attractant. Responses of *E. amylovora* to all the attractants were uniformly inhibited by malate by 91 to 100%. Inhibition of taxis occurs if the attractants share a common receptor site (2). Similarity

of chemical structure, all of attractants being three or four carbon, dicarboxylic acids, lends further support for one receptor site.

The responsiveness of *E. amylovora* to organic acids and aspartate but not to sugars or to the other amino acids appears unique to this organism. Taxis toward organic acids has not been reported in any plant pathogenic bacterium. Such responses are not due to the uniqueness of our strain since the same pattern of taxis was observed with the ATTC strain. The limited range of response of *E. amylovora* may have evolutionary and ecological significance. *Erwinia amylovora* may have had receptor sites for sugars and the other amino acids, but through evolution and due to an ecological need to be different from saprophytes, it has lost these receptor sites. Perhaps it has developed unique chemoreceptors for dicarboxylic acids. All the strong attractants, except maleate, are reportedly present in plants, and malate, the strongest attractant, accumulates in cell vacuoles of apples (21). Conceivably, this compound or a similar one is the "chemical odor" which *E. amylovora* follows to the portal of entry.

Acknowledgment

Literature Cited

1. Adler, J., 1973. A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J. Gen. Microbiol. 74:77-81.
2. Adler, J., 1969. Chemoreceptors in bacteria. Science 166:1588-1597.
3. Adler, J., G. L. Hazelbauer, and M. M. Dahl. 1973. Chemotaxis toward sugars in *Escherichia coli*. J. Bacteriol. 115:824-827.
4. Adler, J., and B. Templeton. 1967. The effect of environmental conditions on the mobility of *Escherichia coli*. J. Gen. Microbiol. 46:175-184.
5. Billing, E., L. A. E. Baker, J. E. Crosse, and C. Garrett. 1961. Characteristics of English isolates of *Erwinia amylovora* (Burr) Winlow *et al.* J. Applied Bacteriol. 24:195-211.
6. Chet, I., Y. Zilberstein, and Y. Henis. 1973. Chemotaxis of *Pseudomonas lachrymans* to plant extracts and to water droplets collected from the leaf surfaces of resistant and susceptible plants. Physiol. Plant Pathol. 3:473-479.
7. Doetsch, R. N., and G. J. Hageage. 1968. Motility in procaryotic organisms: Problems, points of view, and perspectives. Biol. Rev. 43:317-362.
8. Feng, T. Y., and T. T. Kuo. 1975. Bacterial leaf blight of rice plants: VI. Chemotactic responses of *Xanthomonas oryzae* to water droplets exudated from water pores on the leaf of rice plants. Bot. Bull. Acad. S in, (Taipei) 16(2):126-136. (In Engl. with Engl. and Chin. Summ.) Biol Abst. 61(1):4739.
9. Kelman, A., and J. Hruschka. 1973. The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*. J. Gen. Microbiol. 76:177-188.

10. Koch, A., 1971. The adaptive response of *Escherichia coli*. Adv. Microbiol. Physiol. 6:147-217.
11. Mesibov, R., G. W. Ordal, and J. Adler. 1973. The range of attractant concentration for bacterial chemotaxis and the threshold and size of response over this range. J. Gen. Physiol. 62:203-223.
12. Mesibov, R., and J. Adler. 1972. Chemotaxis toward amino acids in *Escherichia coli*. J. Bacteriol. 112:315-326.
13. Miller, T. D., and M. N. Schroth. 1972. Monitoring the epiphytic populations of *Erwinia amylovora* on pear with a selective medium. Phytopathology 62:1175-1182.
14. Moulton, R. C., and T. C. Montie. 1979. Chemotaxis by *Pseudomonas aeruginosa*. J. Bacteriol. 137:274-280.
15. Ordal, G. W., and K. S. Gibson. 1977. Chemotaxis toward amino acids by *Bacillus subtilis*. J. Bacteriol. 129:151-155.
16. Panopoulos, N. J., and M. N. Schroth. 1974. Role of flagellar motility in the invasion of bean leaves by *Pseudomonas phaseolicola*. Phytopathology 64:1389-1397.
17. Powell, D., 1965. Factors influencing the severity of fire blight infections on apple and pear. Mich. State Hort. Sco. Ann. Mtg. Prt. 94:1-7.
18. Raymundo, A. K., and S. M. Ries. 1980. Chemotaxis of *Erwinia amylovora*. Phytopathology 70:1066-1069.
19. Raymundo, A. K., and S. M. Ries. 1981. Motility of *Erwinia amylovora*. Phytopathology 71:45-49.
20. Reinhardt, J. F., and D. Powell. 1960. Culture media for viability studies and storage of *Erwinia amylovora*. Phytopathology 50:685-686.
21. Robinson, T., 1975. Water-soluble organic acids. pp. 42-51. *In* The organic constituents of higher plants, 3rd edition. Burgess publishing Co., Minneapolis, Minn., 347 pp.
22. Sherris, J.C., N. W. Preston and J. G. Shoesmith. 1957. The influence of oxygen and arginine on motility of a strain of *Pseudomonas* sp. J. Gen. Microbiol. 16:86-96.
23. Smith, J. L., and R. N. Doetsch. 1968. Motility in *Pseudomonas fluorescens* with special reference to survival and negative chemotaxis. Life Sci., Part II. Physiol. Pharmacol. 7:875-886.
24. Van der Drift, C., J. Duiverman, H. Bexkens, and A. Krijnen. 1975. Chemotaxis of a motile *Streptococcus* toward sugars and amino acids. J. Bacteriol. 124:1142-1147.

* Present address, University of the Philippines, Los Baños.

Response of Rice with Different Gene Resistance to *Xanthomonas campestris* pv. *Oryzae*

T. W. Mew, C. M. Vera Cruz
International Rice Research Institute
Los Baños, Philippines.

G. L. Raina
Rice Research Station
Kapurthala, Punjab, India

Abstract

Response of four rice cultivars that have different major genes for resistance to isolates of *Xanthomonas campestris* pv. *oryzae* varied. All isolates were virulent to IR8 but differed in vertical virulence to IR20, Cas 209, and IR1545. According to a disease scale of 1 to 9, based on leaf area affected, interaction between cultivar-isolate combination was shown at 14 days after inoculation (DAI). Unless the rice-isolate combination was compatible, there was no significant change at 21 DAI. Random selections of isolates either virulent or not virulent to IR20, but all virulent to IR8 and not virulent to IR1545 were evaluated for their variation in virulence on the three cultivars. Ranking of the isolate based on lesion length indicated that the ranking order was not consistent for any isolate on IR8 and IR1545, which are susceptible and resistant, respectively, to these isolates. Significant difference between isolates that were virulent and not virulent to IR20 was noted both on high and low inoculum densities. Vertical virulence of these isolates was also distinguished on IR20 at various plant ages.

Introduction

Bacterial blight of rice caused by *Xanthomonas campestris* pv. *oryzae* is one of the most widespread rice diseases in Asia. Since the major epidemics in the 1960s, breeding for bacterial blight resistance has become an integral part of rice improvement in Asia. In breeding for resistance to bacterial blight, as with other diseases, two independent variables are important, the host (rice cultivars) and the pathogen (pv. *oryzae*). Many rice cultivars were identified that were resistant in one country but not in another (1, 2, 3, 7, 10, 11). Whether this was due to a pathogenic specialization of pv. *oryzae* was not certain until fairly recently. Controversial results were reported previously, in tropical Asia. Some studies indicated, the variation pattern in virulence was continuous (2, 9, 10). Others showed a vertical rice-bacterium relationship (3, 7, 12). Our research in the past 5 years has shown that the bacteria may have

specialization in pathogenicity on rice cultivars that differ in major genes for resistance (7). Similar findings were observed in Japan after a resistant rice cultivar, Asakase, became susceptible to bacterial blight in a farmer's field (6).

This study was an attempt to further elucidate the differential response of the resistance of rice cultivars to virulence of the bacterial isolates.

Materials and Methods

Isolates of pv. *oryzae* and Reaction of Rice Differentials

Twenty-five isolates collected at IRRI in 1980 were used to test their variation on four rice cultivars differing in resistance-susceptibility. Except for Cas 209, cultivars IR8, IR20, and IR1545-339 (hereafter referred to as IR1545) were used throughout the study. IR8, which has no functional gene known for bacterial blight resistance in the Philippines, was susceptible to isolates of all race groups (7). IR20, which is homozygous for *Xa-4*, was resistant to race 1 but susceptible to race 2 while IR 1545-339, which is homozygous for *Xa-5*, was resistant to both races 1 and 2. Cas 209, a new differential cultivar, was susceptible to race 1 but resistant to race 2.

To further characterize the virulence, five isolates of two groups were selected. Random selection of five isolates of each group was based on a lesion distribution previously studied (10). Therefore, PX061, PX052, PX084, PX085, and PX080 of group 1 and PX063, PX079, PX082, PX087, and PX088 of group 2 were selected as a result of lesion length induced on IR8 for group 1 and IR8 and IR20 for group 2. PX082 was not included in the test involving plant ages.

The growth rate of these isolates in peptone sucrose broth at 28 to 30°C was not different by turbidity reading on the Coleman Nepho-colorimeter from 1 to 4 days after incubation.

Growth of the Plants

Seed of each cultivar were pregerminated for 4 days in petri dishes in a seed-germinator maintained at 30°C and 90% R.H. Three pregerminated seeds of each cultivar were transplanted in the greenhouse in a 15-cm diameter clay pot filled with sieved soil fertilized with a rate of 90-60-60 of N-P-K fertilizer. Additional ammonium sulfate was applied at 30 kg N/ha. 1 week before inoculation.

Forty-day-old plants were usually used. For the experiment involving different ages, seeds were sown 3 times at 10-day-intervals so that inoculation could be done at the same time.

Inoculum Preparation and Inoculation

The stock culture of each isolate was maintained on slants of peptone sucrose agar (PSA) at -10°C. Reisolation from artificially infected IR8 leaves was done to preserve the virulence of the isolates. Inoculum was prepared by culturing the isolates of PSA medium for 72 hours. The growths were each suspended in 10 ml of distilled water and shaken vigorously in a mixer. The density of the resulting suspension was

determined in a Coleman Nepho-colorimeter with a 590 nm filter. The original concentration had an absorbance reading of 1.0 (ca. 10^8 cells/ml). Serial dilution was then prepared from the initial concentration to make 10^6 cells/ml.

Inoculation was done by clipping the leaves 1 to 2 cm from the tips of the differentials.

Experimental Design and Disease Measurement

All experiments were in a greenhouse. The split-plot design for two factors and split-split plot design for more than two factors were used. The treatments were replicated 3 times.

Inoculated leaves were observed daily for symptom development. Disease reactions were assessed visually at 2 weeks after inoculation using the Standard Evaluation System (SES) (5) for rice on percent leaf area affected.

Results

Differential Response of Rice Cultivars to Bacterial Isolates

When 25 isolates of pv. *oryzae* from IRRI were tested for their virulence, the 4 rice cultivars responded differentially to them (Fig. 1). Distinct differential response was observed at 14 and 21 DAI; the difference of the latter was not significant from 14 DAI. When IR8 and IR20 were compared at 14 DAI, 24 of the 25 isolates were virulent to IR8 and two race groups of the bacterial isolates were noted to infect IR20; one caused a disease score (SES) of more than 7 and the other less than 3. Similar comparisons were made between IR20 and IR1545, IR20 and Cas 209, and IR1545 and Cas 209. There appeared to be only one isolate (BB903) that was virulent to IR1545.

Interactions between the four rice cultivars and the representative isolates are shown in Table 1. Isolate BB903 was virulent to all the cultivars, BB909 to IR8 and Cas 209, and BB904 to IR8 and IR20.

Comparison of Selected Isolates From Race 1 and Race 2 on Infection of IR8, IR20, and IR1545.

Variability of the isolates on lesion production was shown in the above data. Random selection of 5 isolates from race 1 and 4 from race 2 were further compared for their virulence.

Inoculum Density. On IR8, the ranking order of the isolates on lesion length was not consistent to any isolate (Table 2). At a high inoculum density (ca. 10^8 cells/ml), PX088 of race 2 was the top ranking isolate while at a lower inoculum density, PX061 of race 1 had the highest ranking. The ranking order on both inoculum densities was not significant except for PX080 of race 1, which had consistently shown the lowest ranking order.

The rank against IR20, however, showed that the isolates in race 2 had a consistently higher ranking order than those in race 1 at both inoculum levels (Table 2). IR20 was resistant to all isolates of race 1. The rank of the isolates of the two races was inconsistent at the two inoculum levels. There was no significant difference among the ranking orders of the isolates in either race 1 or race 2.
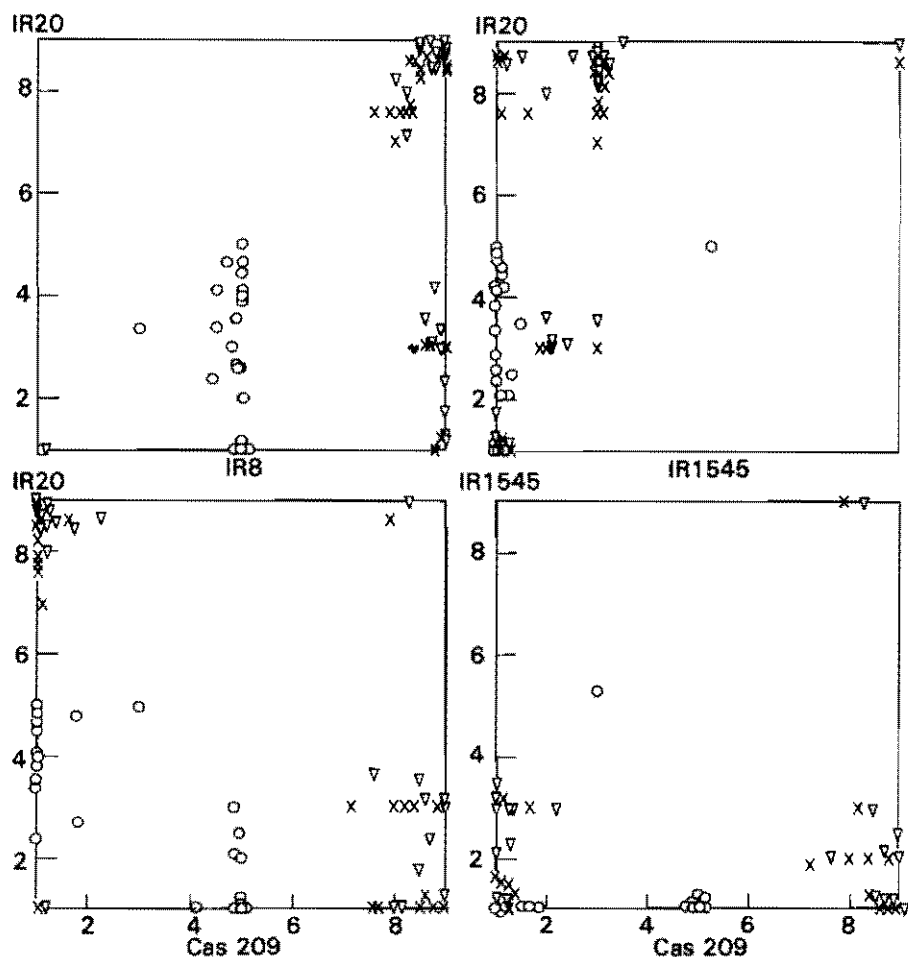
Fig. 1. Comparisons of four rice cultivars' responses to isolates of *Xanthomonas campestris* pv. *oryzae* on a disease scale from 1 to 9 on leaf area affected. 0 = 7 days after inoculation; X = 14 days; and ▽ = 21 days.

Table 1. Response of rice cultivars that have different resistance to selected isolates of *Xanthomonas campestris* pv. *oryzae* at IRRI[1] farm in 1980.

Cultivar	Bacterial blight scores								
	BB 903			BB 909			BB 904		
	7 DI	14 DI	21 DI	7 DI	14 DI	21 DI	7 DI	14 DI	21 DI
IR 8	5.0	9.0	9.0	4.9	9.0	9.0	5.0	8.7	9.0
IR20	5.0	8.6	9.0	2.1	3.0	3.0	5.0	8.6	8.7
Cas 209	3.0	7.9	8.3	5.0	8.4	9.0	1.0	1.0	1.0
IR1545	5.3	9.0	9.0	1.1	2.0	2.0	1.0	1.0	1.7

[1] The International Rice Research Institute at Los Baños, Laguna, Philippines.

[2] Based on the Standard Evaluation Systems for Rice (IRRI). 1 = less than 1°/o leaf area affected, and 9 = over 50°/o leaf area affected.

[3] DI = days after inoculation.

When the isolates were evaluated on IR1545, which is resistant to isolates of both races, variable ranking of the isolates was noted (Table 2). Although there was a significant difference in the ranking sequence of isolates, the rank was not consistent to any specific isolate and the reaction was nevertheless resistant.

Plant Age. When IR8 plants were 45 days old, PX080 of race 1 was observed to be in the lowest ranking sequence and became significantly different from other strains of the two races in 55-day-old plants (Table 3). A similar trend was observed on 35-day-old IR8 plants, except with PX080. On IR20, significant difference between race 1 and race 2 isolates was noted on all three ages of the rice plants (Table 3). IR1545, however, responded inconsistently to none of the isolates of the two races at the three ages of plant growth as did the ranking order (Table 3).

Discussion

For many years, pathogenic specialization of *Xanthomonas campestris* pv. *oryzae* has not been well understood. Controversial results were reported on whether there was such a phenomenon of the rice bacterial blight pathogen in tropical Asia. Available information has suggested that variation in virulence from one country to another, and from one locality to another in the same country, was based on the test of some rice cultivar (2, 10, 11, 12). Early, Mew and Vera Cruz (6) showed a weak interaction between rice cultivars and the bacteria. Later, a strong interaction was demonstrated when Cas 209 was identified (7).

A comprehensive study compared the two sets of rice differential cultivars developed independently in Japan and at IRRI in response to the bacterial isolates from Japan and the Philippines (4). Vertical virulence of the bacterial isolates was shown. As each of the cultivars has major gene(s)

Table 2. Ranking of isolates of the two race groups of *Xanthomonas campestris* pv. *oryzae* according to lesion length on three cultivars

Cultivar	Inoculum dosage					
	10^8 cfu/ml			10^6 cfu/ml		
	Isolate[b]	Rank[c]	Lesion(cm)	Isolate[b]	Rank[c]	Lesion(cm)
	PX088 (2)	1 a	26.8	PX061 (1)	1 a	22.8
	PX087 (2)	2 a	25.6	PX087 (2)	2 a	21.7
	PX082 (2)	3 a	24.8	PX085 (1)	3 a	21.4
	PX084 (1)	4 a	24.6	PX082 (2)	4 a	21.1
IR8	PX061 (1)	5 a	23.8	PX052 (1)	5 a	20.9
	PX079 (2)	6 a	23.9	PX084 (1)	6 a	19.9
	PX085 (1)	7 a	23.5	PX079 (2)	7 a	19.2
	PX052 (1)	8 a	22.1	PX088 (2)	8 a	19.3
	PX063 (2)	9 a	22.1	PX063 (2)	9 a	17.3
	PX080 (1)	10 b	15.1	PX080 (1)	10 b	0.0
	PX088 (2)	1 a	22.5	PX088 (2)	1 a	18.6
	PX082 (2)	2 a	22.4	PX082 (2)	2 a	16.2
	PX087 (2)	3 a	21.2	PX079 (2)	3 a	16.0
	PX079 (2)	4 a	20.0	PX063 (2)	4 a	15.9
IR20	PX063 (2)	5 a	18.0	PX087 (2)	5 a	15.5
	PX080 (1)	6 b	4.0	PX061 (1)	6 b	3.2
	PX084 (1)	7 b	3.4	PX052 (1)	7 b	2.9
	PX085 (1)	8 b	3.3	PX085 (1)	8 b	3.0
	PX061 (1)	9 b	3.2	PX084 (1)	9 b	2.5
	PX052 (1)	10 b	2.9	PX080 (1)	10 b	2.1
	PX082 (2)	1 a	3.6	PX061 (1)	1 a	2.4
	PX061 (1)	2 a	3.6	PX079 (2)	2 a	2.2
	PX088 (2)	3 ab	3.0	PX088 (2)	3 a	2.2
	PX080 (1)	4 ab	3.0	PX087 (2)	4 a	2.1
IR1545	PX052 (1)	5 ab	3.0	PX082 (2)	5 a	2.0
	PX079 (2)	6 ab	2.6	PX052 (1)	6 a	1.9
	PX063 (2)	7 ab	2.5	PX063 (2)	7 ab	1.7
	PX087 (2)	8 ab	2.5	PX080 (1)	8 ab	1.6
	PX084 (1)	9 ab	2.3	PX085 (1)	9 ab	1.4
	PX085 (1)	10 b	1.7	PX084 (1)	10 b	0.4

[a] IR8, susceptible to both groups of isolates. IR20, susceptible to group 2 but resistant to group 1, and IR1545, resistant to both

[b] Figure in parenthesis denotes race group

[c] Ranking orders followed by a common letter are not significantly different at the 5% level by the Duncan multiple range test.

different from the others for vertical resistance, the results appear to indicate a gene-for-gene relationship of the resistance in rice and of the virulence in the bacterial pathogen.

Our results further confirmed that there is a strong interaction between pv. *oryzae* isolates and the rice cultivars that have different resistance (Table 1). The number of isolates in the present study may be too small to show the actual distribution, yet those that were virulent to IR20 were slightly dominant over other isolates. Only one isolate was virulent to all cultivars (Fig. 1).

Table 3. Effect of plant age on ranking the isolates in two race groups of *Xanthomonas campestris* pv. *oryzae* according to lesion caused by the infection.

Cultivar		Plant Age							
	35 DS[a]			45 DS			55 DS		
	Isolate[b]	Rank[c]	Lesion (cm)	Isolate[b]	Rank[c]	Lesion (cm)	Isolate[b]	Rank[c]	Lesion (cm)
	PX052 (1)	1 a	23.5	PX085 (1)	1 a	27.9	PX084 (1)	1 a	26.4
	PX084 (1)	2 ab	22.9	PX088 (2)	2 a	27.7	PX061 (1)	2 a	26.1
	PX063 (2)	3 abc	22.4	PX079 (2)	3 a	27.4	PX085 (1)	3 a	24.6
	PX085 (1)	4 abc	21.0	PX084 (1)	4 a	27.3	PX052 (1)	4 a	23.8
IR8	PX088 (2)	5 abc	21.0	PX061 (1)	5 a	26.6	PX063 (2)	5 a	23.4
	PX061 (1)	6 abc	20.0	PX052 (1)	6 a	26.0	PX079 (2)	6 a	23.4
	PX080 (1)	7 bc	20.0	PX063 (2)	7 ab	23.4	PX088 (2)	7 a	23.3
	PX079 (2)	8 bc	19.4	PX087 (2)	8 b	21.8	PX087 (2)	8 b	14.9
	PX087 (2)	9 c	18.5	PX080 (1)	9 b	20.0	PX080 (1)	9 b	14.3
	PX088 (2)	1 a	17.4	PX079 (2)	1 a	15.6	PX079 (2)	1 a	18.7
	PX079 (2)	2 a	16.5	PX088 (2)	2 a	14.8	PX088 (2)	2 a	18.6
	PX063 (2)	3 a	16.2	PX063 (2)	3 a	12.9	PX063 (2)	3 a	15.6
	PX087 (2)	4 a	15.1	PX087 (2)	4 a	12.4	PX087 (2)	4 a	10.3
IR20	PX061 (1)	5 b	4.3	PX061 (1)	5 b	5.3	PX061 (1)	5 b	5.4
	PX085 (1)	6 b	4.0	PX084 (1)	6 bc	4.3	PX085 (1)	6 bc	4.2

Table 3, continued

Cultivar	Plant Age								
	35 DS[a]			45 DS			55 DS		
	Isolate[b]	Rank[c]	Lesion (cm)	Isolate[b]	Rank[c]	Lesion (cm)	Isolate[b]	Rank[c]	Lesion (cm)
	PX084 (1)	7 b	3.8	PX080 (1)	7 bc	3.9	PX084 (1)	7 c	3.9
	PX052 (1)	8 b	3.8	PX052 (1)	8 c	3.5	PX052 (1)	8 c	3.5
	PX080 (1)	9 b	3.2	PX085 (1)	9 c	3.4	PX080 (1)	9 c	3.9
	PX061 (1)	1 a	3.4	PX088 (2)	1 a	3.5	PX063 (2)	1 a	3.5
	PX085 (1)	2 ab	3.2	PX080 (1)	2 a	3.4	PX080 (1)	2 a	3.2
	PX084 (1)	3 ab	2.9	PX063 (2)	3 a	3.2	PX084 (1)	3 a	3.1
	PX079 (2)	4 ab	2.9	PX079 (2)	4 a	3.2	PX088 (2)	4 a	3.0
IR1545	PX080 (1)	5 abc	2.8	PX061 (1)	5 ab	3.1	PX061 (1)	5 a	3.0
	PX063 (2)	6 abc	2.6	PX084 (1)	6 abc	2.8	PX079 (2)	6 a	2.9
	PX088 (2)	7 abc	2.5	PX085 (1)	7 bc	2.3	PX052 (1)	7 a	2.8
	PX087 (2)	8 bc	2.2	PX052 (1)	8 c	2.1	PX087 (2)	8 a	2.5
	PX052 (1)	9 c	2.0	PX087 (2)	9 c	2.1	PX085 (1)	9 b	2.7

[a]Days after sowing. [b]Figure in parenthesis denotes race group. [c]Ranking order followed by a common letter are not significantly different at the 5% level by the Duncan multiple range test.

The specificity of the bacterial isolates to infect compatible cultivars, although it was not clearcut as to presence-or-absence of the lesions, was nevertheless demonstrated. The differential response of the rice cultivars was not influenced by plant age and inoculum density at 14 DAI. The pathogenic specialization of pv. *oryzae* on rice cultivars that have different major genes for resistance is therefore further confirmed.

Literature Cited

1. Aldrick, S. J., I. W. Buddenhagen, and A. P. K. Reddy. 1973. The occurrence of bacterial blight in wild and cultivated rice in Northern Australia. Aust. J. Agric. Res. 24:219-227.
2. Buddenhagen, I. W., and A. P. K. Reddy. 1972. The host, the enviroment, *Xanthomonas oryzae*, and the researcher. P. 289-295, in Rice Breeding. The International Rice Research Institute, Los Baños. Philippines. 738 p.
3. Ezuka, A., and O. Horino. 1974. Classification of rice varieties and *Xanthomonas oryzae* strains on the basis of their differential interactions. Bull. Tokai-kinki Natl. Agric. Exp. Stn. 27:1-19.
4. Horino, O., T. W. Mew, G. S. Khush, and A. Ezuka. 1980. Resistance of Japanese and IRRI differential rice varieties to pathotypes of *Xanthomonas oryzae* in the Philippines and Japan. IRPS 53. 1-11.
5. International Rice Research Institute. 1980. Standard evaluation system for rice. 44 pp. IRRI, Los Baños, Laguna, Philippines.
6. Kuhara, S., T. Kurita, Y. Tagami, H. Fujii, and N. Sekiya. 1965. Studies on the strains of *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson, the pathogen of the bacterial leaf blight of rice, with special reference to its pathogenicity and phage-sensitivity. Bull. Kyushu Agric. Exp. Stn. 11:263-312.
7. Mew, T. W., and C. M. Vera Cruz. 1979. Variability of *Xanthomonas oryzae:* Specificity in infection of rice differentials. Phytopathology 60:152-155.
8. Mew, T. W., C. M. Vera Cruz, and R. C. Reyes. 1981. Interaction of *Xanthomonas campestris* pv. *oryzae* races to a resistant rice, Cas 209. Phytopathology (in press).
9. Mew, T. W., C. M. Vera Cruz and M. Machmud. 1981. Variation in virulence of *Xanthomonas campestris* pv. *oryzae* races causing bacterial blight of rice. Ann. Phytopath. Soc. Japan (in press).
10. Ou, S. H., F. L. Nuque, and J. P. Silva. 1971. Pathogenic variation among isolates of *Xanthomonas oryzae* of the Philippines. Plant Dis. Rep. 55:22-26.
11. Rao, Y. P., S. K. Mohan and O. R. Reddy. 1971. Pathogenic variability in *Xanthomonas oryzae*. Plant Dis. Rep. 55:593-595.
12. Reddy, O. R., and S. H. Ou. 1976. Pathogenic variability in *Xanthomonas oryzae*. Phytopathology 66:906-909.

Pathotypes of *Xanthomonas campestris* **pv.** *graminis*

T. Egli
CIBA-GEIGY Ltd.
Basil, Switzerland

D. Schmidt
Swiss Federal Research Station for Agronomy
Nyon, Switzerland

Bacterial wilt is a tracheobacteriosis of forage grasses that was first investigated and described in the mid 1970s (1). The disease is widespread in Europe, and has also been observed in New Zealand (5, 6). The pathogen, for which the designation *Xanthomonas campestris* pv. *graminis* has been proposed (7), has been shown to be differentiated into various *formae speciales*. To date, four such *formae speciales* - or pathotypes - have been found which can be clearly differentiated by their host-specific pathogenicity.

The *Lolium* pathotype, which occurs widely in nature, has a broad spectrum of host plants. These include the genera *Lolium, Festuca, Dactylis,* and *Trisetum,* from which the pathogen has repeatedly been isolated. It is possible that the host spectrum is even wider, since certain species of *Phleum, Poa, Deschampsia, Phalaris,* and *Alopecurus* can also be infected artificially with bacteria of this pathotype (3).

The host plant spectrum is more narrow for the three other pathotypes of *X. campestris* pv. *graminis,* namely the *Phleum, Poa,* and *Arrhenatherum* pathotypes. In principle, the host spectrum covers only the grass genus from which the bacterium has been isolated. The *Phleum* genus appears to be in general very susceptible to the *Phleum* pathotype; all the *Phleum* species tested were found to be highly sensitive to isolates of *Phleum pratense.* In the case of *Poa,* there are considerable differences at the species level regarding susceptibility to the *Poa* pathotype (isolates from *Poa trivialis*). For the *Arrhenatherum* pathotype (isolates from *Arrhenatherum elatius*), species-relevant differentiation has not yet been clarified since so far it has been possible to conduct infection tests with only one species (*A. elatius*).

After several host passages, it was not possible to see any adaptation of the bacteria to grasses outside their own true host spectrum. This suggests that the host specificity of the pathotypes of *X. campestris* pv. *graminis* is a stable characteristic.

It was observed also that all strains of the *Lolium* pathotype showed the same pathogenicity pattern independently of the host species from which they had been isolated and independently of their geographic origin.

In principle, bacterial plant diseases can be controlled in various ways (2). In the case of bacterial wilt of forage grasses breeding for resistance is undoubtedly the control method of choice (4). The findings reported herein on the pathogenic variability of *X. campestris* pv. *graminis* may be useful for breeding programs designed to develop resistance against bacterial wilt in forage grass varieties.

Literature Cited

1. Egli, T., M. Goto, and D. Schmidt. 1975: Bacterial wilt, a new forage grass disease. Phytopathol. Z., 82:111-1 1

2. Egli, T., and E. Sturm. 1981. Bacterial plant diseases and their control. In'Chemie der Pflanzenschutz und Schädlings bekampfungsmittel'Vol, , 6, pp. 345-388, Ed. R. Wegler (Springer-Verlag: Berlin, Heidelberg, New York).

3. Herzog, J., 1978. Phytopathologische Untersuchungen an einer Bakteriose von Futtergrasern. Diploma Thesis. ETH, Zürich (unpublished).

4. Schmidt, D., and B. Nüesch. 1980. Resistance to bacterial wilt (*Xanthomonas graminis*) increases yield and persistence of *Lolium multiflorum*. EPPO Bull. 10 (3), 335-339.

5. Van den Mooter, M., J. Swings, M. de Cleene, P. Leyns and J. de Ley. 1981. *Xanthomonas campestris* pv. *graminis* from forage grasses in Belgium. Parasitica (in press).

6. Wilkins, P. W., J. K. Exley. 1977. Bacterial wilt of ryegrass in Britain. Plant Pathol. 26, 99.

7. Young, J. M., D. W. Dye, J. F. Bradbury, C. G. Panagopoulos, and C. F. Robbs. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N. Z. J. of Agric. Res., 21:153-177.

Pathogenicity of Three Strains of Citrus Canker Organism on Grapefruit

R. E. Stall, J. W. Miller, G. M. Marco,
B. I. Canteros de Echenique

University of Florida, Gainesville, FL
Dept. Agriculture and Conservation Serv., Gainesville, FL
I.N.T.A., Corrientes, Argentina

Abstract

Three strains of *Xanthomonas campestris* pv. *citri* have been identified in South America. The three strains (A, B, and C) were distinguished pathogenically by infiltration of 14-day-old leaves of grapefruit seedlings with inocula of 10^3-10^4 cells ml-^{1}. The A strain was the most aggressive. With equal colony forming units in inocula, 6 times more lesions occurred with the A strain than the B strain. The B strain bacteria were much more difficult to isolate than those of the A strain. The C strain did not cause lesions in grapefruit leaves, but did cause small collapsed areas, typically of a hypersensitive reaction. Identification of the strains is important in citrus canker eradication programs.

Introduction

Citrus canker is caused by the bacterium, *Xanthomonas campestris* pv. *citri* (Hasse) Young *et al.* and is one of the most feared diseases of citrus (7). The causal bacterium was first described by Clara Hasse (9) after canker was brought to the United States in 1911 (4). Early workers (7) could not control canker copper sprays, and consequently, eradication of *X. camp.* pv. *citri* was advocated. Eradication was executed successfully in the United States, South Africa, Australia, and New Zealand (3, 4, 6).

Eradication of citrus canker was attempted in Sao Paulo Province, Brazil after it was introduced there in 1957 (17). Other outbreaks have occurred there since the first one. The organism has become endemic in some other provinces of Brazil and in Paraguay. Citrus canker is presently epidemic in Argentina. The bacterium causing this infestation is aggressive on grapefruit and certain sweet orange trees and is thought to be the same strain of the pathogen that occurred in the Unites States. The organism was thought to originate in Asia and is called the Asiatic, or A strain.

Another form of citrus canker existed in South America many years prior to 1957. It is restricted to Argentina, Paraguay, and Uruguay. Although most citrus types are susceptible to the causal strain, the bacterium is thought to be less aggressive than the A strain. In the field the disease is

only a problem on lemon and lime trees. The pathogen of this disease is called the B strain of *X. camp.* pv. *citri* (5).

A third form of citrus canker was reported in Brazil by Nemakata (14). The bacterium of this form is pathogenic primarily on Mexican lime. Based on physiological, serological, and pathological differences from the A strain, Nemakata and de Oliveira (15) suggested the bacterium be named *X. citri* f. sp. *aurantifolia*. It is commonly called the C strain.

Differentiation of these strains in the field is nearly impossible because symptoms are nearly identical and host ranges overlap. Confusion associated with identification of the strains has caused much uncertainty of the need for eradication campaigns to stop the spread of the aggressive form of citrus canker. The purpose of this paper is to report pathogenic differences of the three strains on grapefruit seedlings. The results may be useful in identification of the strains.

Materials and Methods

Isolation

Many attempts to isolate *X. camp.* pv. *citri* were made with lesions from grapefruit (A strain) and lemon (B strain). The isolation procedure was to crush a lesion in 1 ml of sterile tap water and to make 3 serial 10-fold dilutions. From each dilution 0.05 ml was transferred to nutrient agar plates. The liquid was spread over the plate with a sterile glass rod. The puncture method of Goth (8) also was used for some isolations. Plates were incubated at 28° C.

Source of Isolates

A culture (B-43) from a grapefruit tree in Corrientes Province in Argentina was selected as representative of the A strain. A culture (XC-5) of the B strain was obtained from a lemon tree in Entre Rios Province in Argentina. This culture was of the large colony type. A lyophilized culture of *X. citri* f. sp. *aurantifolia* (XC-512) was obtained from Dr. Victoria Rossetti, Biological Institute, Sao Paulo, Brazil and was used as the C. strain. All bacteria were maintained in nutrient broth at 4°C.

Host Plants

Seedlings of Duncan grapefruit were used as host plants. These seedlings were growing in pots 12 cm in diameter and were from 30 to 45 cm tall at inoculation. The plants were kept before and after inoculation in a growth room with artificial lighting and a temperature of 28°C.

Inoculations

Bacteria were cultured on nutrient agar plates for 24 h and then washed from the surface and suspended in sterile tap water. Each suspension was adjusted to an OD of 0.3 at 600 nm with spectrophotometer. The density of bacteria was determined to be about 5×10^8 cells ml-[1]. Lower concentrations were prepared by appropriate dilutions. Half-leaves were inoculated by infiltration of inoculum into the mesophyll by the method of Klement *et al.* (10).

Data

Quantitative levels of pathogenicity were estimated from the number of lesions that developed per cm² of leaf. Lesions were counted about 4 weeks after inoculation and the area of leaf inoculated was determined by the dot method (12).

Results

Isolations

The A strain was isolated consistently from leaves of grapefruit and other citrus types, including lemon. Yellow colonies of *X. camp.* pv. *citri* were easily distinguished from saprophytic bacteria within 3 to 5 days after placing bacterial suspensions on nutrient agar plates.

The B strain was not easily isolated on nutrient agar. No colonies were obtained from over 75 isolations attempted from field lesions. Saprophytic bacteria were abundant on the plates. Suspensions of some crushed lesions were diluted 1 per 100 and infiltrated into young grapefruit leaves. Numerous lesions always developed which was evidence that many viable cells existed in suspensions from the lesions.

Isolations were subsequently made from the grapefruit leaves inoculated with the B strain. Competition from saprophytic bacteria was minimal in these isolations. Numerous small colonies of bacteria appeared on the plates 10-14 days after suspensions were placed on them. A few large colonies also developed and appeared to be *Xanthomonas* (Fig. 1). The large colonies grew rapidly when subcultured on nutrient agar. These large colonies were pathogenic and one of them (XC-5) was used in subsequent comparisons of aggressiveness of A and B strains.

Isolations of the C strain were not possible because diseased tissue was not available.

Comparison of Strains A and B

Five grapefruit seedlings which had 4 flushes of growth were selected when apical buds were just beginning to open. Fourteen days later, one-half of the oldest leaf of each flush, separated by the midvein, was infiltrated with inocula consisting of 6.0×10^3 cells ml-¹ of B-43 (A-strain). The other half of each leaf was infiltrated with inocula of 6.3×10^3 cells ml-¹ of XC-5 (B-strain).

The number of lesions that developed with B-43 was greater than the number with XC-5 (Table 1). The number of lesions in the youngest leaf inoculated with strain A was 6 times more than the number that developed with the B strain. No lesions formed in old leaves inoculated with strain B, but a few lesions did form in old leaves inoculated with strain A. The number of lesions was inversely correlated with age of leaves.

Comparison of Strains A and C

Two-week-old leaves of grapefruit seedlings were inoculated with B-43 (strain A), or XC-512 (strain C). Suspensions of both bacteria were about 10^4 cells ml-¹. Typical lesions of canker formed in leaves inoculated with the A strain about 7 days after inoculation, but no lesions formed in leaves
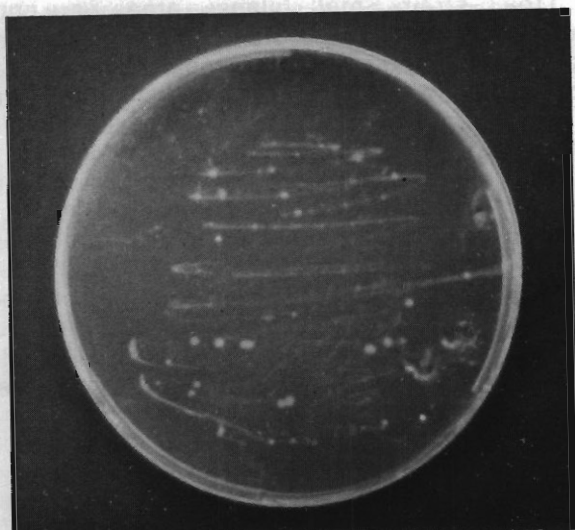
Fig. 1. Illustration of growth of *Xanthomonas citri* strain B on plates of nutrient agar. Numerous small colonies form a streak over plates with a few large colonies distributed among the slow-growing small colonies.

Table 1. The number of lesions per cm^2 in grapefruit leaves of different ages after inoculation with strain A and B of *Xanthomonas camp.* pv. *citri* .

Flush no.	Pathogenic strains	
	A	B
	Lesions/cm^2	Lesions/cm^2
5[a]	17.5[b]	2.9
4	6.8	0.0
3	2.4	0.0
2	2.4	0.0
1	1.0	0.0

[a] Leaves of the fifth flush were the youngest.

[b] Mean of five leaves.

inoculated with the C-strain. Small collapsed areas were noted with a 10X hand lens in leaves inoculated with XC-512. The number of collapsed areas was about the same as the number of lesions in leaves inoculated with B-43. The collapsed areas were thought to be the result of a hypersensitive reaction (HR) (10). The culture of the C strain caused typical lesions of canker on seedlings of Mexican lime and Sour orange.

Leaves of grapefruit seedlings were inoculated with high numbers of bacteria of B-43, XC-5, and XC-512 to test for HR. In addition, a suspension of *X. camp.* pv. *vesicatoria* from tomato was injected into grapefruit leaves as an HR check. The suspensions of all bacteria were adjusted to about 5×10^8 cells ml^{-1}. Confluent necrosis of leaf tissues inoculated with the C strain and *X. camp.* pv. *vesicatoria* began 2 days after inoculation and complete necrosis occurred by 4 days. This was considered evidence for HR. Necrosis in grapefruit leaves did not begin until seven days after inoculation with the A and B strains.

Discussion

Identification of strains of *Xanthomonas campestris* pv. *citri* is essential for citrus producers in South America. Strain B is not a serious problem and is controlled with copper sprays (5). Therefore, eradication of trees infested with strain B is not necessary. Strain C is serious only on Mexican lime, and there is no fear of spread to other citrus types. However, the A strain is very aggressive and has a wide host range among citrus types. Control programs for the A strain on susceptible citrus trees have not been determined at this time. Therefore, removal of trees infested with the A strain is still being practiced. Before removal of trees, citrus producers want an accurate identification of the strain of *X. camp.* pv. *citri* that is present.

Nemakata and de Oliveira (15) reported serological differences between strains A and C. Serological tests have been used in Brazil to identify strains. Serological differences between A and B strains have also been reported (13). Serological determinations of strains, however, must be made with reservations. Serotypes are not always related to pathotypes in studies involving large numbers of isolates (1, 11, 16).

The difference in pathogenicity between A and B in our test was quantitative and is consistent with observations of the diseases in the field. However, the differences between other isolates of A and B may not be the same as found with the two isolates used in this work. The culture of the B strain used here may not be typical of cells of the B strain in the field. The isolate grew well on nutrient agar in contrast to a predominance of cells from nature that did not grow well on nutrient agar. A comparison of the natural populations of the A and B strains must await successful culture of all cells of the B strain. Culturing is necessary to determine inoculum levels.

Identification of the B strain possibly can be made by testing for growth on nutrient agar. This method was used in a survey of strains of canker of lemon in Corrientes Province, Argentina, where the A strain has become endemic and in Entre Rios Province, Argentina, where that A strain is limited in distribution. *Xanthomonas camp.* pv. *citri* was isolated on

nutrient agar from all samples of lemon from Corrientes and only 2 of 55 samples of lemon canker from Entre Rios. It was concluded that cancrosis of lemon was caused by the A strain in Corrientes and by the B strain in Entre Rios. The A strain predominated over the B strain when introduced into an area.

The difference in pathogenicity between the A and C strains was qualitative. The qualitative difference was great and easy to determine. With qualitative differences in pathogenicity, however, mutants for change of strain occur in high numbers. For example, change from tomato to pepper strains of *X. camp.* pv. *vesicatoria* occurred at 4×10^{-4} mutants per cell per division. The difference between those strains also was qualitative. Mutants for change of strain would best be detected by pathogenicity tests.

Literature Cited

1. Charudattan, R., R. E. Stall, and D. L. Batchelor. 1973. Serotypes of *Xanthomonas vesicatoria* unrelated to its pathotypes. Phytopathology 63:1260-1265.
2. Dahlbeck, D., and R. E. Stall. 1974. Mutations for change race in cultures of *Xanthomonas vesicatoria.* Phytopathology 69:634-636.
3. Doidge, E. M., 1929. Further studies on citrus canker. S. Afr. Agr. Dept. B. 51:1-31.
4. Dopson, R. N., 1964. The eradication of citrus canker. Plant Dis. Reptr. 48:30-31.
5. DuCharme, E. P., 1951. Cancrosis B of lemons. Citrus Magazine 13:18-20.
6. Dye, D. W., 1969. Eradicating citrus canker from New Zealand. N. Z. J. Agr. 118:20-21.
7. Fawcett, H. S., 1936. Citrus diseases and their control. McGraw Hill Book Company, Inc., New York. 656 p.
8. Goth, R. W., 1965. Puncture method for isolating bacterial blight of bean. Phytopathology 55:930-931.
9. Hasse, C. H., 1915. *Pseudomonas citri* the cause of citrus canker. J. Agr. Res. 4:97-100.
10. Klement, Z., G. L. Farkas, and L. Lovrekovitch. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in the tobacco leaf. Phytopathology 54:474-477.
11. Lucas, L. T., and R. G. Grogan. 1964. Serological variation and identification of *Pseudomonas lachrymans* and other phytopathogenic *Pseudomonas* nomenspecies. Phytopathology 59:1908-1912.
12. Marshall, J. K., 1968. Methods for leaf area measurement of large and small leaf samples. Phytosynthetica 2:41-47.
13. Messina, M. A., 1976. Los métodos serológicos en el estudio de la bacteria que produce la "cancrosis cítrica" en la Argentina. I. Hallazgo de un nuevo serotipo. Metología empleada en el estudio completo. INTA, Concordia, Argentina, Serie Técnica No. 46, 10 pp.
14. Nemakata, T., 1971. Estudos comparatrios entre *Xanthomonas citri* (Hasse) Dow., agente causal do "cancro citrico" e *Xanthomonas citri* (Hasse) Dow., n. f. sp. *aurantifolia*, agente causal da "cancrose do limoeiro Galego". Tese apresentada a Escola Superior de Agriculturci" Luis de Queiroz." 65 pp.
15. Nemakata, T., and A. R. de Oliveira. 1972. Comparative serological studies between *Xanthomonas citri* and a bacterium causing canker on Mexican

lime. Pages 151-152 in: H. P. Maas Geesteranus, ed. Proc. Third Int. Conf. Plant Path. Bacteria. Centre Agr. Pub. and Doc., Wageningen, The Netherlands. 365 pp.

16. Otta, J. D., and J. English. 1971. Serology and pathology of *Pseudomonas syringae.* Phytopathology 61:443-452.

17. Rossetti, V., C. C. Lassa, and T. Nemakata. 1969. Dez anos de observacoes sobre o cancrosis citrico, no estado de Sao Paulo. O. Biologico 35:45-48.

Variation, Virulence of *Pseudomonas syringae* pv *phaseolicola* on Beans in Colombia

R. A. Buruchara, M. A. Pastor-Corrales

CIAT
Cali, Colombia

Abstract

Thirty isolates of *Pseudomonas syringae pv. phaseolicola* (Burkholder, 1926) Young, Dye, and Wilkie, 1978 (ISPP List 1980), from Colombia were separated into race 1 and 2 on the basis of their pathogenicity for *Phaseolus vulgaris* cv. Red Mexican UI-3. Race determination was based on both leaf and pod reaction. Standard isolates of race 1 and 2 were used for comparison. Twenty Pasto isolates were classified as race 2, whereas 7 isolates from Popayán, 1 from Palmira, and 2 from Tenerife were identified as race 1.

The isolates were also classified in their order of virulence, by the diameter of watersoaking lesions caused on pods during a period of 5 days on cultivars Seminole, G. N. Nebraska #1 Sel 27, and Wisc HBR 72 upon inoculation using a needle. The most virulent isolates were from Pasto. Cultivar Wisc HBR 72 (with high foliar resistance) had a susceptible pod reaction (mean lesion diameter 3.1 mm; dispersion 0.5-5.3 mm) comparable to the susceptible cultivar Seminole (mean lesion diameter 2.8 mm; dispersion 0.5-4.8 mm). G. N. Nebraska #1 Sel 27 had a mean lesion diameter of 1.8 mm and a dispersion between 0.4 and 2.8 mm. The order of the isolates in virulence was similar in all three cultivars and gave a correlation coefficient of 0.87 between the 3 cultivars.

Introduction

Halo blight of beans (*Phaseolus vulgaris* L.) incited by *Pseudomonas syringae* pv. *phaseolicola* (Burkholder, 1926) Young, Dye and Wilkie, 1978 (ISPP List 1980), is one of the bacterial diseases responsible for low bean yields in some of the bean growing regions (4). Occurrence of the disease has been reported in Africa, Australia, Canada, Europe, Latin America, New Zealand and United States (23, 24, 32). In Latin America the disease occurs in bean growing areas with moderate temperatures such as in some regions of Brazil (2), Chile (7), Colombia (31) and Guatemala (31).

Pathogenic variation in *Ps. syringae* pv. *phaseolicola* was for the first time demonstrated by Jensen and Livingstone (12). In 1964, Walker and Patel (29) reported the existence of 2 races (race 1 and race 2) in the United States. Their race distinction was based on bean cultivar 'Red Mexican UI 3' which is resistant to race 1 but susceptible to race 2. Since then, reports

on the occurrence of the two races in different countries have been documented (1, 8, 10, 17, 21, 30). Coyne *et al.* (5) in 1979 reported a more virulent strain than the previously reported race 2 of Walker and Patel (29). However, Schroth *et al.* (19) in 1971, suggested that, there exist many strains of *Ps. syringae* pv. *phaseolicola* with varying degrees of virulence. They also found that isolates of neither race 1 nor race 2 were homogenous with respect to virulence when tested on certain varieties. In 1979, Szarka and Velich (26) also observed that, *Ps. syringae* pv. *phaseolicola* does not consist of two races only but of a series of strains which can be ranged by their increasing pathogenicity.

The aim of this study was to determine the pathogenic variation and virulence of *Ps. syringae* pv. *phaseolicola* collected from selected bean growing regions of Colombia.

Materials and Methods

Sources, Isolation, and Verification of Isolates

Thirty isolates collected from some regions (Palmira, Pasto, Popayán, and Tenerife) of Colombia were used in these studies. In addition, race 1 and 2 isolates received from Dr. D. Hagedorn (University of Wisconsin), isolates HB 16 from Dr. M. L. Schuster (University of Nebraska), and isolate OHB from Dr. S. V. Beer (Cornell University) were included for comparison (Table 1).

To isolate bacteria from the host, lesions and parts bordering them were cut from leaves of naturally infected plants. They were surface sterilized with 0.5% sodium hypochloride for 2 min rinsed twice with sterile distilled water and comminuted in a test tube containing sterile distilled water. The bacteria suspension was streaked on nutrient agar (beef extract, 3 g; peptone (Difco), 3 g; agar (Difco) 15 g; and distilled water, 1000 ml), and incubated at 27°C.

Cultures were purified by a series of single colony transfers and then verified as *Pseudomonas syringae* pv. *phaseolicola* by biochemical and pathogenicity tests (14). These included colony morphology, fluorescent pigment production on King's medium B (13), oxidate test, levan production, catalase reaction, production of hydrogen sulphide, arginine dihydrolase test, and pathogenicity test on susceptible bean cultivar 'Red Kidney'. The isolates were maintained on nutrient agar slants at 4°C and also in lyophilized form.

Seed Source

Seed of all bean cultivars and lines used were obtained from the Bean Pathology and the Genetic Resources Sections of Centro Internacional de Agricultura Tropical (CIAT). Seed increase was made from single plant selection. Plants were grown in the greenhouse in 15 cm pots containing a mixture of soil and sand (5:1) which had been sterilzed previously. Two to three plants were grown per pot.

Inoculation Procedures

In all inoculations, bacterial suspensions were prepared from 48 h old cultures, grown on yeast dextrose calcium carbonate agar (YDC) medium

Table 1. Source, host, and identifications of the *Pseudomonas syringae* pv. *phaseolicola* isolates.

Source	Host	Identification
Popayán (Colombia)	*Phaseolus coccineus*	CBP-177, PC-2, PC-3
		PC-4, PC-5, PC-6
Popayán (Colombia)	*P. vulgaris*	CBP-178
Tenerife (Colombia)	*P. vulgaris*	CBP-172, CBP-173
Palmira (Colombia)	*P. vulgaris*	CBP-176
Pasto (Colombia	*P. vulgaris*	PPP-1, PPP-2, PPP-3
		PPP-4, PPP-5, PPP-6
		PPP-7, PPP-8, PPP-11
		PPP-12, PPP-13, PPP-14
		PPP-15, PPP-16, PPP-17
		PPP-18, PPP-19, PPP-20
		PPP-21, PPP-22
Dr. Hagedorn (Univ. of Wisc. USA)	*P. vulgaris*	CBP-196 (Race 1), CBP-197 (Race 2)
Dr. Schuster (Univ. of Nebraska, USA)	*P. vulgaris*	HB-16
Dr. S.V. Beer (Cornell Univ. USA)	*P. vulgaris*	CBP-198 (OHB)

(yeast extract (Difco), 10 g; dextrose (Difco), 20 g; $CaCo_3$, 3.5 g; agar (Difco), 20 g; and distilled water, 1000 ml). The suspension was then adjusted turbidimetrically using a spectronic 20 calorimeter (Bausch and Lomb Co.) to a concentration of 5×10^7 colony forming units (CFU) per milliliters.

Leaf reaction of bean cultivars was determined by using the water-soaking method as described by Schuster (20). The abaxial surface of either young unifoliate leaves or half-expanded first trifoliate leaves was sprayed with the bacterial suspension using a de Vilbiss atomizer attached to a compressed airline at 15 psi, until water-soaking appeared. Plants were kept in the cool part of the greenhouse where temperatures averaged 22°C.

Pod reaction was determined using the needle inoculation method (11, 18, 25, 28). Young growing green pods were used. A sterile needle was dipped into the bacterial suspension and then inserted at different points (3-5) along the pod's legth. The latter were placed in 250 ml Erlenmeyer flasks containing a small amount of distilled water and loosely plugged at the mouth with cotton wool to create a humid condition. The contents were left at room temperature (22 to 25°C) for 5 days. The diameter of water-soaking reaction around the point of inoculation was measured with the use of a stereoscope. Resistance was defined as the appearance of necrotic

spot at the point of inoculation. Fifteen to 30 readings were made for each isolate per cultivar. The experiment was repeated twice.

Seed inoculation was made by partial vacuum using a modification of Goth's method (9). Seeds in muslin bags were submerged in a bacterial suspension in a glass dessicator connected to a vacuum suction pump. They were then exposed to a partial vacuum of 415 mm of mercury for 5 minutes, after which, the negative pressure was released suddenly. The seeds were air-dried at room temperature for 3 days and then planted and grown in the growth chamber where temperature was maintained at 20°C.

Results

Biochemical Tests

On the basis of biochemical and pathogenicity tests, the isolates collected and isolated, were identified as *Pseudomonas syringae* pv. *phaseolicola*. On King's medium B, the cultures produced a diffusible fluorescent pigment, they caused levan formation on nutrient agar containing 5% (W/V) sucrose, gave positive catalase test, negative oxidase test, negative arginine dihydrolase test and did not produce hydrogen sulphide gas from nutrient broth. All the isolates were pathogenic when inoculated on to susceptible bean cultivar Red Kidney.

Race Determination and Pathogenicity Tests

Race determination was performed by inoculation of individual isolates on leaves and excised pods of cultivar Red Mexican UI3, which is resistant to race 1 but susceptible to race 2 (15). Cultivar Seminole was used as a susceptible (10) control and lines G. N. Nebraska #1 Sel 27 and Wisc HBR 72 resistant and highly resistant respectively to race 1 and 2 (16) were also included. Seed inoculation by partial vacuum was also used for comparison. A large amount of water-soaking at the site of inoculation indicated a susceptible reaction. Systemic chlorosis caused by toxin translocation was noted. Plants were rated as resistant if they showed brown necrotic lesions with some traces of water-soaking at the site of inoculation. This is the type of resistance referred to as tolerance by Patel and Walker (15). A highly resistant reaction was one where plants showed a brown necrotic (hypersensitive) reaction on the point of inoculation with no water-soaking.

All isolates except CBP 176 were pathogenic to cultivar Seminole. Large amount of water-soaking appeared on inoculated leaves 5 to 7 days after inoculation and some of the plants developed systemic chlorosis. The isolates that induced brown necrotic lesions on Red Mexican UI 3 were classified as race 1, but those that incited water-soaking were regarded as race 2. Twenty Pasto isolates were found to be race 2 whereas 7 isolates from Popayán, 1 from Palmira, and 2 from Tenerife belonged to race 1 (Table 2). Corresponding pod inoculation gave similar results of race characterization. However, isolate CBP 172 reacted as race 1 on plant inoculation but race 2 on pod inoculation.

The line G. N. Nebraska #1 Sel 27 was resistant (tolerant) to all race 1 isolates and most of the race 2 isolates. However, some Pasto isolates (PPP

Table 2. Race determination of 30 isolates of *Pseudomonas syringae* pv. *phaseolicola* from Colombia based on their pathogenicity to bean *(Phaseolus vulgaris L.)* cultivar "RED MEXICAN UI-3".

Isolates	Origin	Host	Race determination	
			Plant	Pod
CBP-177, PC-2	Popayan	*P. coccineus*	1	1
PC-3, PC-4, PC-5, PC-6	Popayan	*P. coccineus*	1	—a
CBP-178	Popayan	*P. vulgaris*	1	1
CBP-172	Tenerife	*P. vulgaris*	1	2
CBP-173			1	1
CBP-176	Palmira	*P. vulgaris*	1	1
PPP-1, 2, 3, 4, 5, 6, 7, 8, 11, 12, 13, 14, 15, 16 17, 18, 19, 20, 21, 22	Pasto	*P. vulgaris*	2	2

a = not tested

7, PPP 18, PPP 20) incited a susceptible water-soaking reaction without any sustemic chlorosis. The line Wisc HBR 72 showed the highest degree of resistance for leaf reaction to all isolates tested. However, the line showed a susceptible pod reaction.

Most of the seeds of the cultivar Seminole inoculated by partial vacuum disintegrated in the soil whereas the controls (seeds inoculated with distilled water) germinated and grew normally. Cultivar Red Mexican UI 3 was resistant to race 1 isolates. Most of the race 2 isolates caused water-soaked lesions on the cotyledons, stems, and leaves. Plants were stunted and some of them showed systemic chlorosis.

Virulence Determination

Szarka and Velich (26) observed that virulence of *Pseudomonas syringae* pv. *phaseolicola* can be characterized by the diameter of water-soaked spots around the point of inoculation. In our studies, cultivar Seminole was used to determine the variation in virulence among the collected isolates. The lines G. N. Nebraska #1 Sel. 27, Wisc HBR 72, and Red Mexican UI 3 were also used for comparison.

The mean diameter and range of the water-soaked spots for each cultivar are presented on Table 3. The line Wisc HBR 72 (with high foliar resistance) had susceptibility as comparable to that of Seminole. The frequency distribution of the isolates on the basis of mean diameter of water-soaked spots of the 4 lines is shown in Figure 1. Isolate PPP 20, had the highest mean diameter value, followed by PPP 18, PPP 16 and PPP 22. The order (high to low) for most of the isolates in virulence was similar in the lines G.N. Nebraska #1 Sel 27, Wisc HBR 72 and Seminole. A high correlation coefficient (r) between the lines on the order of isolates virulence with

Table 3. Average diameter and range (mm) of watersoaked spots induced by 30 isolates of *Pseudomonas syringae* pv. *phaseolicola* on pods of 3 tested bean *(Phaseolus vulgaris* L.*)* lines.

Line	G. N. Nebraska No. 1 Sel 27	Seminole	Wisc HBR 72
Average	1.9	2.8	3.1
Range	0.4 - 2.8	0.5 - 4.8	0.5 - 5.3
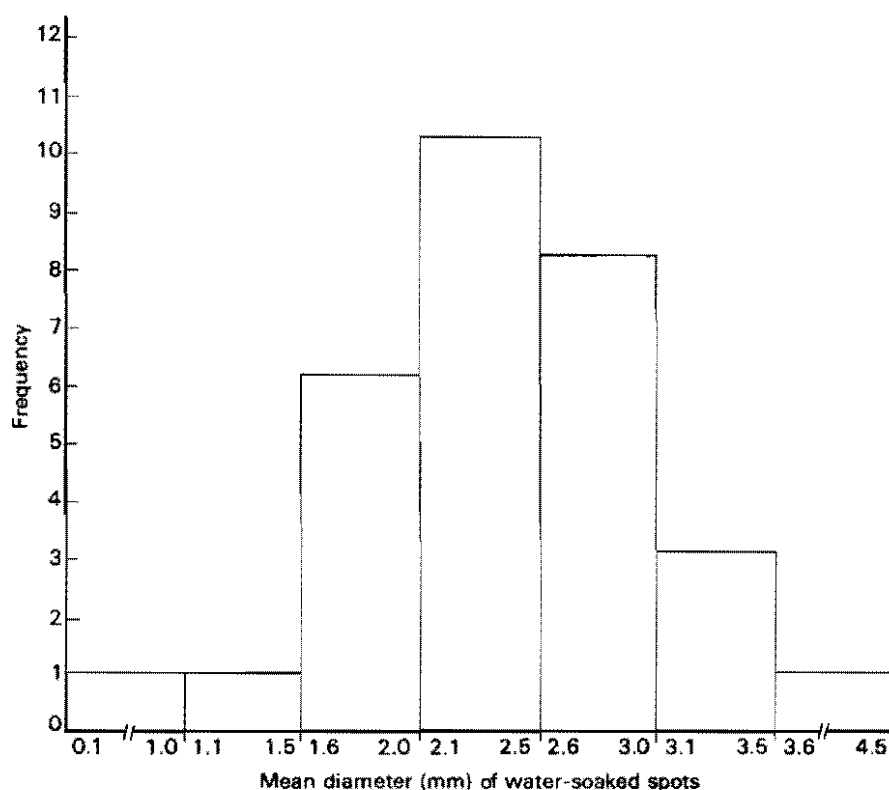
Fig. 1. Histogram showing frequency distribution of 30 isolates of *P. syringae* pv. *phaseolicola* in relation to diameter of watersoaked spots caused on 4 bean *(Phaseolus vulgaris* L.) lines.

respect to the means of the diameter of water-soaked spots was observed (Table 5).

The virulence of the isolates varied in two ways. Some of the isolates were more (or less) virulent than others on the 3 lines; isolate PPP 20 was consistently more virulent than isolates PPP 12 (Figure 2). This type of

variation was observed with most of the isolates. But, virulence of other isolates depended on the cultivar, isolate PPP 22 was less virulent that isolate PPP 18 on the line G. N. Nebraska #1 Sel 27 but was more virulent on Wisc HBR 72. These types of variations were observed both within and between the isolates of the 2 races identified (Figure 2).
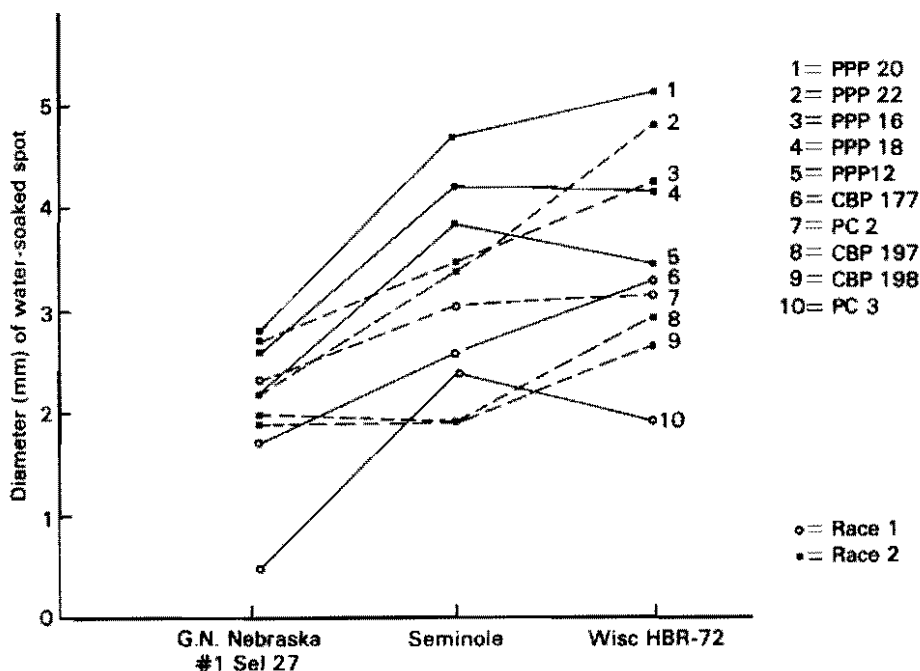
Fig. 2. Variation in diameter of watersoaked spots on pods of 3 bean (*Phaseolus vulgaris* L) lines inoculated with isolates of *P. syringae* pv. *phaseolicola*.

Discussion

The resistance of the line G. N. Nebraska #1 Sel 27 to races 1 and 2 of *Pseudomonas syringae* pv. *phaseolicola* was previously reported by Coyne and others (6). But the water-soaking reaction incited by some of the Pasto strains in our studies suggested that they were able to overcome some of the genes' controlling resistance to race 2. Similar observations have been made by Coyne and others (5) in U.S.A. and Poryazos (16) in Bulgaria. The line, however, showed some degree of pod resistance (Table 3).

Line Wisc HBR 72 was highly resistant to all isolates and gave a hypersensitive leaf reaction, a form of induced resistance (4). The independent reactions of the plant components observed stress the importance of evaluating and selecting plants with both leaf and pod resistance.

The race studies showed that both race 1 and 2 occur in Colombia. There was a close correlation between the leaf and pod methods used to determine races on cultivar Red Mexican UI 3. The designation of isolate CBP 172 as race 1 by leaf reaction and 2 by pod reaction may be due to an intermediary character of the isolate's virulence, which is not uncommon (10).

Goth (9) observed that seed inoculation by partial vacuum was superior to soaking them in a bacterial suspension. He found, however, that heavily infested seed disintegrated in the soil. Lack of germination observed with Seminole and poor germination caused by some race 2 strains on cultivar Red Mexican UI 3, may have been due to similar effects. The higher susceptibility of bean plants at an early age (15) may have also played a role.

Virulence studies showed that a number of the Colombian isolates were more virulent than the standard isolates used for comparison (Tables 3 and 4). The pod reaction of the lines used gave a good correlation in rating

Table 4. Average diameter (mm) of the watersoaked spots induced by standard isolates of *Pseudomonas syringae* pv. *phaseolicola* on pods of 3 bean *(Phaseolus vulgaris* L.) lines.[a]

Line	G. N. Nebraska No. 1 Sel 27	Seminole	Wisc HBR 72
Race 1	0.6	1.0	0.9
Race 2	1.9	1.9	3.0
HB-16	1.6	2.0	2.1
CBP 198 (OHB)	2.0	1.9	2.7

[a] Average of no less than 20 values

Table 5. The correlation coefficients (r) between 4 bean *(Phaseolus vulgaris* L.) lines on the order of isolates in virulence with respect to the mean diameters of water-soaked spots on pods.

Lines/Cultivars	Correlation coefficients (r)
Red Mexican UI3 : Seminole 1	0.53
Red Mexican UI3 : G.N. Nebraska No. 1 Sel 27	0.57
Red Mexican UI3 : Wisc HBR 72	0.53
Seminole : G. N. Nebraska No. 1 Sel 27	0.86
Seminole : Wisc HBR 72	0.87
G. N. Nebraska No. 1 Sel 27 : Wisc HBR 72	0.87

isolates in their order of virulence although some reactions with some of the isolates showed to be host dependent. The data obtained indicated that differentiated race 1 and 2 consisted of isolates which were not homogeneous when tested with different lines. These results agree with those obtained by previous workers (19, 26). It may suffice to develop and standardize a method that can determine the whole range of virulence variability among the isolates or strains of *Pseudomonas syringae* pv. *phaseolicola*. This is important, because the information obtained thereof is essential for a successful breeding program.

Acknowledgment

Literature Cited

1. Bannerot, N., 1964. Repport thactivite, SCAP Versailles, France.
2. Costa, A. S., 1972. Investigacoes sobre molestias do feijoeiro no Brazil. En Anais do I Simposio Brazilero de Fiejao, Vol. 2, pp. 337-338. Univ. Fed. Vicosa, Minas Gerais, Brazil.
3. Coyne, D. P., and M. L. Schuster. 1974. Breeding and genetic studies of tolerance to several bean (*Phaseolus vulgaris* L.) bacterial pathogens. Euphytica 23:651-656.
4. Coyne, D. P., and M. L. Schuster. 1979. Bacterial diseases of legumes: A summary of breeding and resistance. *In:* M. H. Dickson (ed.) Annual Report Bean Improvement Coop. 22:21-22. (Mimeo). N. Y. Agri. Expt. Sta. Geneva, N.Y.
5. Coyne, D. P., M. L. Schuster and Carol Erwin. 1979. Reaction of *Phaseolus vulgaris* germplasm to a new virulent strain *Pseudomonas phaseolicola*. *In* M. H. Dickson (ed.) Annu. Rept. Bean Improvement Coop. 22:20-21 (Mimeo.), N.Y. Agr. Expt. Sta. Geneva, N.Y.
6. Coyne, D. P., M. L. Schuster and C. C. Gallegos. 1971. Inheritance and linkage of the halo blight systemic chlorosis and leaf water-soaked reaction in *Phaseolus vulgaris* L., variety crosses. Plant Dis. Rep. 55:203-207.
7. Dubin, H. J., and L. R. Ciampi. 1974. *Pseudomonas phaseolicola* en Chile. Fitopatología 9:91-92.
8. Epton, H. A. S., and B. J. Deverall. 1965. Physiological races of *Pseudomonas phaseolicola* causing halo blight of beans. Plant Pathology 14:53-54.
9. Goth, R. W., 1966. The use of partial vacuum to inoculate bean seeds with phytopathogenic bacteria. Plant. Dis. Rept. 50:110-111.
10. Hale, C. N., and J. D. Taylor. 1973. Races of *Pseudomonas phaseolicola* causing halo blight of beans in New Zealand. J. Agr. Res. 16:147-149.
11. Hill, K., D. P. Coyne, and M. L. Schuster. 1972. Leaf, pod and systemic chlorosis reactions in *Phaseolus vulgaris* to halo blight controlled by different genes. J. Amerc. Soc. Hort. Sci. 97:494-498.
12. Jensen, J. H., and J. E. Livingstone. 1944. Variation in symptoms produced by isolates of *Phytomonas medicaginis* var. *phaseolicola*. Phytopathology 34:471-480.

13. King. E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescein. J. Lab. Clin. Med. 44:301-307.

14. Lelliot, R. A., E. Billing and A. C. Hayward. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.

15. Patel, P. N., and J. C. Walker. 1965. Resistance of *Phaseolus* to halo blight. Phytopathology 55:889-894.

16. Poryazov, I., 1975a. About the disease reaction to *Pseudomonas phaseolicola* (Burkh) Dowson of some tolerant or resistant bean lines. *In* D. P. Coyne (ed.), Annu. Rept. Bean Improvement Coop. 18:58-60. (Mimeo), Univ. of Nebraska, Lincoln.

17. Poryazov, I., 1975b. Physiological races of *Pseudomonas phaseolicola* (Burkh) Dowson in Bulgaria. *In* D. P. Coyne (ed.), Annu. Rpt. Bean Improvement Coop. 18:57-58. (Mimeo). Univ. of Nebraska, Lincoln.

18. Russel, P. E., 1976. Potential sources of resistance to halo blight of beans. *In* M. H. Dickson (ed.) Annu. Rpt. Bean Improvement Coop. 19:65-67. (Mimeo.), Univ. of Nebraska, Lincoln.

19. Scroth, M. N., V. B. Vitanza, and D. C. Hildebrand. 1971. Pathogenic and nutritional variation in the halo blight group of fluorescent pseudomonads on beans. Phytopathology 61:757-882.

20. Schuster, M. L., 1955. A method of testing resistance of bean bacterial blights. Phytopathology, 45:519-520.

21. Schuster, M. L., D. P. Coyne, and E. D. Kerr. 1965. New virulent strain of halo blight bacterium overwinters in the field. (Abstr.) Phytopathology 55:1075.

22. Schuster, M. L., D. P. Coyne, and E. D. Kerr. 1966. Pathogenicity tests of Nebraska isolates of halo blight bacteria in legumes. *In* D. P. Coyne (ed.), Annu. Rpt. Bean Improvement Coop. 9:35-36. (Mimeo). Univ. of Nebraska, Lincoln.

23. Schuster, M. L., and D. P. Coyne. 1981. Biology, epidemiology, genetics and breeding for resistance to bacterial pathogens of *Phaseolus vulgaris* L. *In* J. Janick (ed.) Hort. Rev. Vol. 3. 468 pp.

24. Schwartz, H. F., 1979. Halo blight and miscellaneous bacterial diseases. *In* H. F. Schwartz and G. E. Gálvez (eds.), Bean Production Problems: diseases, insects, soil and climatic constraints of *Phaseolus vulgaris* L. CIAT, Cali, Colombia. 424 pp.

25. Szarka, J., and I. Velich. 1976. Virulence of Hungarian and standard strains of *Pseudomonas phaseolicola* on beans (*Phaseolus vulgaris* L.). *In* M. H. Dickson (ed.), Annu. Rpt. Bean Improvement Coop. 19:81-83. (Mimeo.), Univ. of Nebraska, Lincoln.

26. Szarka, J., and I. Velich. 1979. A study of the aggressivity of isolates belonging to the *Pseudomonas phaseolicola* (Burkh.) Dowson. *In* M. H. Dickson (ed.), Annu. Rpt. Bean Improvement Coop. 22:64-65. (Mimeo). N. Y. Agr. Exp. Sta. Geneva, N.Y.

27. Szarka, J., and I. Velich. 1980. Classification of isolates of *Pseudomonas phaseolicola* (Burkh.) Dowson by means of the diameter of watersoaked lesions caused on pods of beans. *In* M. H. Dickson (ed.), Annu. Rpt. Bean Improvement Coop. 23:105. (Mimeo.), N.Y. Agr. Exp. Sta, Geneva, N.Y.

28. Taylor, J. D., 1970. Bacteriophage and serological methods for the identification of *Pseudomonas phaseolicola* (Burkh.) Dowson. Annals. Appl. Biol. 66:387-396.

29. Walker, J. C., and P. N. Patel. 1964. Inheritance of resistance to halo blight of beans. Phytopathology 54:952-954.

30. Wharton, A. L., 1967. Sources of infection by physiological races of

Pseudomonas phaseolicola in dwarf beans. Plant Pathology 16:27-31.

31. Zaumeyer, W. J., 1973. Goals and means for protecting *Phaseolus vulgaris* in the tropics. *In* P. H. Graham (ed.) Potentials of field beans and other food legumes in Latin America. CIAT Ser. Seminars 2E, Feb. 26-March 1, 1973. CIAT, Cali, Colombia.

32. Zaumeyer, W. J., and R. H. Thomas. 1957. A monographic study of bean diseases and methods for their control. USDA Tech. Bul. 255 pp.

Studies on Genetic Resistance of Beans to *Pseudomonas· phaseolicola* in Kenya

G. K. Kinyua, D. M. Mukunya, E. M. Van Breukelen

University of Nairobi
Nairobi, Kenya

Abstract

Halo blight of beans caused by *Pseudomonas phaseolicola* (Burk.) Dows is one of the most important diseases of beans in Kenya. It is widely distributed, resulting in very low yields from early infected beans. Thirty isolates of *P. phaseolicola* were obtained from 12 bean growing districts. Seven belonged to Race 1 while 23 belonged to Race 2. The predominating Race 2 caused severe drooping of primary leaves in Red Mexican UI 3 seedlings. Two lines GLP 16 and GLP x92 resistant to Race 2 were crossed with susceptible lines and their F_2 progeny analyzed for resistance of susceptibility reactions. Resistant bean seedlings reacted mainly with necrotic spots and partial chlorosis to *P. phaseolicola* Race 2. Large water soaked lesions and entire chlorosis constituted the major symptoms on leaves of susceptible seedlings. Resistance to *P. phaseolicola* Race 2 in GLP 16 and GLP x92 was found to be governed by one recessive gene. A ratio of 1 (Resistant) to 3 (Susceptible) was obtained in the F_2 seedlings.

Introduction

Halo blight of beans caused by *Pseudomonas phaseolicola* (Burk.) Dows is one of the most important diseases of beans. It is widely distributed in Kenya and its attack on beans in the early stages results in very low yields. *P. phaseolicola* has been known to occur in two Races (2). Race 2 has been especially virulent, causing stunting and systemic chlorosis. Race I causes mild symptoms and occurs in lower frequency than Race 2. In Romania, 64% of *P. phaseolicola* isolates tested belonged to Race 2 (3). A similar study in Quebec showed that 26 isolates out of 30 belonged to Race 2 (13).

While these two races are well documented (3, 4, 5, 7, 12), opinions differ as to the existence of other races of *P. phaseolicola*. Schroth, Vintanza, and Hilderbrand, as cited by Hubbeling (5), concluded that there was an indefinite number of races within *P. phaseolicola*. They stated that neither Race I nor 2 was homogeneous with regard to virulence when tested on a number of bean cultivars. In 1977 and 1978 Schuster *et al.* found a new Race 3 which attacked GN UI 59 and California Pink, both of which are resistant to Race I and 2 (8).

Genetic resistance in beans to halo blight has been studied extensively (4, 5, 6, 11). Genetic resistance to the different races of *P. phaseolicola* have been identified and incorporated in bean cultivars. Complications due to existence of races do arise since many genes reported are recessive and confer resistance to only one race.

The present investigation comprises differentiation of different isolates of *P. phaseolicola* collected in Kenya and a preliminary study of resistance to two local cultivars with respect to the more virulent Race 2.

Materials and Methods

Collection of Isolation of *P. phaseolicola*

Infected bean plants were collected from 12 different bean growing districts in Kenya. Pure cultures of *P. phaseolicola* were obtained as follows. A portion of an infected leaf or pod was cut into small pieces and one piece placed in a test tube containing sterilized water for five minutes. This allowed the bacterium to ooze out of infected tissue into the water. The suspension was then plated on nutrient agar in a Petri dish. Four plates per sample were prepared and kept in an incubator set at 20°C.

After incubation, a Petri dish without much contamination was selected and a single colony from the plate was transferred into a test tube containing sterilized water, resuspended, and plated on nutrient agar. After incubation for 48 h, a single colony was picked from this plate and inoculated into two test tubes containing nutrient agar. After growth for 48 h the test tubes were stored in a refrigerator as stock cultures. All cultures were labelled for identification of location and date of collection, Pathogenicity studies on a susceptible cultivar, GLP 4 seedlings, and production of livern colonies on 5% sucrose nutrient agar confirmed isolates to be *P. phaseolicola.*

Differentiation of Races

The differentiation of *P. phaseolicola* isolates into Race 1 and 2 was based on the reaction of Red Mexican UI 3. Peeled seeds were dipped in a bacterial suspension corrected at 10^7 cells/ml and planted in sand trays placed on a laboratory bench. Isolates were classified as Race 1 if none or very small necrotic lesions developed on cotyledons and leaves or Race 2 if large lesions, stunting, and systemic chlorosis were observed.

Testing for Resistance

Many cultivars were inoculated for their reactions to *P. phaseolicola* in the greenhouse using the peeled seed method. Using a scale of 0-6 (0=no reaction, 6 = severe reaction and system chlorosis) to differentiate the reactions obtained at the primary leaf stage, five cultivars were selected for genetic studies. GLP 2, GLP 3, and GLP 4 were found to be very susceptible to Race 2 but GLP 16 and GLP x 92 were found to be resistant. Crosses between the three susceptible and the two resistant cultivars were made using the rubbing method with emasculation as described by Buishand (1). Reciprocal crosses were also obtained, except for those with GLP 16 as the female parent whose progeny did not set seed. F_2 seeds were obtained from testing genetic inheritance of resistance in GLP 2 and GLP x92.

The F$_2$ parental and Red Mexican UI 3 seeds were inoculated as described above with a Race 2 isolate and planted in tin pots placed on a laboratory bench. Scores were taken one week after inoculation.

Results

Races of *P. phaseolicola*

Thirty different isolates of *P. phaseolicola* were obtained from different bean growing districts in Kenya (Table 1), indicating the widespread nature of the pathogen. Seven of these isolates were unable to attack Red Mexican UI 3 and were therefore designated as Race 1. These isolates caused very small necrotic lesions on leaves of the differential Red Mexican UI 3. The other isolates attacked Red Mexican UI 3, causing severe drooping of the primary leaves, usually followed by rotting of the entire seedlings. These were therefore classified as belonging to Race 2.

Race 2 was more frequent than Race 1. Race 2 was obtained from all the districts surveyed. Race 1 was found in five districts, Nyeri, Kirinyaga, Bungoma, Kiambu, and Kisii.

Table 1.   Reactions of Red Mexican UI 3 to *Pseudomonas phaseolicola* isolates from different parts of Kenya

District	Isolates	Reaction[a]	Race
Muranga	1	S	2
Embu	2	S/S	2
Nyeri	2	S/R	2/1
Bungoma	2	S/R	2/1
Wundanyi	1	S	2
Meru	1	S	2
Kakamega	1	S	2
Naivasha	1	S	2
Kirinyaga	8	7S/1R	2/1
Kiambu	6	5S/1R	2/1
Kisii	5	2S/3R	2/1

[a]R = Resistance;   S = Susceptible.

Genetic Studies

All susceptible parents, GLP 2, GLP 3, and GLP 4, produced large lesions on cotyledons immediately after germination of inoculated seeds. The inner side of the cotyledons turned black, followed by a rapid multiplication of the bacterium at the cotyledonary node. A few seedlings broke at this node. On the stem, lesions with bacterial ooze developed. Halos typical of

leaf symptoms in susceptible beans were also formed on leaves. Drooping, rotting, and death of some seedlings occurred and those that survived developed systemic chlorosis and greasy lesions on leaves. Severe symptoms included pre-emergence damping off and production of abundant bacterial ooze from leaves and stems of seedlings.

The resistant parents, GLP 16 and GLP x92, produced very tiny necrotic lesions on leaves immediately after germination. Some partial chlorosis was seen on the leaves. Most seedlings of the resistant cultivar GLP x92 had no visible symptoms.

The F_2 progenies tested showed a broad spectrum of reactions from resistant to susceptible types. With the 0-6 scale, plants placed between 0-2 were rated resistant while plants showing 3-6 severity reading, were considered susceptible. These readings were compared with those of parents for ease of interpretation.

Analysis of parental and F_2 progenies are summarized in Table 2. It was observed that most progeny from the crosses of resistant x susceptible bean lines were susceptible. This indicated that genes responsible for resistance in GLP 16 and GLP x92 were recessive. Calculations to

Table 2. Summary of Parental and F_2 seedling reaction to *Pseudomonas phaseolicola* Race 2.

Cross or parent	Resistant	Susceptible	Ratio Expected	X^2	P
GLP 2	0	905			
GLP 3	0	986			
GLP 4	0	888			
GLP 16	984	0			
GLP x 92	938	0			
GLP 2 x GLP 16	262	742	1:3	0.64	44.70
GLP 3 x GLP 16	273	666	1:3	8.31	0.46*
GLP 4 x GLP 16	260	767	1:3	0.06	85.72
GLP 2 x GLP x92	176	512	1:3	0.12	70.40
GLP 3 x GLP x92	211	362	1:3	42.72	0.00**
GLP 4 x GLP x92	213	567	1:3	2.22	14.98
GLP x92 x GLP 2	73	213	1:3	0.04	89.60
GLP x92 x GLP 3	123	286	1:3	5.61	2.98
GLP x92 x GLP 4	138	342	1:3	3.60	6.58

* Significant at 5% level
** Significant at 1% level

determine the number of genes involved (16) indicated that a ratio of 1:3 is highly probable for six of the crosses, thus indicating presence of a single gene pair for resistance in the respective lines.

Discussion

On the basis of Red Mexican UI 3 reaction, *P. phaseolicola* was found to exist in Kenya as Race 1 and Race 2. The high frequency (75%) reported in Kenya for Race 2 agrees with other reports (3, 13). Probability of other races existing in Kenya cannot be ruled out as other differentials used by other workers (5, 8) were not available for testing.

The segregation of F_2 seedlings from crosses of GLP 2 x GLP 16, GLP 3 x GLP 16, GLP 4 x GLP 16, GLP 2 x GLP x92, GLP 3 x GLP x92, GLP 4 x GLP x92, and their reciprocals (Table 2) showed that susceptibility to *P. phaseolicola* Race 2 was dominant over resistance. The susceptible seedling were more frequent than the resistant ones. Other workers in beans have found recessive genes governing resistance to *P. phaseolicola* (4, 6). These similar results show that resistance in GLP 16 and GLP x92 to *P. phaseolicola* Race 2 could be governed by one gene since a ratio of 1 (Resistant) to 3 (Susceptible) was obtained in their F_2 seedling reactions. Other workers have found one gene governing resistance to *P. phaseolicola* (7, 8, 11, 17.)

In three crosses GLP 3 x GLP 16, GLP 3 x GLP x92, and GLP x92 x GLP 3, the ratios for F_2 seedlings could not be fitted into any hypothesis giving a definite number of genes for resistance. This lack of fit could be attributed to variation in the environmental factors and also epistasis. In all the F_2 data that did not fit 1:3 ratio, there was excess of resistant seedlings. The genes for susceptibility could have been masked so that an excess of resistant seedlings was obtained. Disease escape also could have contributed to the deviation of observed ratios from the expected ones. This would have resulted in a higher number of resistant seedlings as in the case of unclear crosses mentioned. However, disease escape would only have had a negligible effect because the inoculation method was very effective and guaranteed maximum infection.

The conclusion that resistance in GLP 16 and GLP x92 is governed by one gene is interesting from the standpoint of breeding for halo blight resistance in local dry beans in Kenya. Most popular local varieties are very susceptible to halo blight and any type of resistance genes would be very useful. As the resistance has not proven stable nor been tested with other races of the pathogen, caution should be exercised in any breeding program using this source of resistance.

Literature Cited

1. Buishand, T. J., 1953. The crossing of bean (*Phaseolus vulgaris* L.) Euphytica 5:41-50.
2. Epton, H. A. S., and B. J. Deverall, 1965. Physiological races of *Pseudomonas paseolicola* causing halo blight of beans. Plant Pathology 14:53-54.

3. Gallegos, B. S., A. Campost and M. A. Cripsin. 1966. Genetic study of bean resistance to *Pseudomonas phaseolicola* (Burk). Dows. Agrociencia. 1 56-66.

4. Hill, K., D. P. Coyne, and M. L. Schuster. 1972. Leaf, pod and systemic chlorosis reactions in *Phaseolus vulgaris* to halo blight controlled by different genes. J. Amer. Hort. Sci. 97:494-498.

5. Hubbeling, N., 1973. Field resistance in *Phaseolus* beans to *Pseudomonas phaseolicola*. Med. Fak. Landb. Gent. 38:1351-1363.

6. Patel, P. N., and J. C. Walker. 1966. Inheritance of tolerance to halo blight in beans. *Phytopathology* 50:681-682.

7. Robinson, R. A., 1969. Diseases resistance terminology Rev. Appl. Mycol. 48:593-606.

8. Schuster, M. L., D. P. Coyne, and C. Smith. 1977. New strains of halo blight bacterium in Nebraska. Bean Impr. Co-op. 22:19-20.

9. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Ames. Iowa State University Press. 593 pp.

10. Szarka, J., and I. Velitch. 1976. Virulence of Hungarian and standard strains of *Pseudomonas phaseolicola* on beans (*Phaseolus vulgaris* L.) Bean Impr. Co-op. 19:81-83.

11. Taylor, J. D., L. L. Budley, and N. L. Innes. 1978. Inheritance of resistance to halo blight of beans (*Phaseolus vulgaris* L.) Ann. of Appl. Biol. 90:101-110.

12. Walker, J. C., and P. N. Patel. 1964. Inheritance of resistance to halo blight of beans. Phytopathology 54:952-954.

13. Wallen, V. R., 1968. Identification of races of *Pseudomonas phaseolicola* from Quebec bean fields. Can. Plant. Dis. Surv. 48:97-98.

Physiological and Pathogenic Variations in *Xanthomonas campestris* pv. *manihotis*

H. Maraite, J. Weyns, O. Yimkwan, P. Lipembra, D. Perreaux

Université Catholique de Louvain
Louvain-la-Neuve, Belgium

Abstract

A collection of 53 *X. campestris* pv. *manihotis* strains from Brazil, Cameroon, Colombia, Ivory Coast, Java, Kenya, Mauritius, Nigeria, South Africa, Sumatra, Taiwan, and Uganda was built up and analyzed. All strains showed gelatin hydrolysis, protein hydrolysis, formation of acid from D (-) arabinose, glucose, and mannose, but not from rhamnose and ribose. Pectate gel was not hydrolysed. Variations among the strains, but without relation to the geographical origin, were observed concerning sodium chloride tolerance, H_2S production, and production of acid from glycerol, maltose, and raffinose. All 39 tested African strains showed a low amylase activity, while among the 8 South American ones, only a strain from Bahia and a strain from Rio de Janeiro did so. Strong amylase activity was related with growth on SX agar and formation of acid from dextrin.

Five strains were inoculated in an air conditioned glasshouse to 13 cassava cultivars from Africa, Asia, and South America, with various degrees of field resistance. Significant differences in virulence were observed among the strains. Some, on the other hand, were particularly virulent on specific cultivars and less on others, suggesting some pathogenic specialization.

Smooth colonial variants occurring occasionally during subculturing may show reduced virulence compared to the parental strain.

Introduction

Cassava (*Manihot esculenta* (Crantz) bacterial blight (CBB), caused by *Xanthomonas campestris* pv. *manihotis* (Berthet & Bondar) Dye, is characterized by the production on the leaves of angular spots evolving into blight areas and by a systemic infection of the stem leading to necrosis of vasular tissues, exudation of bacterial ooze, wilt, and tip die-back (13, 15).

Since its report in 1912 from Brazil (2), the bacteria has been detected in most of the cassava growing countries in the tropics (4), especially during the last decade, when the disease caused extensive losses leading to food shortage in areas such as the Bandundu province in Zaire (15). Cassava improvement programs were initiated in several countries.

The widespread occurrence of the pathogen at the time of first identification, suggests that it had been present for at least several years prior to its detection (23).

In view of this wide distribution, it is not surprising that discrepancies are noted in the descriptions of the physiological characteristics of the pathogen (1, 6, 9, 13, 14). By comparing strains from distinct geographical areas, Lozano and Sequeira (13) observed differences in the serological relationships and carbohydrate utilization and Maraite and Weyns (16) in the amylase activity. Differences in virulence among strains have also been noted (7, 15, 22), but distinct pathogenic races were not yet identified.

Disease control is mainly based on the use of resistant cultivars. It is thus important to know the limits of variation of the pathogen in order to select cultivars with resistance against the most virulent strains and also for the choice of resistant germplasm to control eventually one particular strain. With this in view we are building up a collection of strains from various geographical origins, characterizing these strains in a set of laboratory tests and studying their reaction to cassava cultivars with various degrees of field resistance. Herein we report the most significant results obtained up to now.

Material and Methods

Culture Collection

The origins of the strains are given in Table 1. Dilute suspensions of single colonies appearing on isolation plates, or of the cultures provided by donors, were streaked out on nutrient agar (Difco) and checked for purity. Subcultures of typical single colonies from these streaks were transferred for storage for up to 6 months on Dye's GYCA (10) slopes at room temperature in the dark, or lyophilized for longer preservation. Standard bacteriological (3) and pathogenicity tests were performed as identification checks.

Physiological Characteristics

Stock cultures were revived on GYS plate [glucose, 5 g; yeast extract (Difco), 5 g; $NH_4H_2PO_4$, 0.5 g; K_2HPO_4, 1.5 g; $MgSO_4$. $7H_2O$, 0.2 g; NaCl, 5 g; agar (Difco Bacto), 20 g; water 1 l] and 4 typical colonies transferred to GYCA. The tests were made within 2 weeks from subcultures of these colonies. Incubation temperature was 27°C.

Tests on solid media were performed by a replica plating technique, using 10 x 10 x 2 cm high sterile plastic boxes divided in 25 compartments (Sterilin, U.K.) allowing the comparison of 6 strains in 4 repetitions on one plate. Each compartment received 2 ml of medium, giving a layer about 6 mm thick. Master plates on GYS were incubated for 3 days before replication to the test medium. For tests in liquid medium, 3-day-old subcultures on GYS slopes were washed with sterile water and diluted. One drop of the suspension was added to 2 ml of test medium in 15 mm diameter test tubes, giving a final concentration of about 10^6 cells ml^{-1}.

Gelatin hydrolysis and protein digestion were studied by the agar plate techniques (21, p 33 and p 48, respectively); pectate hydrolysis on Bradbury's modification (5) of the Paton pectate gel medium; tolerance to sodium chloride; and H_2S production according to Dye's (10) methods. For the latter test the cultures were not shaken in order to avoid splashes on

Table 1. Origin of the *X. campestris* pv. *manihotis* strains.

HMB[a] n°	Territory of origin	Date of isolation	Other references or donors[b]
3	Zaïre, Kasaï	1973	Z 39 (15)
6	Zaïre, Bandundu	1973	Z 273 (15)
9	Zaïre, Haut Zaïre	1973	Z 284 (15), NCPPB 3058
10	Zaïre, Bas Zaïre	1973	Z 291 (15), NCPPB 3051
23	Brazil, Sao Paulo	1973	A.L.G. Pereira BM12 (15), ICPBXM147
25	Nigeria	1976	N 578 (16), NCPPB 3060
27,34	Cameroon	1977	C 631, C 671 (16)
36	Nigeria	1977	From a leaf collected in 1977
55	Brazil, Rio de Janeiro	1965	NCPPB 1834, Robbs ENA-300 (20)
58	Nigeria	1978	From a leaf collected in 1978
60,61	Taiwan	1978	From stems sent by L.S.Leu
68	Brazil	1941	NCPPB 1159, Burkholder SM 1 (6)
70	Brazil	1941	NCPPB 1160, Drumond & Hipolito (9)
71	Mauritius	1946	NCPPB 1161, Orian M 3 (18)
72	Colombia	1970	NCPPB 2443, Lozano 23 L (13)
78	Brazil Goias		Takatsu 35 (22)
79	Brazil, Bahia		Takatsu 6 (22)
80	Brazil, Brasilia		Takatsu 7 (22)
81-94	Uganda	1979	From leaves sent by Otim Nape William
174	Java	1979	UQM 1883, Persley I 8
175	Sumatra	1979	UQM 1884, Persley I 10
176	Sumatra	1979	UQM 1881, Persley I 1
177	Sumatra	1979	UQM 1892, Persley I 5
192-199	Uganda	1980	From leaves sent by Otim Nape William
200	South Africa	1981	Strain sent by Manicom (14)
201	South Africa	1981	From a leaf sent by Manicom
202,210	Zaïre	1981	From leaves collected in 1981
203,204	Ivory Coast	1981	From leaves provided by Notteghem
205,206	Kenya	1981	From leaves provided by Onyango
208	Nigeria	1981	From a leaf collected in 1981
209	Cameroon	1981	From a leaf collected in 1980

a Collection of the senior author, Laboratoire de Phytopathologie, U.C.L.
b Isolation number by the authors mentioned in previous publications, donor's or culture collection's reference number.
   NCPPB, National Collection of Plant Pathogenic Bacteria, U.K.
   ICPB, International Collection of Phytopathogenic Bacteria, U.S.A.
   UQM, University of Queensland, Dep. of Microbiology, Australia.

the lead acetate strips. Acid production from carbohydrate was tested on Dye's medium C (10) with, as carbon source: D (-) arabinose, dextrin, D (+) glucose, glycerol, lactose, maltose, mannose, raffinose, L(+) rhamnose or D (-) ribose. Hydrolysis of starch was assessed on medium C supplemented with 0.2 or 2% soluble starch. After 4 days incubation, the bacterial colonies were washed off the agar surface before flooding the medium with iodine solution for 1 min, rinsing with water and measuring the diameter of the clear area after 30 minutes.

Growth on SX agar (21) was measured after 2 weeks incubation.

Pathogenicity Testing

The origin of the cassava cultivars received as cuttings is given in Table 2. Cassava plants were established in an air-conditioned glasshouse with a day temperature of 28 to 30°C and a night temperature of 25 to 26°C, by propagation of green cuttings in sterilized water and transplanted to a light compost. Vigorous, 50 to 70 cm high plants were used for inoculation. Two leaflets of the 3rd leaf from the top were inoculated at 10 points by means of a multipin inoculator, contaminated by a bacterial suspension of about 10^9 cells ml-[1]. The stem was inoculated at the internode between the 3rd and the 4th leaf by pucture with a needle contaminated by passing it through a 48 h-old-bacterial culture on GYS. Generally, 6 plants per cultivar and per strain were inoculated.

Table 2. Origin of the cassava cultivars

Cultivar	Origin[a]	Field characteristics
Amer 6 mois	INERA, Zaire	moderately susceptible
5733		tolerant (15)
Isunikakiyan	IITA, Nigeria	susceptible
30211		resistant
30395		resistant
30555		resistant
58308		resistant
60444		highly susceptible (19)
M. Col 638	CIAT, Colombia	tolerant
M. Ecu 82		resistant
M. Pan 12B		resistant
M. Mex 59		susceptible (Lozano, personal communication)
Yi-chu-shian	PPC, Taiwan	resistant (12)

[a] abbreviations:

INERA = Institut National pour l'Etude et la Recherche Agronomique;
IITA = International Institute of Tropical Agriculture;
CIAT = Centro Internacional de Agricultura Tropical;
PPC = Plant Protection Center.

The disease severity on the inoculated leaves was rated as follows: 0, no symptoms; 1, angular watersoaked spots; 2, some blight areas; 3, blight of the leaflet; 4, blight of the whole leaf; 5, inoculated leaf shed. For stem infection, the following scale was used: 0, cicatrization, no brown areas around the inoculation point; 1, areas of browning or exudation around the inoculation point < 40 mm; 2, area > 40 mm and/or up to 2 uninoculated leaves showing wilt symptoms; 3, more than 2 wilted leaves; 4, tip die-back; 5, whole plant dead.

Results and Discussion

Physiological Characteristics

All strains showed gelatin hydrolysis after 6 days, protein digestion after 4 days, but no pectate hydrolysis, even after 2 weeks of incubation. The major differences detected among strains, concerning physiological characteristics, are presented in Table 3 for 15 representative strains.

Maximum concentration of sodium chloride permitting growth varied between 3 and 5% without any relation to geographical origin of the strains. These figures are higher than those previously (16) reported, because of the higher bacterial inoculum. Turbidity similar to the inoculated control YS broth was generally observed at salt concentrations of 1 to 2% lower than the figure quoted.

Intensity and speed of H_2S production also varied greatly among the tested strains, the first signs of darkening of the lead acetate strips being generally observed only after 4 days of incubation, although turbidity was already high after 2 days. Blackening increased rapidly afterwards up to the end of the test on the 14th day.

The differences in H_2S production may explain the discrepancies noted in the literature concerning the H_2S production by pv. *manihotis* (13, 14).

All strains produced acid from arabinose, glucose, and mannose. A slight and delayed acid production was observed for some strains on dextrin, glycerol, maltose, and raffinose, without any relation to geographical origin. No acid production was detected on rhamnose and ribose. By using the replica plating technique on medium C, we noticed for some carbohydrates an alkalization of the medium, after a more or less pronounced acidification. This may account for discrepancies observed when the results were compared to those obtained in test tubes by a modification of the Hugh and Leifson O. F. test (3).

Starch hydrolysis was demonstrated for all strains on the 0.2% starch medium. The diameter of the clear area after iodine flooding of the plates varied, however, from 2 mm (strain 9) to more than 20 mm (for instance, strain 72). The clear area is larger on a thinner medium layer. Clearcut differences were evident on 2% starch, all the African strains being negative, while the South American ones, except 55 from Rio de Janeiro and 79 from Bahia, were positive (Table 4). The strains from Java and Sumatra showed a low amylase activity. The strains from Taiwan were by mistake reported as similar to those from Africa (17). The amylase activity most probably determined the growth on SX agar containing 1% starch. Growth on this medium is thus not a good criterion for the identification of

Table 3. Characteristics of 15 strains of *X. campestris* pv. *manihotis*.

Origin	No. HMB	NaCl tolerance g/100 ml	H₂S production from (a)				Production of acid from (b)								Starch hydrolysis. detected at (c) g/100 ml		Grown on SX agar (d)
			YS	YS + cysteine	YS + Na₂S₂O₃	YS + peptone	arabinose	dextrin	glucose	glycerol	lactose	maltose	mannose	raffinose	0.2	2	
Africa																	
Cameroon	34	5	1	1	1	2	+	—	+	±	—	—	+	±	±	—	—
Nigeria	25	3	1	1	2	1	+	—	+	—	—	—	+	±	+	—	—
South Africa	200	4	3	3	3	3	+	—	+	—	—	—	+	±	+	—	1
Uganda	93	4	3	3	2	3	+	—	+	±	—	—	+	—	+	—	—
	195	4	1	1	1	2	+	—	+	±	—	—	+	±	+	—	—
Zaire	6	4	2	3	3	2	+	—	+	±	—	+	+	—	+	—	—
	9	4	1	1	1	1	+	—	+	±	—	—	+	±	±	—	—
Asia																	
Sumatra	175	4	3	3	3	3	+	—	+	—	—	+	+	±	+	—	1
South America																	
Brazil	23	3	1	1	2	1	+	±	+	—	—	—	+	±	+	+	3
	55	3	2	3	3	2	+	—	+	—	—	—	±	±	+	—	—
	68	4	1	3	3	2	+	—	+	—	—	—	+	±	+	+	2
	70	4	1	1	1	3	+	±	+	—	—	+	+	±	+	+	2
	79	4	1	1	1	1	+	—	+	+	—	±	+	±	+	—	—
	80	3	3	3	3	3	+	±	+	—	±	—	±	±	+	+	2
Colombia	72	3	1	3	3	2	+	±	+	—	—	—	+	±	+	+	2

a Darkening of the acetate strip after 1 week: 1 = light darkening, 2 and 3 = strip black on 1 and 2 cm from the tip, respectively.

b — = no indicator change after 1 month, + = pH 5.5 within 2 weeks, ± = slight acidification within 1 month.

c With a 4 days incubation on a 6 mm thick medium, diameter of clear area after iodine staining, — = no clear area, + = 1 to 5 mm, + = > than 5 mm.

d Diameter of the colonies after 2 weeks incubation: — = < 3 mm, 1 = 3 to 5 mm, 2 = 5 to 9 mm, 3 = > 9 mm.

Table 4. Distribution of strains with strong amylase activity

Origin	Number of strains	
	tested	with strong amylase activity[a]
Africa		
Cameroon	3	0
Ivory Coast	2	0
Kenya	2	0
Mauritius	1	0
Nigeria	4	0
South Africa	2	0
Uganda	19	0
Zaire	6	0
	39	0
Asia		
Java	1	0
Sumatra	3	0
Taiwan	2	2
	6	2
South America		
Brasil	7	5
Colombia	1	1
	8	6

[a] Hydrolytic area visible after iodine flooding of 4-day-old cultures on Dye's medium C containing 2°/o soluble starch.

pv. *manihotis*. The strains with high amylase activity were those which showed acid production from dextrin. It must be noted that strains 68 and 70 demonstrated high amylase activity, while Burkholder (6) and Drumond and Hipolito (9), who isolated these strains, did not observe starch hydrolysis in their tests.

Cassava originated in South America and it is plausible that pv. *manihotis* has also been distributed from there to the other continents. Because of its systemic colonization of the stem, this bacteria can indeed be propagated with the cuttings used as planting material (23). It is remarkable that all analyzed African strains showed a low amylase activity similar to that found up to now only for the strains from Bahia and Rio de Janeiro on the East Coast of South America. Further comparative studies with a still wider range of origins and test methods may possibly allow the determination of the origin of the pv. *manihotis* strains and perhaps other pathogens and pests imported into Africa.

Variations in Pathogenicity

On all tested cultivar-strain-combinations, angular watersoaked leaf spot were observed within 6 days after inoculation and these had evolved into

blight areas on the 13th day. The distribution around the mean of the blight severity indices observed for the 5 strains on the 8th day after inoculation is shown in Fig. 1A. The highest severity index was recorded with strain 78 on the highly susceptible cultivar 60444, but the differences in disease severity induced by the various strains on the range of cultivars were small and generally made up on the 13th day after inoculation, allowing, under our conditions, no sure identification of the cultivars with high field resistance.

On stem inoculation, clearer differences among cultivars and strains became evident. The IITA selections 30555, 30211, and the CIAT M. Ecu 83, with fair resistance in the field, appeared also as the most resistant in these glasshouse tests: while M. Mex 59, Isunikakiyan, Amer 6 mois and 60444, reported as susceptible, showed severe CBB symptoms (Fig. 1B). The virulence of the strains, expressed by the mean disease index induced on the 13 cultivars, decreases in the following order: 78>60 >10 >25 > 72. Strains 9 and 68 showed an even lower virulence in other experiments. In screening for resistance, it is thus wise to use the most virulent strains occurring in a country (7).

Besides the mean disease index on a cultivar, the amplitude of variation around the mean of the indices induced by the various strains must be considered. On Amer 6 mois, strains 78 and 72 induced similar symptoms, but on Isunikakiyan they induced severe and mild ones, respectively. Inversions in the mean virulence sequence were detected on some cultivars. Strain 10 induced on cultivar 30555 the lowest disease index, and on 5733 the highest one. The latter reaction was already observed in inoculation experiments done in 1973 (15). Cultivars selected for resistance against one particular strain in a country, may thus show severe symptoms when exposed to a still more virulent or different foreign strain. The results also suggest that besides differences in virulence (7, 15, 22), some pathogenic specialization exists. Further studies on a larger number of plants per cultivar and a wider range of strains are needed to confirm this point. The clear distinction of races appears, however, to be difficult. CBB resistance is indeed believed to be due to polygenes, mainly with additive effects but to some extent also with non-additive effects (11). The genotype of the actual improved cultivars is largely unknown. Cultivars with a low mean disease index on stem inoculation and with a narrow amplitude of variation of disease susceptibility to a set of isolates, should be safest for CBB control.

In general, the virulence of the tested strains was not altered during storage and subculturing for several years. During purity checks at transfers of pv. *manihotis* stock cultures, colonial variants similar to the rough and smooth colonies reported by Corey and Starr (8) for *X. phaseoli,* were detected for some strains (Maraite, unpublished). These colonial variants generally became clearly visible only after 4 to 7 days of incubation of a diluted streak on GYS and were easily overlooked in young and dense cultures used for inoculation.

In comparative leaf and stem inoculations, a subculture of a smooth colony of strain 23 showed a strongly reduced virulence, compared to the subculture of a typical mucoid one. The virulence of a subculture of a rough
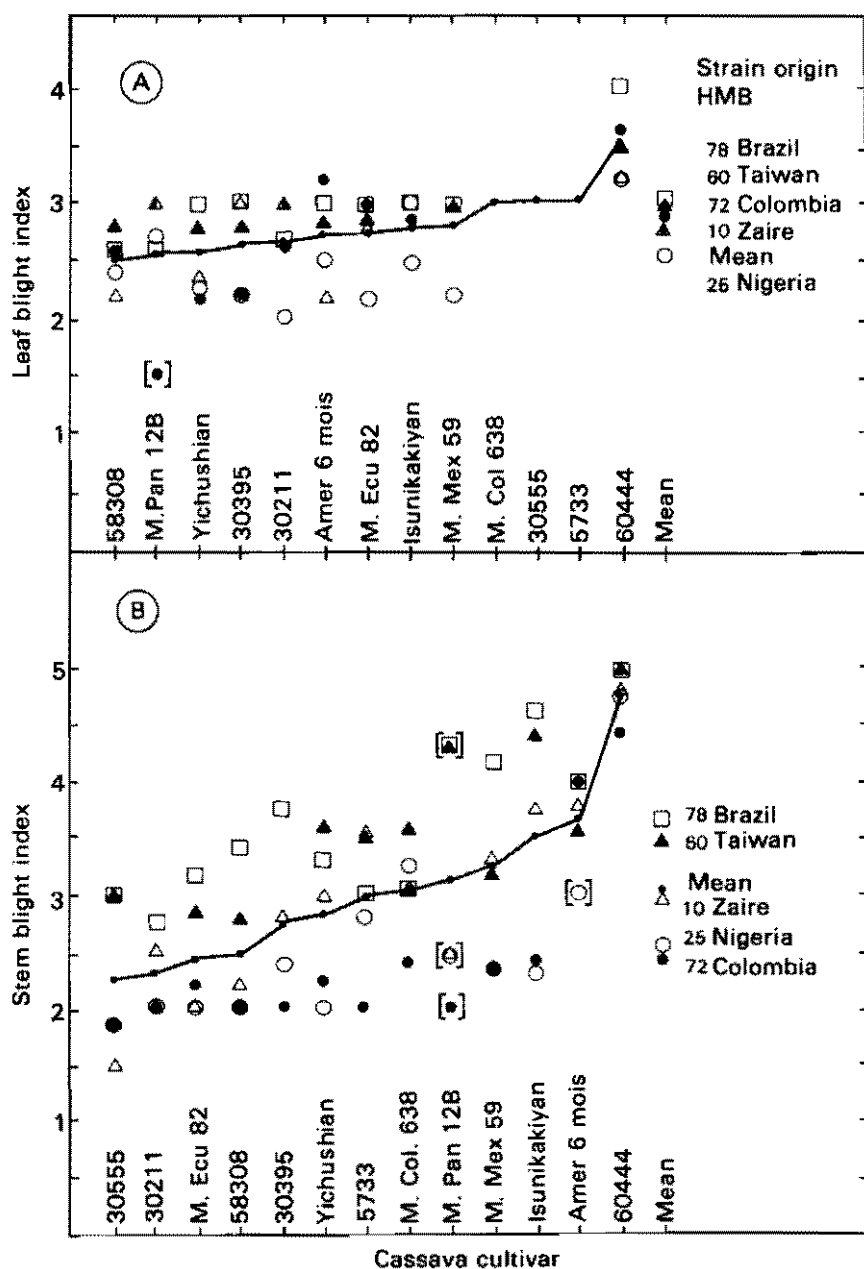
Fig. 1. Interaction of five strains of *Xanthomonas campestris* pv. *manihotis* with a set of cassava cultivars of different degrees of field resistance. (A) Blight severity on leaves, 8 days after inoculation. (B) Severity of blight symptoms on stems, 32 days after inoculation. Means of generally 5-6 plants/cultivar/strain, except for values in brackets.

colony of strain 83 had, nevertheless, an unaltered virulence. Starch hydrolysis by both variants was similar to the parental strains. In transfers of strains for virulence studies, it is thus important to choose the typical mucoid colonial type.

Acknowledgement

Literature Cited

1. Amaral, J. F. Do, and L. G. De Vasconcellos. 1945. Novos estudos do agente etiologico da bacteriose da mandioca. Arq. Inst. Biol., Sao Paulo 10:361-368.
2. Bondar, G., 1912. Una nova molestia bacteriana das hastes da mandioca. Chacaras e Quintaes, Sao Paulo 5:15-18.
3. Bradbury, J. F., 1970. Isolation and preliminary study of bacteria from plants. Rev. Pl. Path. 49:213-218.
4. Bradbury, J. F., 1977. *Xanthomonas manihotis.* CMI Description of Pathogenic Fungi and Bacteria. 559 pp.
5. Bradbury, J. F., 1978. Isolation and identification of cassava bacterial pathogens. Notes for practical work at the cassava bacterial blight workshop. 26-30 June 1978, IITA, Ibadan, Nigeria. 9 pp.
6. Burkholder, W. H., 1942. Three bacterial plant pathogens: *Phytomonas caryophylli* sp. n., *Phytomonas alliicola* sp. n. and *Phytomonas manihotis* (Arthaud-Berthet et Bondar) Viegas. Phytopathology 32:141-149.
7. Centro Internacional de Agricultura Tropical. 1978. Cassava Program. Pp. CI-C68. *In* 1977 CIAT Annual report, Cali, Colombia.
8. Corey, R. R., and M. Starr. 1957. Colony types of *Xanthomonas phaseoli.* J. Bacteriol. 74:137-140.
9. Drumond, O. A. and O. Hipolito. 1943. Notas sobre a bacteriose da mandioca. Pp. 101-118. *In* Manual da mandioca. Chacaras e Quintaes, Sao Paulo.
10. Dye, D. W., 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N. Z. J. Sci. 5:393-416.

11. Hahn, S. K., 1979. Breeding of cassava for resistance to cassava mosaic disease (CMD) and bacterial blight (CBB) in Africa. pp. 211-219. In H. Maraite and J. A. Meyer (ed.) Diseases of tropical food crops. U.C.L., Louvain-la-Neuve, Belgium.

12. Leu, L. S., 1979. Cassava bacterial blight in Taiwan. Pp. 119-129. In H. Maraite and J. A. Meyer (ed.) Diseases of tropical food crops. U. C. L., Louvain-la-Neuve, Belgium.

13. Lozano, J. C., and L. Sequeira. 1974. Bacterial blight of cassava in Colombia: Etiology. Phytopathology 64:74-82.

14. Manicom, B. Q., M. B. Becker, and C. Deschodt. Physiological and biochemical identification of cassava bacterial blight in South Africa (in press).

15. Maraite, H., and J. Meyer. 1975. *Xanthomonas manihotis* (Arthaud-Berthet) Starr, causal agent of bacterial wilt, blight and leaf spots of cassava in Zaire. PANS 21:27-37.

16. Maraite, H., and J. Weyns. 1979. Distinctive physiological, biochemical, and pathogenic characteristics of *Xanthomonas manihotis* and *X. cassavae*. Pp. 103-117. In H. Maraite and J. A. Meyer (ed.). Diseases of tropical food crops, U. C. L., Louvain-la-Neuve, Belgium.

17. Maraite, H., and J. Weyns. 1980. Comparison du *Xanthomonas manihotis* at du *X. cassavae*, p. 5. In Rapport des activites de recherches de 1979. Unité de Phytopathologie, U. C. L., Louvain-la-Neuve, Belgium.

18. Orian, G., 1947. Pp. 37-43. In Report of the Department of Agriculture. Mauritius.

19. Perreaux, D., E. R. Terry, and G. J. Persley. 1979. Studies on screening methods of cassava resistance to bacterial blight. Pp. 13-16. In E. R. Terry, G. J. Persley, and S. C. A. Cook (Ed.) Cassava bacterial blight in Africa. Centre for Overseas Pest Research, London, U.K.

20. Robbs, C.F., R. de L. D. Ribeiro, O. Kimura, and F. Akiba. 1972. Variacoes em *Xanthomonas manihotis* (Arthaud-Berthet) Starr. Rev. Soc. Brasil. Fitopatologia 5:67-75.

21. Schaad, N. W., 1980. Laboratory guide for identification of plant pathogenic bacteria. American Phytopathological Society. St. Paul, 72 pp.

22. Takatsu, A., S. Fukuda, and S. Perin. 1979. Epidemiological aspects of bacterial blight of cassava in Brazil. Pp. 141-150. In H. Maraite and J. A. Meyer (ed.). Diseases of tropical food crops, U. C. L., Louvain-la Neuve, Belgium.

23. Terry, E. R., G. J. Persley, and S. C. A. Cook (eds.) 1979. Cassava bacterial blight in Africa. Centre for Overseas Pest Research, London, U. K., 72 pp.

Taxonomy and Etiology of Soft Rot Erwinia Affecting Potatoes in Southern Chile

L. Ciampi-Panno

Austral University of Chile
Valdivia, Chile

Abstract

Studies were undertaken to detect the possible presence of species of pathogenic bacteria belonging to the *Carotovora* group of *Erwinia* affecting either potato plants in the field or potato tubers during storage. Thirteen isolates out of 130 cultures assayed were soft rot *Erwinia*. Six were Eca and five Ecc. Two isolates remain uncertain and they may be considered as "low temperature" isolates of Ecc due to their particular biochemical and growth characteristics.

Introduction

The taxonomic status of the genus *Erwinia* has greatly improved in recent years. Three groups make up the genus *Erwinia:* the *amylovora, carotovora,* and *herbicola* groups. Among them, species belonging to the carotovora groups are referred to as the soft-rot bacteria, namely *E. carotovora* var. *carotovora* (Ecc); *E. carotovora* var. *atroseptica* (Eca), and *E. chrysanthemi* (Echr). Geographic distribution, specific host range, and biochemical features are important characteristics for the identification of species belonging to the *carotovora* groups (Perombelom and Kelman, 1980).

In Southern Chile soft rot of potato tubers during storage and black leg of potato plants in the field are important diseases caused by pectinolitic soft-rot coliform bacteria. Early studies on identification of species was based on disease symptoms (Mujica, 1941). Only in 1973 was *Erwinia carotovora* (Jones) Holland fully described as affecting potato plants and tubers in Southern Chile (Ciampi, 1972; Ciampi, 1973). However, no studies have been made to fully identify subspecies of the *carotovora* group.

The objective of this work was to detect the possible presence of species of pathogenic bacteria belonging to the *carotovora* group affecting either potato plants in the field or potato tubers during storage.

Materials and Methods

Isolations of virulent strains were made from 18 potato plants with typical black leg symptoms; 6 plants with brown spots along the stem and

17 tubers with soft rot decay. The isolations from plant materials were performed using methods described by Ciampi (1972 b).

Pure cultures obtained from the isolations were tested for pectinolitic activity on Beraha (1968) semi-solid agar medium, using sodium polypectate (Sigma Chemical Co.) as pectic base. All the isolates that showed pectinolitic activity were tested for potato soft rot in moist chambers using methods described by Ciampi (1972b).

The strains that showed pectinolitic activity and caused soft rot of potato tubers in moist chambers were used for biochemical identification tests used in distinguishing the soft rot Erwinias (Pérombelom and Kelman, 1980). The following tests were performed: Sensitivity to erythromycin; reducing substances from sucrose; gas from d-glucose; acid from lactose; palatinose and α methyl-glucoside and growth at 37 and 40°C.

Type cultures were also used as known controls. The isolates were obtained from the culture collection of the Plant Pathology Department, Univ. of Wisconsin, Madison (Dr. A. Kelman collection). The isolates used were: Echr SR 120; EccSR44, and Eca SR44.

Results

Symptomatology of Collected Diseased Plants and Tubers

Under field conditions potato plants affected with black leg showed typical blackening of the basal part of the stem. The rot involved also stolons and mother tubers. In some cases the rot extended to aerial parts of the plant and the foliage showed wilt symptoms and yellowing of the leaflets. Depending upon the stage of the disease, stem rot occurred along and up the stem, with blackening of the edges of the stem.

Tubers collected in vegetable stores and farmer's markets showed two kinds of rot. In most cases the tubers showed a hard dark rot, with little odor. The rot extended to the interior with black-brown and white cream zones. This rot was observed especially in those tubers affected by physical damage (e. g. harvesting tools). The second kind of rot was a more typical soft-rot. Interior tissues were soft, slimy, clear colored and foul smelling. In all cases the cuticule was not affected.

Pectinolitic Activity on Beraha Semi-solid Pectate Medium

The cultures tested showed two kinds of reactions. First, the non-pectinolitic organisms grew on the medium but no liquefaction occurred; also a change from green to blue color was observed around the colonies. On the other hand, pectinolitic organisms caused a liquefaction of the medium and a change from green to yellow. This was observed after 24 h of growth at room temperature 20°C ± 2°C.

Symptoms of Inoculated Sliced Potato Tubers

All the cultures tested in moist chambers under laboratory conditions showed similar symptoms. The center of the rot was white and cream colored with black margins. The tissues were changed to a soft mass by pectic enzymes. Starch was not affected and the cuticule remained unchanged. Along the margins of the rot it was possible to detect a black strip which marked the boundary between healthy and diseased tissues.

Biochemical Characteristics of Erwinia Isolates

Ten biochemical reactions and growth characteristics of 16 isolates tested are presented in Table 1. Type cultures (Echr 120; Eca SR44 and Ecc SR100) are presented in italics; Erwinias isolated from Chilean material are presented in numbers.

Discussion

Field data indicates that potato plants affected by soft rot *Erwinia* at the base of the stem showed classical black leg symptoms. Among other characteristics, blackening of the stem and destruction of roots and stolons indicated the presence of soft rot *Erwinia*. However, plant symptoms are insufficient for species separation since Eca and Ecc may be found affecting potato plants in the field (Pérombelom and Kelman, 1980). Among the virulent strains isolated, three were obtained from plants affected with black-leg symptoms; one was identified as Ecc and two as Eca (Table 1). Therefore, this finding corroborates the observations published by Stanghellini and Meneley (1975) that both Ecc and Eca can cause black-leg on potato plants.

Tuber rot symptoms were recorded during recolection of diseased tubers. Evidence indicates that tubers wounded by harvesting tools develop a dark semi-hard rot. Tissues under these conditions are affected by large amounts of aerobic bacterial species. On the other hand, the foul-smelling rot accompanied by slime affected tissues was observed in undamaged tubers. This more classical *Erwinia* rot indicated few bacterial saprophytic species expressing a more anaerobic kind of rot.

All the isolates obtained (Eca and Ecc) showed identical symptoms when inoculated as pure cultures on potato slices in moist chambers. This confirms the concept that potato soft rot symptoms caused by pure cultures are important characteristics for diagnosis of genera, but not relevant for species differentiation. Eca was isolated four times from tubers as well as Ecc. This fact indicated that Eca and Ecc isolated from Chilean potato parts can cause disease on either potato plants or tubers.

Beraha semi-solid pectate medium proved to be adequate for the purposes of this research. Pure cultures were tested for pectin degradation on this semi-solid base (Echr, Ecc, and Eca grew in similar ways on this medium.) The characteristics of the cultures growing on this rich pectate medium are an excellent system for discarding the non-pectinolitic cultures. Some *Bacillus* isolates did not cause liquefaction of this medium; however, they were able to grow on potato slices in moist chambers. Also, some green fluorescent *Pseudomonads* were isolated, causing some degree of pectate degradation. Therefore, this medium worked in a semi-selective way, making it possible to obtain all *Erwinia* isolates and some *Pseudomonas*.

Moist chambers are excellent systems to detect virulent soft rotting potato bacterial pathogens. Furthermore, they give the possibility of separating *Erwinia* isolates from other soft rot causing bacteria (e.g. strains of *Bacillus* and *Pseudomonas*). The particular look of the rot produced by virulent *Erwinia* makes this task very easy. Thus, after pure cultures are

Table 1. Cultural and biochemical characteristics of thirteen Erwinia isolates obtained from potato plants and tubers in Southern Chile.

	(a) Echr	(b) Eca	E6	E14	E15	E16	E18	E21	E12	E17	(c) Ecc	E8	E9	E10	E11	E13
Gram stain	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Growth at 37oC	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Growth at 40oC	+	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on pectate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tuber rot	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sensitivity to erythromycin	+	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Gas from glucose	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Acid from lactose	−	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Acid from palatinose	−	+	+	+	+	+	+	+	+	+	−	−	−	−	−	−
Acid α-Methil-Glucoside	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Reducing substances from sucrose	−	+	+	+	+	+	+	+	−	−	−	−	−	−	−	−
Isolated from (d)		P	P	T	T	T	P	T	T	T	T	P	T	T	T	T

(a) Ech = *Erwinia chrysanthemic* SR 120
(b) Eca = *Erwinia caratovora* var. *atroseptica* SR 100
(c) Ecc = *Erwinia caratovora* var. *caratovora* SR 44
(d) P = plant; T= tuber.

made from isolations, growth on Beraha medium is determined. Then, soft rot in moist chambers provide the next step, which leads to the elimination of undesirable isolates and to keeping virulent strains of pectinolitic organisms belonging to the *carotovora* group of the genus *Erwinia* (Echr, Eca, and Ecc).

Thirteen isolates were soft rot *Erwinia* from about 130 pure cultures tested. Our soft rot Erwinias were isolated from potato plants with typical black leg symptoms and potato tubers with soft rot decay. No Erwinias or other plant pathogenic bacteria were isolated from plants with brown spots along the stem. Our soft rot Erwinias were Eca (six) and Ecc (five) sensu Pérombelon and Kelman (1980). Two isolates remain uncertain (E12 and E17 in Table 1). They may be considered as intermediate strains in observation since "the different kinds of bacteria are not separated by sharp divisions but by slight and subtle differences in characters so that they seem to blend into each other and resemble a spectrum" (Gordon *et al.* 1977).

Therefore, strains E12 and E17 may be considered close to Eca because they use palatinose and failed to grow at 37 and 40°C. On the other hand, they may be considered close to Ecc because they did not produce acid from α- methyl-glucoside and no reducing substances from sucrose were detected. However, considering the particular climate characteristics of Southern Chile (rainy winters and cool summers) and biochemical features as well as the fact that they were isolated only from potato tubers, these two isolates may be considered as "low temperature strains of Ecc."

Finally, this is the first report for Chile of Eca causing both black leg and soft rot in potato stems and tubers; also, it is the first report of Ecc causing black leg in potato plants. There is no evidence at this moment of the presence of Echr. This research is the starting point of a broader and more detailed taxonomic study, especially to investigate the importance of low temperature strains of Ecc in Southern Chile.

Literature Cited

1. Beraha, L., 1968. A rapid method for the preparation of a semi-solid agar medium for detection of pectolitic enzyme activity in *Erwinia carotovora*. Plant Dis. Rep. 52:167.
2. Ciampi, L., 1972. Estudio taxonómico de los agentes etiológicos causantes de pudriciones húmedas en tubérculos de papa. Ag. Técnica 32:176-181.
3. Ciampi, L., 1972b. Enfermedades bacterianas de la papa en Chile, ecología y distribución. Fitopatología 7:5-14.
4. Ciampi, L., and S. González. 1973. Estudio taxonómico de los agentes etiológicos causantes de pudriciones húmedas en plantas de papas. Ag. Técnica 33:16-20.
5. Gordon, R. E., J. L. Hyde, and J. A. Moore Jr. 1977. *Bacillus firmus-Bacillus lentus*: A series or one species. Int. J. Syst. Bacteriol. 27:256-262.
6. Mujica, F., 1941. Nómina de las enfermedades y pestes de las papas cuya existencia se ha comprobado en el país. Bol. San. Veg. 1:70-72.
7. Pérombelom, M. C. M., and A. Kelman. 1980. Ecology of the soft rot *Erwinia*. Ann. Rev. Phytopathol. 18:361-387.
8. Stanghellini, M., and J. Meneley. 1975. Identification of soft rot *Erwinia* associated with black-leg of potato in Arizona. Phytopathology 65:86-87.

Pectolitic *Bacillus* spp.; Opportunistic or Pathogen

L. Ciampi-Panno

Universidad Austral de Chile
Valdivia, Chile

Abstract

During a study of soft-rot of potato tubers and potato black leg, *Bacillus* species were quite often isolated. These species of pectolytic spore-formers are normally found as part of the soil bacterial flora. They cannot degrade starch, a compound also not degraded by *Erwinia* plant pathogens. High temperatures and wounds from potato harvesting tools allow infection by *Bacillus* spp. These findings suggest that pectolytic species of *Bacillus* are opportunistic rather than true pathogens.

Introduction

During the study of potato soft-rot and black leg, *Bacillus* species were isolated quite often. Early reports indicate that healthy potato tubers may carry *Bacillus* species in their vascular tissues (Hollies, 1951). In Chile, *Bacillus* is normally found with potato tubers and plants affected by soft-rot bacteria (Ciampi, 1972).

A study was conducted to identify the pectolitic *Bacillus* species associated with soft decay of potato tubers and plants, and to clarify the plant pathogenic role of these bacteria.

Materials and Methods

A culture collection was obtained from diseased plants and tubers, including: 48 isolates from potato plants with black leg symptoms, 14 isolates from six rotted tubers (from diseased plants), and 14 isolates from six plants with brown lesions along the stem.

All 67 isolates, stored as pure cultures, were tested on potato slices inside moist chambers. The sliced tubers used measured 4 cm in diameter by 2 cm thick. The pectinolitic Gram positive and sporeforming bacteria were grouped and kept on soil extract agar slant for species determination.

Identification of the isolates was made using material and methods described by Gordon *et al.* (4). For further details of isolation and test on potato slices see paper on *Erwinia* by the same author.

Results

Symptomatology of Diseased Tubers and Plants

Most potato plants and tubers collected under field hot conditions showed typical black leg and soft rot symptoms. A few plants presented

brown lesions at the end of the main root measuring 1 to 2 cm long by 0.5 to 1 cm wide. Other plants presented black lesions along the stem, most of them 10 to 15 cm from the stem end. These lesions were 5 to 10 cm long by 1 cm wide.

Symptoms of Inoculated Sliced Potato Tubers

All 67 isolates were tested on potato slices inside moist chambers. Rot caused by *Bacillus* spp. was easily differentiated from the soft rot caused by *Erwinia* spp. (Fig. 1). The endospore forming isolates showed a brown rot which spread on the surface of the potato up to the edges after 48 h at 25°C.

Under the conditions used for inoculation, potato starch was not affected and only pectic substances seem to be affected.

After three days at 25°C all the tubers were 50% rotted. Older inoculated tubers have a tendency to become dark and black in the surface.

Pectinolitic Activity of Bacillus Isolates on Beraha Semi-solid Pectate Medium

All the *Bacillus* isolated showed superficial growth on this medium. The pectate-rich base was not liquified and no acidification of it occurred. On the other hand, *Erwinia* isolates developed a deep digestion of the pectin with strong acidification of the medium (yellow color). All the *Bacillus* isolates also showed an alcalinization (blue color) during growth on this medium.

Biochemical and Cultural Characteristics of Isolates of Bacillus

All the *Bacillus* isolated were Gram positive motile rods with cylindrical and central endospores. The sporangia were not definitely swollen and globules were absent in protoplasm of stained cells. All the cultures showed growth at pH 5.7; produced a positive Voges-Proskauer reaction and fermented glucose, maltose, and xylose. The pH in the Voges-Proskauer broth was 4.9 to 5.2. Arabinose was not fermented, starch tested on plates was not hydrolized, and the cultures did not grow anaerobically. Reduction of nitrates to nitrites was not detected. According to these characteristics, the isolates were identified as *Bacillus pumilus* Meyer and Gottheil, 1901.

Discussion

Diseased plants and tubers under field conditions may have been invaded by other pectinolitic bacteria besides *Erwinia* soft-rotters. The isolation of *Bacillus pumilus* from diseased potato tubers and stems with black-leg symptoms is an indication that this species may play an important plant pathogenic role.

From 18 plants with typical black-leg symptoms, three *E. carotovora* and *B. pumilus* were isolated. The greater abundance of *Bacillus* over *Erwinia* is not explained at this time. Probably, work with a specific medium for isolation of *Erwinia* such as CVP medium (Cuppells and Kelman, 1974), could lead to detection of more *Erwinia* isolates. However, the lack of proper chemicals made this impossible. Another explanation of the low
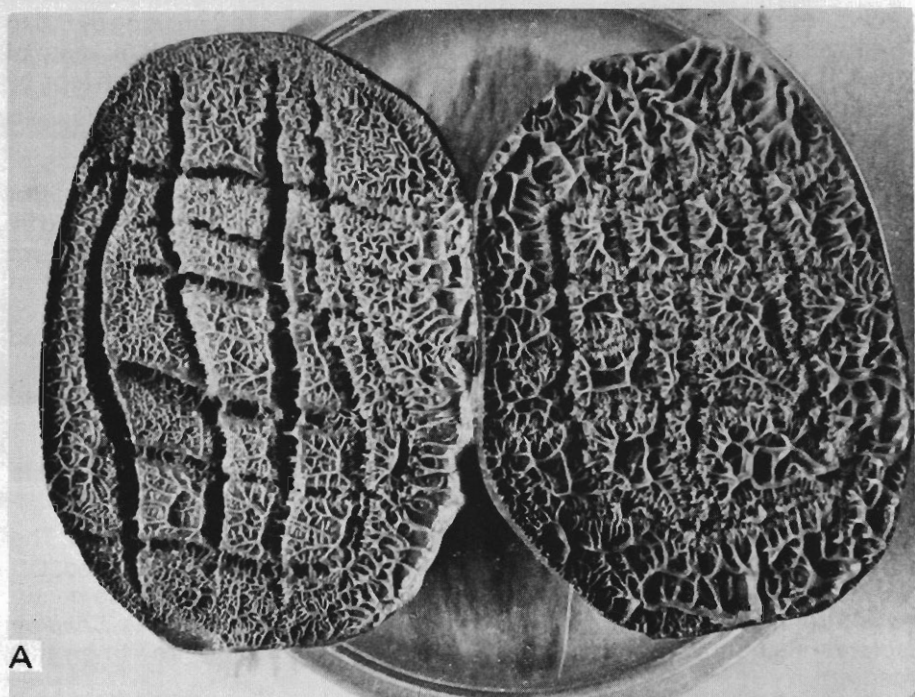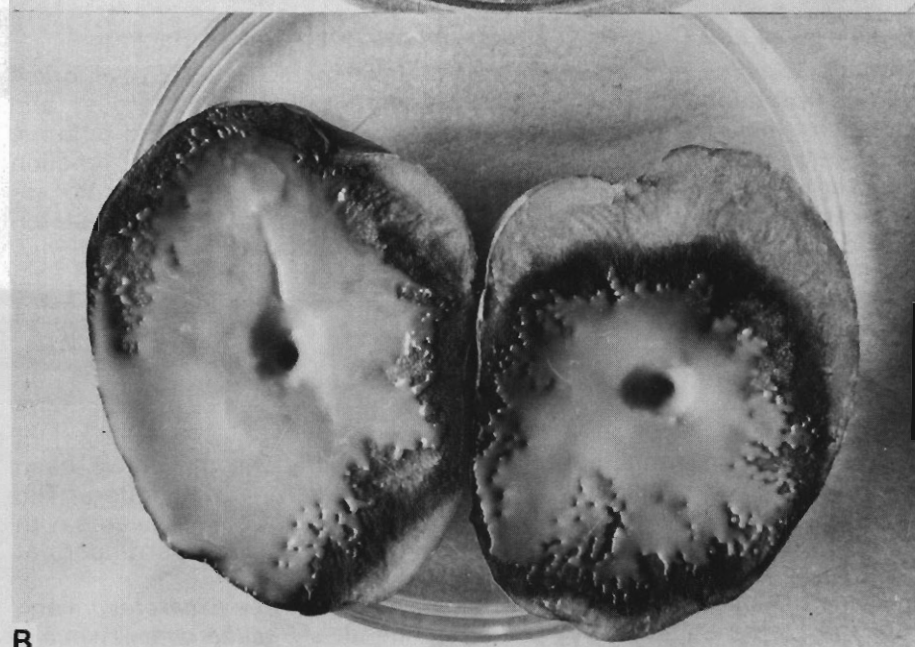
Fig. 1. Potato tubers inoculated with *Bacillus pumilus* (a) and *Erwinia carotovora* (b) kept inside moist chambers during 48 h at 25°C.

numbers of *Erwinia* could be the advanced stages of the potato stems with black-leg symptoms. The wilted stems of the plants were 50% rotted and affected by many different species of microorganisms. Under these conditions, decayed tissue is mostly invaded by saprophytes. Probably, in early stages of the disease, *Erwinia* members are more numerous than *Bacillus* and secondary invaders.

An important step in separating pectinolitic species is to inoculate potato slices inside moist chambers. Symptoms on the surface of sliced potatoes usually provide an excellent means of grouping the different genera of organisms involved in black-leg and potato soft-rot. This is evident in Figure 1. *E. carotovora* rot is typical and very different from *B. pumilus* rot.

Another way to differentiate pectinolitic organisms is by inoculating young cultures with Beraha semisolid pectate medium. *B. pumilus* isolates are unable to cause liquefaction of this medium under our laboratory conditions. On the other hand, *E. carotovora* isolates caused a deep crater and acidification when growing on this medium.

Microscopic preparations showed that growth of *B. pumilus* isolates occurred in intercellular spaces and in collapsed cells of potato tissue. Potato cells were loose, indicating that the pectic components of the middle lamella were liquified. Also, under these conditions, starch was not hydrolized and remained unaffected. Even though *B. pumilus* representatives caused rot of potato tissue by degrading pectic substances, they were unable to liquify sodium polypectate (polygalacturamic acid-sodium salt from SIGMA) on plates. This could indicate that *Bacillus* species have particular and different pectic enzymes from those of *Erwinia*.

B. pumilus species were isolated only from plants with typical black-leg lesions during warm days in summer time. No plant pathogenic bacteria were isolated from eight potato stems with brown lesions at the base of stem and roots. This clearly indicates that tissues of potato stems and tubers, after a soft-rot process initiated by *Erwinia* species, harbor a particular flora which has a role in degradation and mineralization of organic matter from plant tissues (Ciampi, 1981).

The advanced stages of rotted potato stems and the warm weather conditions suggest that *B. pumilus* is an important organism involved in the degradation or organic matter, and is not a true plant pathogen. However, the question whether *Bacillus* species are capable of indicating per se a plant pathogenic process remains uncertain.

Literature Cited

1. Ciampi, L. R., 1972. Enfermedades bacterianas de la papa en Chile, ecología y distribución. Fitopatología 7:5-14.
2. Ciampi-Panno, L. 1981. Bacterias fitopatógenas y el nicho degradativo. Fitopatología 16:20-27.
3. Cuppels, D., and A. Kelman. 1974. Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. Phytopathology 64:468-475.
4. Gordon, R. E., W. C. Haynes, and C. Hor-Nay Pang. 1973. The genus *Bacillus*. Agriculture Handbook 427. U.S.D.A.
5. Hollis, J. P., 1951. Bacteria in healthy potato tissue. Phytopathology 41:350-356.

Acknowledgment

Plasmids in Virulent and Avirulent Strains of *Erwinia stewartii*

D. L. Coplin, R. D. Frederick, M. H. Tindal, S. L. McCammon

Ohio State University Agri. Research and Development Center, Wooster, OH, U.S.A.

Abstract

Plasmids in 39 strains of *Erwinia stewartii* were examined by agarose gel electrophoresis. Most virulent strains had 11 to 13 plasmids ranging in molecular mass from 2.8 to 210 megadaltons (Mdal) and contained plasmids of 2.8, 8.8, 16.8, 29.5, 43, 49, 70, and 210 Mdal. *Bam*HI restriction patterns of total plasmid DNA from six virulent strains were almost identical, indicating that many of the same plasmids were present in these strains. Avirulent strains tended to have fewer plasmids than virulent strains. Bacteriophage Mu pf7701, a *kan*[f] *kil*[-] derivative of Mu *cts*62 was inserted into pDC190, the 68 Mdal plasmid of SS104. Transfer of the Mu pf7701 hybrid plasmid, designated pDC191, to two virulent strains, produced a class of weakly virulent transconjugants. These transconjugants contained pDC191 and a new 51 Mdal plasmid and were missing the 68 and 21.6 Mdal resident plasmids. Our results suggest that a mutation in pDC190 can decrease the virulence of *E. stewartii*.

Introduction

Stewart's wilt of corn caused by *Erwinia stewartii* is an excellent model system for studying the genetics of pathogenicity. We have previously shown than *E. stewartii* has a complex plasmid system which comprises as much as 25% of its genome (5). Plasmids in virulent strains SW2 and SS104 have been characterized by agarose gel electrophoresis (AGE) and electron microscopy. Strain SS104, which we have been using for most of our genetic studies, harbors plasmids with mean molecular masses of 2.7, 2.8, 8.8, 16.7, 21.6, 29.5, 41.5, 43, 48, 68, and 210 Mdal. Some of the plasmids in this strain are conjugative (4). The purpose of this study was to compare the size classes of plasmids in virulent and avirulent strains and, in addition, we present preliminary data that a mutation in the 68 Mdal plasmid of SS104, pDC190, can decrease virulence.

Materials and Methods

Culture media, growth conditions, storage of bacteria, and procedures for filter matings have been described previously (2). Some of the *E. stewartii* strains used in this study are given in Table 1. Other virulent strains (with prefix SW) were isolated from diseased corn plants collected throughout the midwestern, southern, and northeastern states in 1974

Table 1. Origin of Erwinia stewartii strains

Strain	Phenotype	Source
DC211	Wild type	D. Coplin
GC6, LC	Wild, type	A. Karr
SS104	Wild type	ICPB[a]
DC150	Avirulent	M. Turner
ES-4	Avirulent, nonencapsulated	T. Woods
SS10 to SS13	Avirulent, nonencapsulated	ICPB (Lindstrom, 1940)
DC283-19A	SS104 Na1R (Mu pf7701)	S. McCammon (8)
DC336	SS104 Na1R (Mu cts62)	D. Coplin (3)
DC350	Avirulent, non pigmented, Rif[R] variant of SS104	L. N. Gibbins (6)

[a] ICPB denotes the International Collection of Phytopathogenic Bacteria, M. P. Starr, curator.

and 1975. Bacteriophage Mu pf7701, a temperature-inducible, kanamycin resistant (KmR), kil⁻ derivative of Mu cts62, was obtained from Martha Howe (11).

Plasmid DNA was isolated by the rapid alkaline lysis technique of Birnboim and Doly (1) and electrophoresed on 0.5% agarose (Seakem ME) gels in Tris-acetic acid buffer at 5.0 V/cm, as described previously (5). Plasmid RA1 (86 Mdal) and SW2 and SS104 plasmid were used as molecular mass standards. For restriction digests, plasmid DNA was isolated as above, phenol extracted once, and ethanol precipitated twice. Restricted DNA was electrophoresed in 0.7% agarose gels at 1.5 V/cm. Restriction endonucleases and bacteriophage λDNA were obtained from Bethesda Research Laboratories, Inc. and used according to their recommendations.

Virulence of *E. stewartii* was determined on 8-day-old Earliking sweet corn seedlings. Plants were grown in a controlled environment chamber at 30°C under a mixture of cool white fluorescent and incandescent lights (23,000 lux) with a 16 h daylength. Toothpicks were dipped in agar cultures of bacteria and used to wound the seedling 1 cm above the soil line. Symptoms were rated at 5 and 15 days after inoculation, on a scale from 1 to 5 (1=no symptoms, 2=scattered lesions, 3=slight wilting, 4=severe wilt, 5=dead).

Results

Distribution of Plasmids in *Erwinia stewartii*

To identify those plasmids most frequently found in virulent strains, we screened 31 virulent and 8 avirulent strains for plasmids by AGE (Fig. 1, Table 2). These strains represented different geographic areas and times
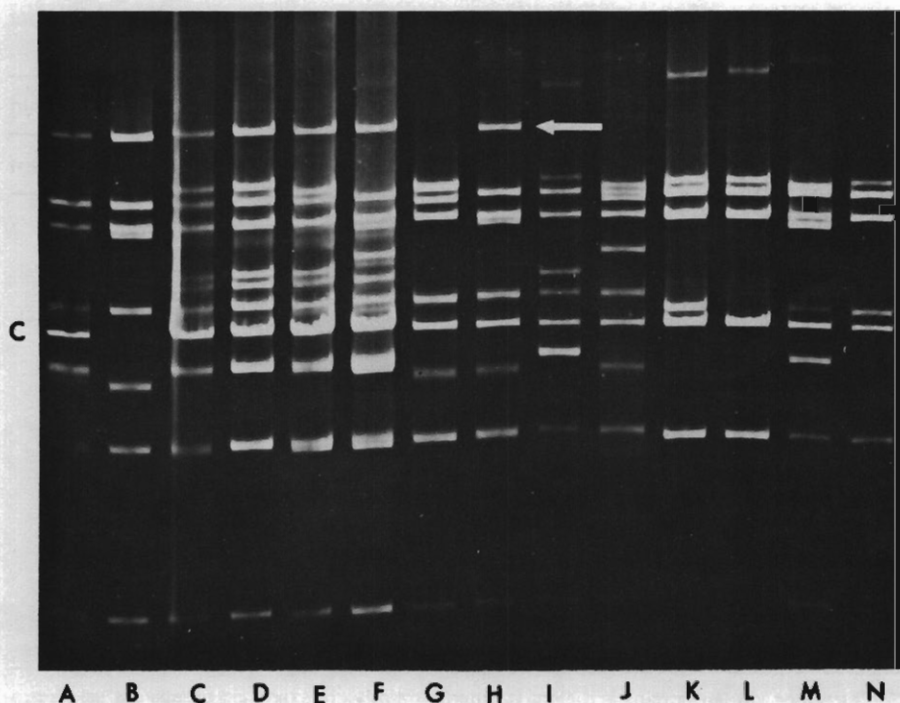
Fig. 1. Agarose gel electrophoresis of plasmid DNA from *E. stewartii* strains (A) SW18, (B) SS104, (C) SW2, DSW14, (E) SW13, (F) SW3, (G) GC6, (H) LC, (I) DC150, (J) ES-4, (K) SS10, (L) SS12, (M) SS11, and (N) SS13. Strains in lanes A-H are virulent and those in lanes I-N are avirulent. The arrow indicates the 68-70 Mdal plasmids and C denotes chromosomal DNA.

of isolation. All strains contained multiple plasmids. Twenty-nine of 31 virulent strains had from 11 to 13 plasmids; the fewest was 8 in one strain. In each case the plasmid profiles were very similar (Fig. 1). Eight size classes of plasmids (2.8, 8.8, 16.8, 29.5, 43, 49, 70, and 210 Mdal) were found in 87% or more of the virulent strains (Table 2). In cases where one of these "common" plasmids was missing, a unique plasmid, which could have been derived from the missing plasmids, was often present. For example, three of four strains missing the 8.8 Mdal plasmid had a 10.2 Mdal plasmid; SWII was missing the 43 Mdal plasmid and had a 28 Mdal plasmid; and SW3 was missing the 210 Mdal plasmid and had a 130 Mdal plasmid. Other size classes of plasmids were also quite frequent; 23, 33, 34.5, and 51 Mdal plasmids were found in 29 to 65% of the strains.

Avirulent strains (SS10 through SS13), which have been in culture over 40 years, had fewer plasmids but those present were typical of the size classes found in contemporary strains. In regard to the "common" plasmids, 6 of 8 avirulent strains were lacking the 70 Mdal plasmid and 5 of 8 were either missing the 29.5 Mdal plasmid or it appeared to have a small deletion.

Table 2. Distribution of plasmids in virulent and avirulent strains of *Erwinia stewartii*

Size class Mdal	% Strains with plasmid[a]		Size class Mdal	% Strains with plasmid	
	Virulent	Avirulent		Virulent	Avirulent
205-215	97	75	34-35	65	12
130	3	0	33-34	65	25
77	3	0	31	3	0
67-70	97	25	29-30	94	38
51-52	29	25	28	3	38
50-51	23	62	23-24	48	38
48-50	87	88	21-22	16	12
46	3	12	20	3	0
43-44	97	100	16-17	100	100
41-43	26	12	10-11	13	0
38-40	16	0	8-9	87	62
36-37	10	12	2.7 - 2.8	100	100

[a] Thirty-one virulent strains and eight avirulent strains were examined by agarose gel electrophoresis using SW2 plasmids as standards.

The fragments generated by *Bam*HI digestion of total plasmid DNA from six virulent strains (Fig. 2) were almost identical. Virulent strains shared 21 of 23 fragment bands, even though they contained several different plasmids. Digests of avirulent strains contained fewer bands but almost all of them were present in the digests of plasmids from virulent strains.

Isolation and Characterization of Plasmid pDC191

We attempted to label plasmids in SS104, by inducing Mu pf7701 replication in strain DC283-19A, which has Mu pf7701 on its chromosome, and then selecting for conjugal transfer of KmR to strain DC350, which is an avirulent derivative of SS104 lacking the 210, 48, 41.5, and 21.6 Mdal plasmids. We expected to find Mu pf7701 transpositions into those plasmids missing in DC350. DC283-19A was induced at 42°C for 60 min, followed by 37°C for 60 min and then mated with DC350 on filters for 3 h. KmR RifR transconjugants were selected. None of the transconjugants became virulent but one harbored an 89 Mdal plasmid and had lost pDC190 (Fig. 3). This new plasmid was designated pDC191 and possibly represents insertion of Mu pf7701 (24 Mdal) into PdC190 (68 Mdal) accompanied by a small deletion (3 Mdal).

Since many of our avirulent isolates were missing 68 to 70 Mdal plasmids, we decided to study the relationship of pDC191 to virulence. pDC191 was transferred by conjugation from DC350 to avirulent strains DC150 and ES-4 and virulent strains DC211 and DC336. In our first
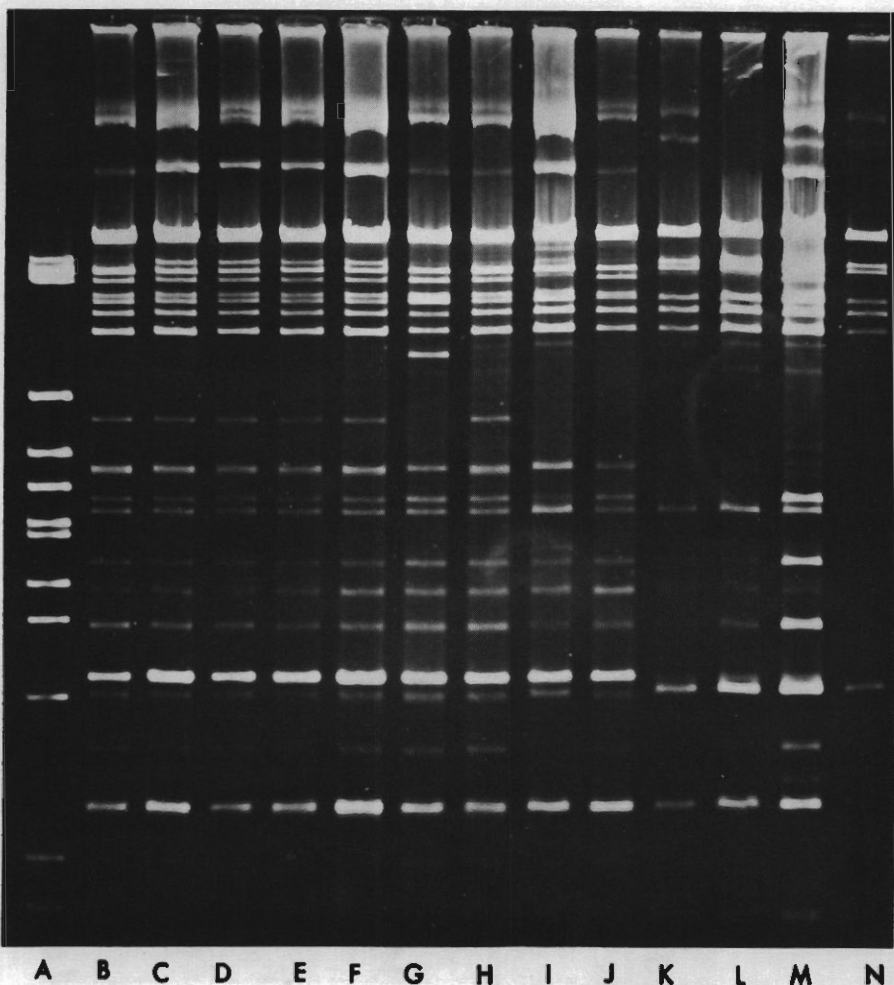
Fig. 2. Agarose gel electrophoresis of *Bam*HI digested total plasmid DNA from *E. stewartii* strains (B) SS104, (C) SW2, (D) SW11, (E) SW3, (F) GC6, (G) LC, (H) DC150, (I) ES-4, (J) SS10, (K) SS12, (L) SS11, and (M) SS13. Lane A contains a mixture of *Eco*RI and *Hind*III fragments. Strains in lanes A-G are virulent and those in lanes H-M are avirulent.

experiments, KmR transferred at $1\text{-}6 \times 10^{-7}$ transconjugants/input donor cell in 2 h matings. However, only 7/18 DC336 and 3/12 DC211 transconjugants contained an autonomous pDC191 plasmid when examined by AGE. In addition to pDC191, these transconjugants also had a new 51 Mdal plasmid and were missing pDC190 and the 21.6 Mdal plasmid (Fig. 3). The virulence of transconjugants was decreased when they harbored pDC191 and lost pDC190 (Fig. 4). The KmR transconjugants which did not have pDC191 probably have Mu pf7701 insertions on the chromosome.
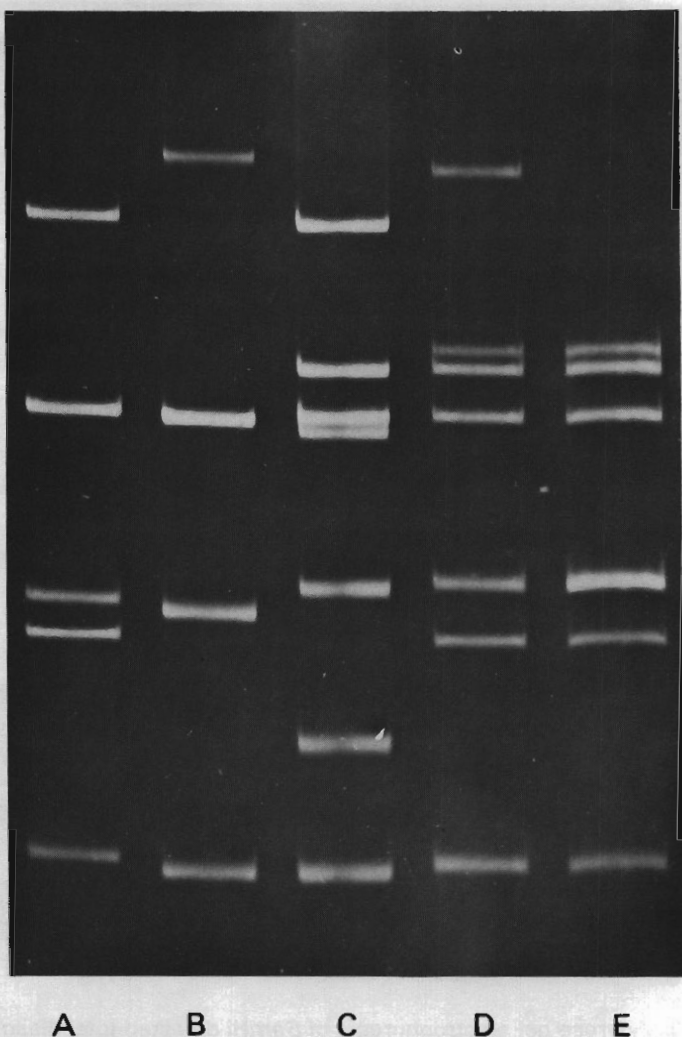
Fig. 3. Agarose gel electrophoresis of plasmid DNA from *E. stewartii* strains (A) DC350, (B) DC350 (pDC191), (C) SS104, (D) DC336 (pDC191), and (E) a DC336 (pDC191) transconjugant, which has been cured of pDC191. C denotes the chromosome. SS104 has a 41.5 Mdal plasmid which is missing in DC 336.

Acquisition of Mu pf7701 alone had no effect on virulence. The disease index for the seven DC336 (pDC191) transconjugants was 1.2 ± 0.1, whereas that for the 11 DC336 (pf7701) strains was 4.7 ± 0.2 and DC336 was 4.8. The pDC191 transconjugants formed occasional lesions but did not cause extensive leaf blight or systemic wilting. Bacteria reisolated from the lesions incited by each of the weakly virulent transconjugants
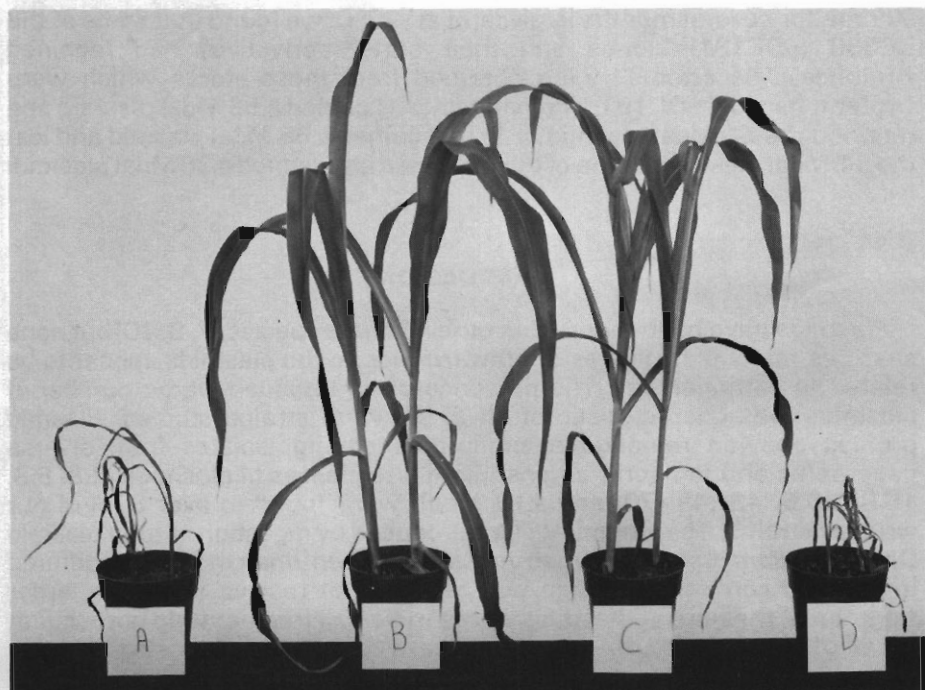
Fig. 4. Earliking sweet corn inoculated with DC336 (pDC191) transconjugants: (A) DC336; (B) a DC336 (pDC191) transconjugant which has (pDC191) and a 51 Mdal plasmid, and is missing pDC190; (C) a spontaneous KmS segregant of 336 (pDC191); and (D) a "revertant" of the segregant strain in C which has lost the 51 Mdal plasmid and reacquired pDC190.

contained the expected plasmid and were still weakly virulent when reinoculated. Similar results were obtained with DC211. The virulence of DC150 and ES-4 was not restored by acquisition of pDC191.

In subsequent experiments, transfer of pDC191 from DC350 to DC336 produced transconjugants which contained 1) a new 63 Mdal plasmid but not pDC191 and were not missing any resident plasmids or 2) pDC191 and a new 36 Mdal plasmid and were missing pDC190. Both types of transconjugants were virulent.

Spontaneous loss of pDC191 was about 0.3% per cell and several KmS segregants were obtained. In addition, a culture of DC336 (pDC191) was enriched for cured strains by heat induction of Mu pf7701 as described above. Twelve cured strains were obtained by screening 700 survivors for KmS. All of the cured strains were missing pDC191 and had retained the 51 Mdal plasmid. Spontaneous segregants remained weakly virulent. Concommitant loss of pDC191 and KmR indicates that Mu pf7701 is present only on pDC191 and the avirulence of the transconjugants is not due to pf7701 insertions at other sites.

All pDC191 transconjugants and their cured derivations were purified several times by streaking and selecting single colonies. However, after

storage for several months in glycerol at -20°C, we found that some of the DC336 (pDC191) clones and their cured derivatives had regained virulence. "Revertants" were obtained from these stocks which were virulent, had lost pDC191, and had either 1) gained a 63 Mdal plasmid and retained the 51 Mdal plasmid or 2) had gained a 68 Mdal plasmid and lost the 51 Mdal plasmid. Some of the latter had also gained a 36 Mdal plasmid.

Discussion

Plasmids have been reported in other *Erwinia* species (7, 9, 10) but none share as many plasmids as *E. stewartii* nor do the plasmids appear to be related to pathogenicity. The presence of an unusually large number of plasmids was characteristic of all *E. stewartii* strains studied. Plasmid profiles showed remarkable similarities among isolates from diverse geographic and temporal origins. Eight size classes of plasmids (2.8, 8.8, 16.8, 20.5, 43, 49, 70, and 210 Mdal) were found in over 87% of our virulent strains. The fragment sizes produced by digestion of total plasmid DNA with *Bam*HI were likewise very similar even when the strains differed in plasmid content. Although our gels did not resolve all of the larger fragments, the finding that differences in *Bam*HI fragments do not account for all of the plasmid DNA that differs between strains suggests that some of the "variable" plasmids may be part of larger "common" cointegrate plasmids. DNA hybridization studies are needed to confirm our notions on the identity of similar-sized plasmids in different strains.

The 68 to 70 Mdal plasmid size class was frequently missing in avirulent strains. By *Bam*HI digestion of pDC190 DNA recovered from agarose gels, we have tentatively assigned the 1.9, 2.6, 4.0, 4.4, 5.5, 13.6 and 15.3 Mdal fragments to this plasmid (data not shown). It is significant that all of these fragments are present in the *Bam*HI digest of GC6 which is the only virulent strain missing the 68 to 70 Mdal plasmid.

Results of this study demonstrate that pDC191 can modify the virulence of its host strains. The most probable interpretation of our results is that pDC191 is defective and upon introduction into a new host displaces the corresponding wild-type resident plasmid, resulting in loss of virulence. At this point the nature of the mutation in pDC191 and how this plasmid affects virulence is not clear. To explain our results we propose the following tentative hypotheses: 1) pDC191 is derived from and incompatible with pDC190 and it contains a mutation that affects virulence. 2) pDC190 is a cointegrate of several or more plasmids. 3) To resolve incompatibility between pDC190 and pDC191 either plasmid may dissociate, integrate into the chromosome, or both. 4) The virulence genes of pDC190 must be on an autonomous plasmid to be expressed.

The site of Mu pf7701 insertion in pDC191 may or may not be related to its effect on virulence. If the insertion occurred in DC283-19A, then Mu pf7701 may have directly inactivated a virulence gene. Alternatively, the recipient DC350 may have a mutation in pDC190 which is partially responsible for its avirulence. Thus, if pDC191 was formed by zygotic induction and transposition of Mu pf7701 after transfer of the prophage to

DC350, then it could contain this mutation and the site of Mu pf7701 insertion would not be related to virulence.

Introduction of pDC191 into a strain that contains pDC190 with selection for pDC191 does not require that the pDC190 be displaced. Instead, either plasmid could dissociate into component plasmids that are compatible, integrate into the chromosome, or both. The latter possibility would explain the formation of new plasmids in DC336 (pDC191) transconjugants. Likewise, integration of pDC190 into the chromosome and its subsequent excision would account for the observed "reversion" of several transconjugants and their cured derivatives and the appearance of new plasmids in these strains. Thus, the effect of pDC191 on virulence appeared to depend on how the incompatibility between it and pDC190 was resolved. Strains with a 68 Mdal plasmid were always virulent. If pDC190 was missing, then strains with the 63 or 36 Mdal plasmids were virulent and those with the 51 Mdal plasmid were avirulent. Loss of the 21.6 Mdal plasmid was not considered important because we have other derivatives of SS104 that are missing this plasmid but still virulent.

Since our DC336 transconjugants which have been cured of pDC191 are still weakly virulent, our assumption that they contain an integrated pDC190 plasmid also implies that the putative virulence gene is not expressed while the plasmid is integrated.

Literature Cited

1. Birnboim, H. D., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids. Res. 7:1513-1523.
2. Coplin, D. L., 1978. Properties of F and P group plasmids in *Erwinia stewartii*. Phytopathology 68:1637-1643.
3. Coplin, D. L., 1979. Introduction of bacteriophage Mu into *Erwinia stewartii* by use of an RK2::Mu hybrid plasmid. J. Gen. Microbiol. 113:181-184.
4. Coplin, D. L., and R. G. Rowan. 1979. Conjugative plasmids in *Erwinia stewartii*, p. 67-73. *In* Station de Pathologie Végétalé et Phytobactériologie. (ed.) Proc. 4th Int. Conf. Plant. Path. Bact. Angers, 1978. I.N.R.A. Boucouze.
5. Coplin, D. L., R. G. Rowan, D. A. Chisholm, and R. E. Whitmoyer. 1981. Characterization of plasmids in *Erwinia stewartii*. Appl. Environ. Microbiol. 45:(In press)
6. Garibaldi, A., and L. N. Gibbins. 1975. Induction of avirulent variants in *Erwinia stewartii* by incubation at supraoptimal temperatures. Can J. Microbiol. 21:1282-1287.
7. Gibbins, L. N., P. M. Bennett, J. A. Sanders, J. Grinstead, and J. C. Connolly. 1976. Acceptance and transfer of the R-factor RP1 by members of the "herbicola" group of the genus "*Erwinia.*" J. Bacteriol. 128:309-316.
8. McCammon, S. L., R. G. Rowan, and D. L. Coplin. 1980. Properties of *Erwinia stewartii* plasmid pDC250 containing bacteriophage Mu pf7701 and Tn10 insertions. Phytopathology 71:240.
9. Panopoulos, N. J., M. V. Guimares, S. Hua, C. Sabersky-Lehman, S. Resnik. M. Lai and S. Schaffer. 1978. Plasmids in phytopathogenic bacteria, p. 238-241. *In* D. Schlessinger (ed.), Microbiology, American Soc. for Microbiology. Washington, D.C.

10. Sparks, R. B., and G. H. Lacy. 1980. Purification and characterization of cryptic plasmids pLS1 and pLS2 from *Erwinia chrysanthemi*. Phytopathology 70:369-373.

11. Thompson, C. J., and M. Howe. 1979. Tn5 induced mutations in bacteriophage Mu. Abstr. No. 544. Ann. Mtg. Amer. Soc. Microbiol.

Progress in Chromosomal Genetics of *Erwinia chrysanthemi*

A. K. Chatterjee, M. A. Brown, J. S. Ziegle, K. K. Thurn

**Kansas State University
Manhattan, KS, U.S.A.**

Abstract

As adjuncts to the existing Hfr mating system in *E. chrysanthemi*, we have sought and detected chromosome transfer by various R and F plasmids and by a generalized transducing temperate bacteriophage, Erch-12, originating in a Dieffenbachia strain of *E. chrysanthemi*. Generalized chromosome mobilization was obtained with R plasmids R68.45 and R100drd56. In contrast, the R plasmid, RP4::Tn7 mobilized a limited number of chromosomal markers. The bacteriophage Erch-12 transduced *ade, arg, his, idg* (a locus that specifies pigment (indigoidine) production), *ilv, leu, rif, ser, thr, trp* and *ura*. The transfer of markers unlinked on the chromosome of *E. chrysanthemi* indicated that the bacteriophage mediated generalized transduction. By performing two-factor conjugational and transductional crosses, linkage was detected between various markers. Based upon the linkage data we have revised the partial chromosomal linkage map of *E. chrysanthemi*.

We have obtained mutations promoting temperature-dependent segregation of a broad host range plasmid, pUT 13 which carries transposons Tn402 (trimethroprim-resistance) and Tn406 (carbenicillin-resistance). The host range of the plasmids includes *Erwinia amylovora, E. carotovora, E. chrysanthemi, Escherichia coli, Pseudomonas savastanoi, P. syringae,* and *Salmonella typhimurium*. Our preliminary findings suggest that the mutant plasmid could serve as a transpositional vehicle in phytopathogenic *Erwinia*. In addition, both pUT 13 and its thermosensitive mutant mobilized chromosomal markers in *E. chrysanthemi*. Apparent transposition of Tn5 (Km^r) from the plasmid, pJB4J1 to the genome(s) of *E. chrysanthemi* (EC16) occurred at a high frequency and produced mutations resulting in auxotrophy or non-utilization of carbohydrates. Thus, these plasmids are potentially useful tools in genetics of *E. chrysanthemi* and other *Erwinia* spp.

Introduction

Erwinia chrysanthemi infects at least 27 different plant species worldwide (14, 15). Although the pectolytic enzyme, polygalacturonic acid *trans*-eliminase, appears to play a crucial role in bacterial maceration of plant tissues (2, 9, 10, 16, 25), the identity of other pathogenic factor(s) largely remains unknown.

Recognizing the potential advantages of genetic approaches in probing pathogenic mechanisms of *E. chrysanthemi*, we developed an Hfr-type mating system (9) which allowed mapping of an assortment of chromosomal loci including a gene (*pat*) that specifies production of

polygalacturonic acid *trans*-eliminase. A partial chromosomal linkage map for this bacterial species was depicted (3) based upon gradient of gene transfer, times of entry of gene loci, and linkage between markers. Our susbsequent studies revealed that the formation of Hfr-type donors in *E. chrysanthemi* was a rare event. In the absence of Hfr strains with different directions of chromosome transfer, we could not precisely map gene loci that were distal to the origin and hence transferred infrequently. Moreover, until recently, it was not possible in *E. chrysanthemi* to perform reciprocal crosses using donor strains of desired genotype.

We have resolved these problems by mobilizing chromosomal segments of *E. chrysanthemi* using various R plasmids and a temperate bacterio-phage. These genetic systems now allow fine structure gene mapping in *E. chrysanthemi* (11). We briefly discuss here some of those findings and summarize the properties of a mutant R plasmid potentially useful in genetic studies on *Erwinia* spp. and other Gram negative phytopathogenic bacteria.

Table 1. Chromosome mobilization ability (Cma) of R plasmids in *Erwinia chrysanthemi* strains EC16 and EC183.[a]

R plasmid	*E. chrysanthemi* strain[b]	Gene loci transferred[c]
R68.45	EC16	ade, gal, gtu, his, leu, lys, thr, trp
R68.45	EC183	ade, rif[r]
R100 drd-56	EC183	arg, ilv, leu, ser, thr
RP4::Tn7	EC183	arg, idg
pUT 13	EC183	ser, trp
pUT 13 ts-1	EC183	ser, trp

[a] Matings were done on membranes for 5-6 h using the procedure of Chatterjee (4).

[b] Wild-type strains and their derivatives were used.

[c] Frequency of transfer generally ranged from 10^{-7} - 10^{-4} per input donor cell depending upon the marker and the plasmid.

Materials and Methods

The details of bacterial strains, cultural conditions, and genetic techniques can be found in Chatterjee (4); Chatterjee and Brown (5, 6) and Chatterjee and Starr (8, 9). The following plasmids were used: F'*ts lac*+::Tn10 (John Roth); RP4::Tn7 and pJB4J1::Tn5 (T. C. Currier); R100*drd*-56 (International Collection of Phytopathogenic Bacteria, Davis; 7), and pUT 13 (R. Meyer; 20).

Linkage Between Chromosomal Markers

The data presented in Figs. 1 and 2 and Table 2 show linkage between various pairs of markers on the chromosomes of *E. chrysanthemi* strains
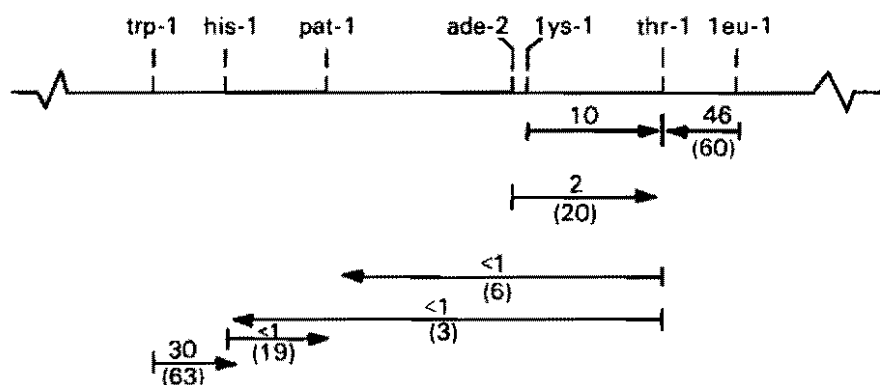
Fig. 1. Linkage between chromosomal loci of *Erwinia chrysanthemi* EC16. Numbers above the arrow are percent coinheritance values in recombinants derived from crosses between R68.45 donor and appropriate recipient strains (4). Those shown parenthetically below the arrow are from recombinants of Hfr crosses (9). The same sets of recipients were used in both types of crosses. Arrowheads represent unselected markers and tails represent selected markers.
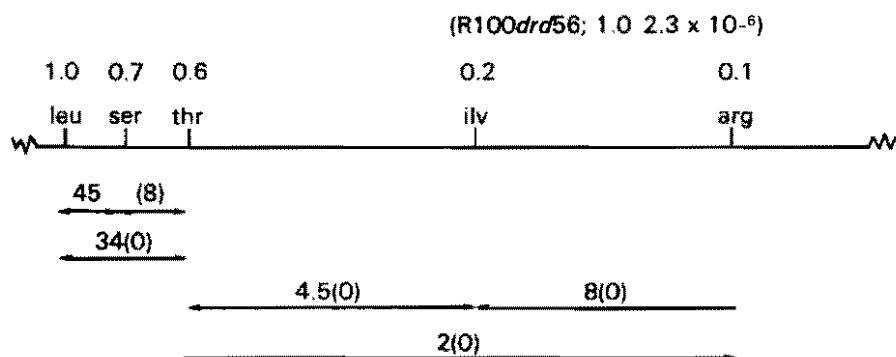
Fig. 2. Cma of R100*drd*-56 in *Erwinia chrysanthemi* strain EC 183 and linkage between chromosomal gene loci. Numbers above the gene loci are the frequencies of transfer normalized with respect to the frequency of the transfer of *leu*. Numbers above arrows are average percent coinheritance values between markers. Those shown parenthetically are from transductional crosses.

EC16 and EC183. Preliminary findings tentatively suggest a high homology in gene order on chromosomes of these strains. Based upon that assumption and our present and earlier findings (3, 9, 11), we show in Fig. 5 a composite chromosomal linkage map of *E. chrysanthemi* strains EC16 and EC183. Our recent findings are consistent with the earlier prediction (3) that the chromosomal linkage map of *E. chrysanthemi* was probably very similar to those of other enterobacteria such as *Escherichia coli* or *Salmonella typhimurium*. By establishing overlapping transductional

Table 2. Linkage between chromosomal markers in *Erwinia chrysanthemi* strain EC183 by transductional and conjugational crosses.[a]

Gene loci (Mode of gene transfer)	Donor (relevant property)	Recipient (relevant property)	Selection	No. tested	Coinheritance Inherited marker	% Coinheritance
I. thr-ser	AC6016	AC6018				
(Transduction)	(Thr$^+$, Ser$^-$)	(Thr$^-$, Ser$^+$)	Thr$^+$	200	Ser$^-$	3.5
	AC6018	AC6016				
	(Thr$^-$, Ser$^+$)	(Thr$^+$, Ser$^-$)	Ser$^+$	199	Thr$^-$	5.0
II. thr-leu						
(Conjugation)	EC183 (R100drd-56)	AC6048	Thr$^+$	50	Leu$^+$	34.0
	(Thr$^+$, Leu$^+$, Str^s)	(Thr$^-$, Leu$^-$, Str^r)	Leu$^+$	50	Thr$^+$	33.0
III. ade-rif						
(Transduction)	EC183	AC6033	Ade$^+$	236	Rif^s	3.3
	(Ade$^+$, Rif^s)	(Ade$^-$, Rif^r)				
(Conjugation)	AC6051 (R68. 45)	AC6033	Ade$^+$	98	Rif^s	50.0
	(Ade$^+$, Arg$^-$, Leu$^-$, Rif^s)	(Ade$^-$, Arg$+$, Leu$^+$, Rif^r)				
IV. arg-idg						
(Transduction)	EC183	AC6055	Arg$^+$	100	Idg$^-$	72
	(Arg$^+$, Idg$^-$)	(Arg$^-$, Idg$^+$)				
(Conjugation)	AC6059 (RP4:: Tn7)	AC6062	Arg$^+$	135	Idg$^-$	93
	(Arg$^+$, Idg$^-$, Nal^s)	(Arg$^-$, Idg$^+$, Nal^r)				

[a] Refer to Chatterjee (4) and Chatterjee and Brown (5) for the details of transductional and conjugational crosses and conditions for the selection of various recombinant classes. Idg$^+$ = pigment (indigoidine) production; Rif^r = Rifampin resistance.

linkages between additional markers we now are assessing the extent of the genetic homology between these phytopathogenic and non-phytopathogenic enterobacteria.

Construction of a Temperature-Sensitive (ts) Plasmid Vehicle

To use drug-resistant translocatable (Tn) elements in genetic studies of *E. chrysanthemi*, we attempted introducing R(RP4::Tn7, pJB4J1::Tn5)and F (F'ts *lac⁺*::Tn10) plasmids into the strains EC16 and EC183 of this bacterial species. While all of these plasmids were readily transferred from *E. coli* to *E. chrysanthemi* strain EC16, only the R plasmid, RP4::Tn7 was received by strain EC183. In preliminary trials, we detected the occurrence of Km^{r}Gm^s clones of EC16 (pJB4J1) and Str^r Tp^{r}Km^s clones of EC16 (RP4::Tn7), suggesting instability of the plasmid vehicle and translocation of Tn5 (Km^r) and Tn7 (Str^{r}Tp^r) in this bacterial host. Subsequently, by using the plasmid, pJB4J1::Tn5, we obtained auxotrophic or carbohydrate fermentation mutants in strain EC16 that also were Km^r and Gm^s. Genetic analysis of such presumptive insertion mutations is in progress. In *E. chrysanthemi* (EC183), RP4::Tn7 was stable since segregants carrying Tn7 markers (Str^{r}Tp^r) were not obtained.

Although F'ts *lac⁺*::Tn10 was normally expressed in *E. chrysanthemi* strain EC16, the inability of the bacterium to grow well at 37°C or higher prevented us from exploiting the temperature-sensitive replication of the plasmid in generating transpositional mutations. It became apparent that for efficient transpositional mutagenesis in *E. chrysanthemi* strain EC183, we needed a vehicle that was unstable under physiological conditions.

In contrast to F plasmid or bacteriophages (as λ or P22), R plasmids belonging to the P incompatibility group, because of their broad host range, are potentially useful in genetic studies on *Erwinia* spp. and other Gram negative phytopathogenic bacteria. We therefore attempted the isolation of a temperature-sensitive mutant of the R plasmid, pUT 13 (20) which is derived from R751 and carries transposons Tn402 (trimetroprim-resistance) and Tn406 (carbenicillin-resistance). Following nitrosoguanidine mutagenesis and D-cycloserine selection in the presence of drugs at 40°C, we obtained a mutant (pUT 13 *ts-1*) that segregated at high frequency upon growth of several bacterial hosts at 30°C or higher (see below). The host range of pUT 13 and pUT 13 *ts-1* included *E. amylovora, E. carotovora, E. chrysanthemi, Escherichia coli, Pseudomonas savastanoi, P. syringae,* and *Salmonella typhimurium* (Table 3).

A temperature-dependent segregation of the mutant plasmid (pUT 13 *ts-1*) occurred in *E. chrysanthemi* (EC183) and *E. coli* (2492) hosts (Table 4; Fig. 6). The frequency of drug-sensitive (Cb^{s}Tp^s) segregants at 30°C ranged from 11% to 45%, depending upon bacterial species employed; upon growth at 37°C, 99.9% of the clones of these bacterial strains were Cb^s and Tp^s. Under similar growth conditions the parent plasmid, pUT 13 was stable in these hosts (Table 4; Fig. 6).

Results and Discussion

Chromosome Mobilization by R Plasmids

To complement the Hfr mating system, we examined R plasmid-

Table 3. Transfer of pUT 13 and pUT 13 *ts-1* from *E. coli* (2492) to *Erwinia*, *Pseudomonas* and *Salmonella* spp.[a]

Recipient	Frequency of transfer (per input donor cell)	
	pUT 13	pUT 13 *ts-1*
E. amylovora (EA178)	1.4×10^{-5}	3.1×10^{-5}
E. carotovora (EC)	2.2×10^{-5}	3.3×10^{-8}
E. chrysanthemi (EC16)	5.6×10^{-5}	1.6×10^{-2}
E. coli (K12)	3.4×10^{-2}	4.0×10^{-1}
P. savastanoi (2009)	1.3×10^{-7}	4.6×10^{-6}
P. syringae (179)	5.2×10^{-5}	6.2×10^{-4}
S. typhimurium (LT2)	2.1×10^{-5}	3.6×10^{-5}

[a] Crosses were done according to Chatterjee (4) on membranes at $30°C$. pUT 13 *ts-1* is a temperature sensitive mutant of pUT 13 (see text for the details).

mediated chromosome mobilization in strains EC16 and EC183 of *E. chrysanthemi*. The data summarized in Table 1 show that the R plasmid, R68.45 transferred an array of chromosomal markers in the strain EC16; the frequency of gene transfer did not vary enough to suggest polarity in chromosome transfer. Comparison of the data on coinheritance (linkage) between markers in recombinants derived from R68.45 donor to those from Hfr donors (Fig. 1), suggested that R68.45-carrying strains, as opposed to the Hfr strain, transferred smaller chromosomal segments. Nevertheless, our data indicated a generalized chromosome transfer by R68.45 and consequently, a potential use of this plasmid in genetic analysis of the strain EC16 of *E. chrysanthemi*.

In contrast, in the strain EC183, the frequency of the transfer of R68.45 was low (about 5×10^{-8} transconjugants per input donor cell) from an *E. coli* strain. Thus far, using ECI83/R68.45 transconjugants as donors, we have detected transfer of two closely linked markers (Tables 1 and 2). Moreover, chromosome mobilization ability (Cma) of R68.45 in the strain ECI83 was markedly unstable since a progressive loss of Cma occurred upon cultivation on a complex (L) medium containing drugs (ampicilin, kanamycin, and tetracycline). Recent studies with R68.45 have shown that the plasmid carries a DNA duplication (approximately 2.1 kb) near the gene that confers Km^r (12, 13, 19, 23). Moreover, the finding of Currier and Morgan (12, 13) with naturally occurring deletions of R68.45 in various *Erwinia* spp. suggested that the duplicated region of the R68.45 genome probably was responsible for the Cma of the plasmid. Thus, the variability in Cma of R68.45 in strains EC16 and EC183 could be ascribed to relative stabilities of the duplicated DNA segments (perhaps an IS sequence; 27) in these bacterial hosts.

In contrast to R68.45, other R plasmids such as R100*drd*-56, RP4::Tn7, or pUT 13 were readily transferred from *E. coli* donors to *E. chrysanthemi*

(EC183). These plasmids as well as pUT 13 *ts*-1 (a temperature-sensitive mutant of pUT 13; see below) mobilized chromosomal markers (Tables 1 and 2). The R plasmid, R100*drd*-56 mobilized several chromosomal genes; the gradient in transfer frequency (Fig. 2) suggested a polarized chromosome transfer. It remains to be determined if this apparent polarity resulted from a transient site-specific interaction of the R plasmid with the chromosome of the strain EC183 or from chromosome transfer by a minority of Hfr cells present in the population of plasmid-containing bacteria. This uncertainty notwithstanding, the system can be used to estimate linkage between markers on the chromosome of *E. chrysanthemi* strain EC183 (Fig. 2).

Our data as well as those of others (17, 18) on Cma of R plasmids in *E. chrysanthemi* reveal two different patterns: (i) generalized Cma, i.e., transfer of a number of chromosomal markers as with R68.45 in EC16 or R100*drd*-56 in EC183; and (ii) specialized Cma, i.e., transfer of few (linked) markers as with RP4::Tn7 and pUT 13 in EC183. These differential patterns probably reflect the occurrence on the host chromosome of sites at which plasmids can interact, albeit transiently, promoting chromosome transfer.

Generalized Transduction

Transduction, i.e., bacteriophage-mediated transfer of bacterial genes, is a powerful tool in fine structure gene mapping. Bacteriophages infect many phytopathogenic bacteria (21, 26). Lysogeny also is prevalent in various phytopathogenic species. Despite that, until recently, gene transfer by transduction was not reported in any such bacterial species. Our approach (5) in developing a transducing system in *E. chrysanthemi* involved inducing a temperate bacteriophage in a wild-type strain of *E. chrysanthemi*, propagating the virus in a susceptible strain (EC183), and testing the transfer of chromosomal markers by UV irradiated and non-irradiated bacteriophage lysates. Since this approach might also be applicable to other phytopathogenic bacterial species, we briefly consider here some of the salient features.

Paulin and Nassan (22) reported lysogeny in wild-type strains of *E. chrysanthemi*. We confirmed that observation, by treating cultures of wild-type strains (obtained from Dr. R. S. Dickey, Cornell University) with the drug, mitomycin C. While all of the 15 strains tested lysed in the presence of the drug (Fig. 3), we detected plaque formation with lysates of three strains. The bacteriophage (Erch-12) that plaqued on the strain EC183 was investigated further.

The UV irradiated lysate produced various classes of prototrophic (*ade*$^+$, *arg*$^+$, *leu*$^+$, *his*$^+$, *ser*$^+$, *thr*$^+$, *trp*$^+$, *ura*$^+$) and drug-resistant (*rif*^{r}) recombinants. With unirradiated lysate most of those recombinant classes were obtained, but compared to irradiated lysates the frequency of transfer was 5 to 50 fold lower, depending upon the marker (Fig. 4; also see Chatterjee and Brown, 5). A relationship existed between the extent of viral inactivation and the frequency of transduction (Fig. 4); highest transductional frequency was generally noted at a UV dosage that inactivated 50 to 99.9% of the input phage particles. That the bacteriophage mediated generalized transduction was indicated by the transfer of an assortment of unlinked markers in the presence or absence of DNase.
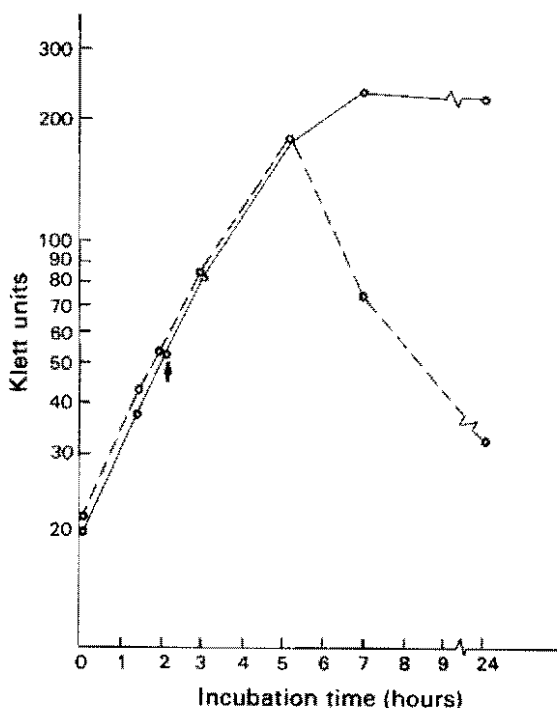
Fig. 3. Effect of mitomycin C on growth of *Erwinia chrysanthemi* strain KS612. Mitomycin C (5 ng/ml, final concentration) was added to an exponentially growing culture (dashed line) at the indicated time (arrow) and incubation was continued at 30°C in dark. Untreated culture (solid line) served as a control.

In *E. amylovora*(EA178) and *E. coli*(K12), loss of the mutant plasmid, but not of the parent plasmid, occurred at a high frequency upon growth at 30°C. In contrast to the remarkable instability of the mutant plasmid in those hosts, in *Salmonella typhimurium* (LT2) both the mutant and parent plasmids were stable at 30°C or 37°C (Table 4). Although the basis for this stability is at present unclear, the following two possibilities might be considered: (i) host factor(s) allow replication (maintenance) of the temperature-sensitive plasmid, thereby phenotypically suppressing the temperature-sensitive (Ts) phenotype; or (ii) the *ts* mutation on the plasmid genome reverts at a high frequency in this host. Investigations in progress should allow us to distinguish between these possibilities.

To determine if Tn402 (Tp^r) or Tn406 (Cb^r) transposed into genomes of various bacterial species, we first looked for Cb^{r}Tp^s or Cb^{s}Tp^r segregants of the plasmid, pUT 13 *ts-1*, and then tested such segregants for changes in phenotypic properties. The data (Table 4) show the occurrence of Cb^{r}Tp^s (but not of Cb^{s}Tp^r) clones in *E. amylovora* (EA178), *E. carotovora* (EC),

Table 4. Segregation of drug-resistance markers upon growth at 30° C or higher in bacteria harboring the plasmid pUT 13 or pUT 13 ts-1.[a]

Bacterial species	Strain	Growth			pUT 13			pUT 13 ts-1				
		Temperature °C	tested	$Cb^r Tp^r$	$Cb^r Tp^s$	$Cb^s Tp^r$	$Cb^s Tp^s$	tested	$Cb^r Tp^r$	$Cb^r Tp^s$	$Cb^s Tp^r$	$Cb^s Tp^s$
Erwinia amylovora	EA 178	30	90	90	0	0	0	180	1	16	0	163
		32	90	90	0	0	0	180	0	4	0	176
E. carotovora	EC	30	90	90	0	0	0	90	86	3[b]	0	1
E. chrysanthemi	EC 183	30	180	176	0	0	4	180	102	0	0	78
		37	180	180	0	0	0	180	0	0	0	180
Escherichia coli	K 12	30	90	90	0	0	0	180	45	1	0	134
		37	90	90	0	0	0	180	1	2	0	177
	2492	30	90	90	0	0	0	100	62	0	0	38
		37	90	90	0	0	0	90	0	0	0	90
Salmonella typhimurium	LT2	30	90	90	0	0	0	90	86	4[b,c]	0	0
		37	90	90	0	0	0	90	78	12[b,c]	0	0

[a] Bacteria (about 10^5 cells/ml) were inoculated to L broth and grown to saturation (about 5×10^9 cells/ml) at indicated temperature. Dilutions were spread on L agar and incubated at 30° C. Individual colonies were patched on L agar, L agar + carbenicilin (200 ng/ml) and L agar + trimethoprim (200 ng/ml). Flollowing 24 hr incubation at 30° C, growth was scored. $Tp^r Cb^s$ segregants were detected in none of these bacterial species.

[b] $Cb^r Tp^s$ clones of these species were also transfer-deficient.

[c] $Cb^r Tp^s$ segregants of *S. typhimurium* were insensitive to the bacteriophage PRD1, while the parent (LT2/pUT 13 *ts-1*) was sensitive to the bacteriophage.
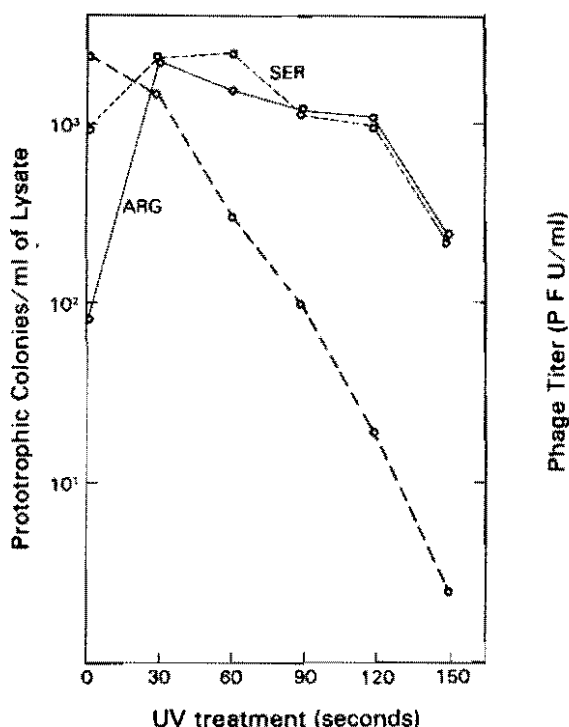
Fig. 4. Effects of UV irradiation on inactivation of the bacteriophage Erch-12 and the frequency of transduction of ser+ and arg.+ For the details of UV irradiation and transductional crosses refer to Chatterjee and Brown (5).

Escherichia coli (K12), and *Salmonella typhimurium* (LT2) upon growth at 30°C or higher. However, at higher growth temperature (i.e., 37°C as opposed to 30°C), Cb^{r}Tp^s segregants occurred more frequently in *S. typhimurium* but not in other bacterial species tested. None of the Cb^{r}Tp^s clones thus far examined are auxotrophic. However, such segregants of *E. carotovora* (EC) and *S. typhimurium* (LT2) were transfer-deficient. Moreover, the segregants of *S. typhimurium* (LT2) were insensitive to the bacteriophage PRD1, although transfer-proficient cells on this bacterial strain harboring pUT 13 or pUT 13 *ts-1* were sensitive to the bacteriophage. These findings tentatively suggest that the Cb^{r}Tp^s phenotype of the segregants of *E. carotovora* and *S. typhimurium* probably resulted from transposition of Tn406 into the genomes of these bacterial species.

In *E. chrysanthemi* (EC183/pUT 13 *ts-1*), despite the loss of the plasmid at high frequency upon growth at 35°C or 37°C, we did not detect Cb^{r}Tp^s or Cb^{s}Tp^r segregants when cells were grown at these temperatures on a non-selective medium (viz. L broth). We then examined the effect of carbenicillin selection on cells grown at 35°C on the occurrence of Cb^{r}Tp^s clones. In such an experiment, of 350 clones tested, 100 were Cb^{r}Tp^s.
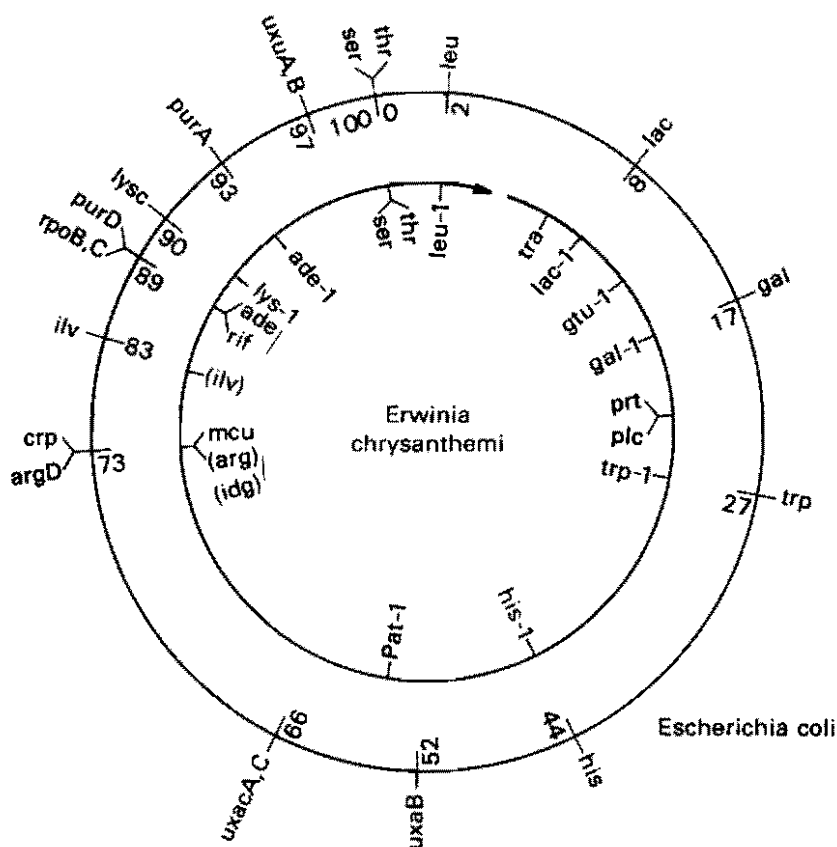
Fig. 5. Comparative linkage maps of *Erwinia chrysanthemi* and *Escherichia coli* K12 (1). *E. coli* map is shown as a closed circle. The numbers represent the map positions of *E. coli* markers. Circularity of the *E. chrysanthemi* chromosome remains to be established. Lines above gene loci indicate transductional linkage between markers. Designation of uncommon markers: *gtu+* = utilization of D-galacturonate; *idg+* = production of the blue pigment (in digoidine); *pat+* polygalacturonic acid *trans*-elliminase production; *mcu+* = utilization of carbohydrates (glycerol, arabinose, ribose, xylose, galacturonic acid, polygalacturonic acid).

None of those segregants, however, were either auxotrophic or deficient in any of the extracellular enzymes. We currently are examining the effects of D-cycloserine enrichment on the recovery of insertion mutations that either are auxotrophic or are unable to utilize various carbohydrates.

In summary, our data show that the broad host range plasmid, pUT 13 and its Ts mutant can be introduced into several Gram-negative phytopathogenic bacterial species. The mutant plasmid (pUT 13 *ts-1*) may prove useful in transpositional mutagenesis in the temperature range of 30 to 35°C. In this context it is noteworthy that Robinson *et al.* (24) obtained a temperature-sensitive mutant (pMR5) of the broad host range R plasmid,

399

Fig. 6. Segregation of pUT 13 and its thermosensitive mutant, pUT 13 *ts-1* in *Erwinia chrysanthemi* strain EC 183 upon growth at various temperatures. Cells of EC183 carrying either pUT 13 or pUT 13 *ts-1* grown for 16 h at 30 C on L agar + carbenicillin (200 ng/ml) + trimethoprim (200 ng/ml) were suspended in L broth. Appropriate dilutions were inoculated to fresh L broth to yield an approximate bacterial population of 10^4-10^5 cells/ml. Cultures were grown at indicated temperature for about 16 h. Serial dilutions were spread on L agar, and incubated at 30°C. Colonies that developed in 24 to 36 h were then replicated onto L agar, L agar + carbenicillin (200 ng/ml), and L agar + trimethoprim (200 Ng/ml). Growth was scored after 24 h incubation at 30°C.

RP1. In an *E. coli* host, the frequency of the segregation of the mutant plasmid (pMR5) was about 77% at 37°C and about 99.9% at 42°C (24). In contrast, the segregation frequency of pUT 13 *ts-1* was 99.9% at 35 or 37°C (Fig. 6; table 4). Since temperature maxima for growth of most phytopathogenic bacteria are considerably lower than that of *E. coli*, we predict greater usefulness of pUT 13 *ts-1* in genetic studies on these bacteria. Moreover, in *E. chrysanthemi* (EC183), we have detected chromosome mobilization by pUT 13 and pUT 13 *ts-1* (Table 1) indicating interaction(s) of these plasmids with the host chromosome. We currently are exploring the possibility of isolating Hfr-type donors in *E. amylovora* and *E. chrysanthemi* by integrative suppression of Ts phenotype of the mutant plasmid.

In concluding this article, we would like to briefly reflect upon the progress that has occurred in genetics of *E. chrysanthemi* during the past

four years since the last Conference at Angers, France, and outline some of the tasks that lie ahead. In terms of genetic system among phytopathogenic bacteria, *E. chrysanthemi* is by far the most developed. A new Hfr strain has been obtained by inserting F'*ts lac*⁺::Tn10 into the chromosome of *E. chrysanthemi* strain pathogenic on *Saintpaulia ionantha* (A. Kotoujansky, Institut National Agronomique, Paris-Grignon, France; personal communication). An assortment of R plasmids are known to mobilize chromosomal markers.

Generalized transduction now allows fine structure mapping of chromosomal genes. Knowledge has been acquired of the location of about 18 different gene loci on the chromosome. A plasmid vehicle is now available which should allow transpositional mutagenesis, construction of additional Hfr strains, and gene isolation. These developments now pave the way for genetic analysis of such pathogenic determinants as cell surface components and pectolytic enzyme. We predict that future developments will occur in those areas as well as in our understanding of factors that determine host-compatibility of this important pathogen.

Literature Cited

1. Bachmann, B. J., and K. B. Low. 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. 44:1-56.
2. Bashan, H. G., and D. F. Bateman. 1975. Relationship cell death in plant tissue treated with a homogeneous endopectate lyase to cell wall degradation. Physiol. Plant Pathol. 5:249-262.
3. Chatterjee, A. K., 1978. Genetics of phytopathogenic bacteria Pp. 3-16. Proc. 4th Int. Conf. on Plant Path. Bact., Angers, France.
4. Chatterjee, A. K. 1980. Acceptance by *Erwinia* spp. of R plasmid R68.45 and its ability to mobilize the chromosome of *Erwinia chrysanthemi*. J. Bacteriol. 142:111-119.
5. Chatterjee, A. K., and M. A. Brown. 1980. Generalized transduction in the enterobacterial phytopathogen *Erwinia chrysanthemi*. J. Bacteriol. 143:1444-1449.
6. Chatterjee, A. K., and M. A. Brown. 1981. Chromosomal location of a gene (idg) that specifies the production of the blue pigment indigoidine in *Erwinia chrysanthemi*. Submitted for publication.
7. Chatterjee, A. K., and M. P. Starr. 1972. Transfer among *Erwinia* spp. and other enterobacteria of antibiotic resistance carried on R factors. J. Bacteriol 112:576-58.
8. Chatterjee, A. K., and M. P. Starr. 1973. Gene transmission among strains of *Erwinia amylovora*. J. Bacteriol. 116:1100-1106.
9. Chatterjee, A. K., and M. P. Starr. 1977. Donor strains of the soft-rot bacterium *Erwinia chrysanthemi* and conjugational transfer of pectolytic capacity. J. Bacteriol. 132:862-869.
10. Chatterjee, A. K., and M. P. Starr. 1978. Genetics of pectolytic enzyme production in *Erwinia chrysanthemi*. Pp. 89-84. Proc. 4th Int. Conf. Plant Path. Bact., Angers, France.
11. Chatterjee, A. K., and M. P. Starr. 1980. Genetics of *Erwinia* species. Annu. Rev. Microbiol. 34:645-676.
12. Currier, T. C., and M. K. Morgan. 1981. A direct DNA repeat in the plasmid R68.45 is associated with deletion formation and concomitant loss of chromosome mobilization ability (Cma). Submitted for publication.

13. Currier, T. C., and M. K. Morgan. 1981. Restriction endonuclease analyses of the Inc PI plasmids RK2, RP1, RP4, R68, and R68.45. Current Microbiol. 5:323-327.

14. Dickey, R. S., 1979. *Erwinia chrysanthemi:* A comparative study of phenotypic properties of strains from several hosts and other *Erwinia* species. Phytopathology 69:324-329.

15. Dickey, R. S., 1981. *Erwinia chrysanthemi:* Reaction of eight plant species to strains from several hosts and to strains of other *Erwinia* species. Phytopathology 71:23-29.

16. Garibaldi, A., and D. F. Bateman. 1971. Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. Physiol. Plant Pathol. 1:25-42.

17. Guimaraes, W. V., N. J. Panopoulos, and M. N. Schroth. 1978. Conjugative properties of IncP, in *Erwinia chrysanthemi* and *Pseudomonas phaseolicola.* Pp. 53—65. Proc. 4th Int. Conf. Plant Path. Bact. Angers, France

18. Lacy, G. H., 1978. Genetic studies with plasmid RP1 in *Erwinia chrysanthemi:* Strains pathogenic on maize. Phytopathology 68:1323-1330.

19. Leemans, J., R. Villarroel, B. Silva, M. Van Montagu, and J. Schell. 1980. Direct repetition of 1.2 M DNA sequence is involved in site-specific recombination by the PI plasmid R68. Gene 10:319-328.

20. Meyer, R. J., and J. A. Shapiro. 1980. Genetic organization of the broad-host-range IncP-1 plasmids R751. J. Bacteriol 143:1362-1373.

21. Okabe, N., and M. Goto. 1963. Bacteriophages of plant pathogens. Annu. Rev. Phytopathol. 1:397-418.

22. Paulin, J. P., and N. A. Nassan. 1978. Lysogenic strains and phage-typing in *Erwinia chrysanthemi,* pp. 539-545. Proc. 4th Int. Conf. on Plant Path. Bact., Angers, France.

23. Riess, G., B. W. Holloway, and A. Puhler. 1980. R68.45, a plasmid with chromosome mobilizing ability (Cma) carries a tandem duplication. Genet. Res. 36:99-109.

24. Robinson, M. K., P. M. Bennett, S. Falkow, and H. M. Dodd. 1980. Isolation of a temperature-sensitive derivative of RP1. Plasmid 3:343-347.

25. Stephens, G. J., and R. K. S. Wood. 1975. Killing of protoplasts by soft-rot bacteria. Physiol. Plant Pathol. 5:165-181.

26. Vidaver, A. K., 1976. Prospects for control of phytopathogenic bacteria by bacteriophages and bacteriocins. Annu. Rev. Phytopathol. 14:451-465.

27. Willetts, N. S., C. Crowther, and B. W. Holloway. 1981. IS21 and chromosome mobilization by R68.45. The Proc. of Society for General Microbiology Quarterly. Vol. 8, p. 88 (S 25).

Identification of F'-Like Plasmids of *Pseudomonas syringae* pv. *phaseolicola*

L. J. Szabo, J. Volpe, D. Mills

Oregon State University
Corvallis, OR, U.S.A.

Abstract

The phytopathogenic bacterium *Pseudomonas syringae* pv. *phaseolicola* strain LR700 harbors a single indigenous 98 x 10⁶ dalton (Mdal) cryptic plasmid, pMC7105. A "plasmidless" derivative of this strain, LR719, has been shown to have pMC7105 integrated into the bacterial chromosome (Curiale, M.S., Ph.D. Thesis, Oregon State University). Preliminary results indicated that pMC7105 could be partially excised from the chromosome of LR719 when three colonies bearing excision plasmids of 35, 60, and 58 Mdal were detected. EcoRI and BamHI fingerprint analyses confirm that these plasmids are essentially comprised of a subset of fragments from pMC7105. More recently, we have isolated 11 additional colonies from LR719 which harbor a single excision plasmid. These plasmids vary in size from approximately 20 to 180 Mdal. A BamHI restriction analysis of a 145 Mdal excision plasmid, pEx8050, revealed that it contains essentially all of pMC7105 and an additional 47 Mdal of DNA which is chromosomal in origin. The episomal nature of pMC7105 may be exploited in developing a genetic system for phytopathogenic pseudomonads analogous to the Hfr system of *Escherichia coli.*

Introduction

The discovery of naturally occurring plasmid in isolates of plant pathogenic pseudomonads (2, 4, 7, 8, 9) has stimulated interest in identifying functions that are associated with particular plasmids. That certain plasmids have a role in determining disease expression was recently demonstrated for *P. syringae* pv. *savastanoi* (2). A 34 x 10⁶ dalton (Mdal) plasmid, pIAA, was shown to encode for two enzymes essential for indoleacetic acid production and gall formation on olive. A 35 Mdal plasmid of *P. syringae* pv. *syringae* also may encode virulence functions since cells cured of this plasmid become avirulent (7, 8). The genetic properties of a plasmid isolated from *P. syringae* pv. *phaseolicola* indicate that it may play an important role in studying both chromosomal and plasmid-encoded functions. This 98 Mdal plasmid, pMC7105, was recently shown to be integrated into the bacterial chromosome in strain LR719 (3). Since LR719 was obtained following treatment with mitomycin C, it is not known whether integration occurred naturally or was induced.

Initially, three excision plasmids were detected among randomly selected colonies of this strain and their molecular sizes are 38, 50, and 58 Mdal (3). Since imprecise excision resulted in the formation of miniplasmids that were 40 to 60 percent smaller than pMC7105, it was reasoned that other excision events could lead to the formation of plasmid-chromosome hybrids, analogous perhaps, to the F-prime (F') plasmids of *Escherichia coli*. F'-like plasmids would provide an excellent genetic system for studying both chromosomal and plasmid encoded functions.

This paper presents preliminary results on the frequency of excision of pMC7105 from the chromosome of *P. syringae* pv. *phaseolicola* and biochemical properties of a large excision plasmid, pEX8050.

Materials and Methods

Bacterial Strains and Media

Three strains of *Pseudomonas syringae* pv. *phaseolicola,* LR719, LR721, and PP805 were used in this study. A 98 Mdal cryptic plasmid, pMC7105 (3), used as a reference standard in this study, was isolated from strain LR721. A closely related strain, LR719, which has pMC7105 integrated in its chromosome, was used to screen for excision plasmids. Strain PP805 was derived from LR719 and it contains a single 145 Mdal excision plasmid, pEX8050.

All cultures were grown and maintained on MaNY medium, a modified MaSNY (4) medium which lacks sucrose. Liquid cultures were grown at 26°C on a gyratory shaker at 200 rpm.

Extraction and Isolation of Plasmid DNA

Screening for excision plasmids from LR719 was accomplished using a modified procedure of Kado and Liu (10). Cells were grown in 10 ml of liquid medium to stationary phase, harvested, and washed once in E buffer [40 mM tris (hydroxymethyl) aminomethane, 40mM acetate,· 2mM ethylenediaminetetraacetic acid, pH 7.9]. The pellet was resuspended with 0.5 ml of E buffer, and lysed by the addition of 1 ml of lysis solution [3% (w/v) sodium dodecylsulfate, 50 mM Tris (hydroxymethyl) aminomethane, solution pH 11.6] and gentle agitation. The lysate was then extracted with a phenol/chloroform solution as previously described (10). Samples of approximately 50 nl were analyzed for plasmid content by gel electrophoresis.

DNA for restriction enzyme analysis was prepared from 0.5 l aliquots of bacteria grown in MaNY medium to an absorbance at 600 nm of 0.6 to 0.8. The cells were lysed and DNA extracted by a modified Currier and Nester (5) procedure in which the shearing step was omitted. Covalently closed circular DNA was isolated on CsCl-ethidium bromide gradients containing 51% CsCl (w/v). Equilibrium centrifugation was carried out at 20°C in a Beckman VTi65 vertical rotor (Beckman Instruments, Fullerton, CA 92632), for 16 to 18 h at 50,000 rpm. Plasmid DNA was removed with a syringe from the side of the gradient tube through a 22 gauge needle. The plasmid DNA was further purified by recentrifugation in a second ethidium bromide-CsCl gradient. The ethidium bromide was removed by several

extractions with ice cold isopropanol and the DNA samples were dialyzed against T buffer [20 mM Tris (hydroxymethyl) - aminomethane, 1 mM ethylenediaminetetraacetic acid, pH 8.0].

Endonuclease Restriction Analysis

Plasmid DNA was cleaved in a 150 nl reaction mixture, containing 2 ng of DNA, the appropriate buffer (6) and 10 to 20 units of restriction endonuclease. After 2 h at 37°C, the reaction was stopped by the addition of 15 nl of a solution containing 3M NaCl, 0.2M ethylenediaminetetraacetic acid. The DNA was precipitated by adding 400 nl of absolute ethanol and incubating at -10°C for 2 to 12h. The precipitate was collected by centrifugation at 10,000 rpm for 20 min at -10°C in a Sorvall SS34 rotor, resuspended in 30 nl of T buffer and heated for 5 min at 70°C.

Gel Electrophoresis

DNA samples were analysed by electrophoresis in a horizontal slab gel containing 0.7% agarose (Grade II, Sigma, St. Louis, Mo 63178) dissolved in running buffer [40mM Tris (hydroxymethyl) aminomethane, 20mM acetate, 1 mM ethylenediaminetetraacetic acid, pH 8.1] (6), at 3V/cm, for 10 h. The gels were illuminated with 302 nm light (Transilluminator, Model C-63, Ultra-Violet Products, 5100 Walnut Grove, San Gabriel, CA91778) and photographed with Polaroid type 55 film (Polaroid Corporation, 575 Technology Square, Cambridge, MA 02139). The number and molecular masses of the DNA fragments were determined from densitometer tracings of negatives on a Gilford Gel Scanner Model 2520 (Gilford Instrument Laboratories Inc., Oberlin, OH 44074). HindIII and XhoI digests of λ DNA were used as molecular weight standards.

DNA-DNA Hybridization

DNA fragments were transferred to diazobenzyloxymethyl (DBM) paper and hybridization conditions were performed as described by Alwine *et al.* (1). pMC7105 probe DNA was labeled with α^{32} [P] - dCTP to a specific activity of 14 x 10^6 cpm/ng using the nick translation kit manufactured by New England Nuclear (549 Albany Street, Boston, MA 02118). Autoradiography was performed using Kodak (Polaroid Corporation, 575 Technology Square, Cambridge, MA 02139) no-Screen-X-Ray film NS-2T, at -80°C for 40 to 90 h.

Results

One hundred colonies of LR719 were screened by agarose gel electrophoresis and 11 were shown to contain a single excision plasmid. In only one experiment has it been difficult to demonstrate that the plasmids were generated by independent excision events. In that experiment each of the three colonies which were randomly selected from a single petri dish harbored a plasmid approximately 145 megadaltons (Mdal) in size. The remaining eight excision plasmids differ significantly in size, ranging from 20 to 180 Mdal. The extreme difference in size of some of these plasmids

relative to the integrated indigenous plasmid (98 Mdal) indicated that excision events could generate both miniplasmids and F-prime-like plasmids which carry chromosomal sequences.

Restriction Endonuclease Analysis of pEX8050

The large excision plasmid pEX8050 (Fig. 1) was selected for further analysis because its size (145 Mdal which is nearly 1.5 fold larger than pMC7105), suggested that it may carry a significant amount of
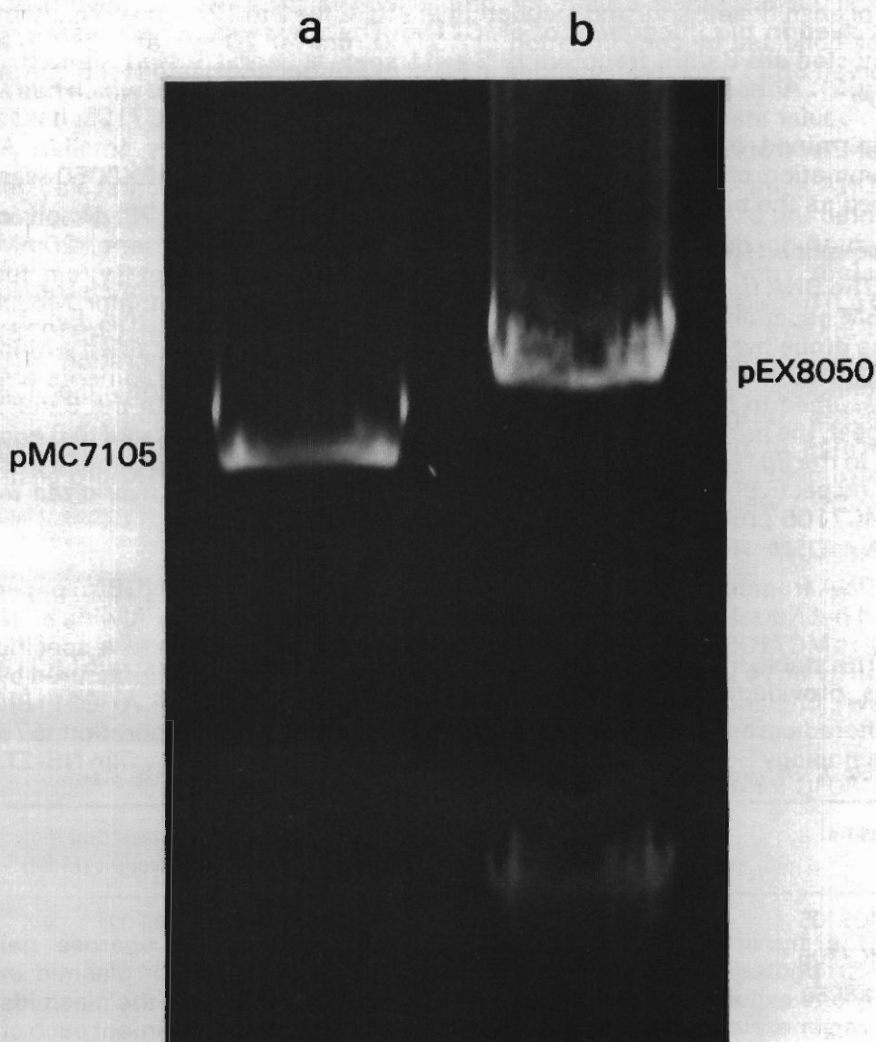
Fig. 1. Agarose gel electrophoresis of plasmid DNA from *P. syringae* pv. *phaseolicola.* (a) pMC7105, 98 Mdal; (b) pEX8050, 145 Mdal.

chromosomal DNA. Furthermore, if the excision event occurred in chromosomal DNA outside of the site of integration, a plasmid restriction fragment which was involved in the integration event may be readily identified by its disappearance from the fingerprint pattern of pMC7105.

The BamHI, Bg1II, and EcoRI fingerprints of pMC7105 and pEX8050 are presented in Figure 2. The wild-type plasmid was cut into 18, 22, and 38 fragments by BamH1, Bg1II, and EcoRI, respectively, whereas these same restriction enzymes cleaved pEX8050 into 32, 32, and 48 fragments, respectively. For each digest of pEX8050, all of the fragments of pMC7105 could be discerned except for one, which is presumed to have been involved in the integration of pMC7105. The fragments which were not detected are BamHI fragment 8, Bg1II fragment 6, and EcoRI fragment 7 (Fig. 2). Although a BamHI fragment of pEX8050 was detected which had a molecular mass approximately the size of fragment 8 of pMC7105, it was determined from tracings of the negatives to be slightly smaller. A summation of the molecular weights of the fragments of pEX8050 was used as the basis for determining its molecular size (Table 1).

Sequence Homology Between pMC7105 and pEX8050

The DNA fragments from the gel shown in Fig. 2 were blot transferred to DBM paper and hybridized with ^{32}P-labeled pMC7105 probe DNA (Fig. 3). The probe hybridizes to all of the fragments of pMC7105 contained within pEX8050 and one new fragment in each digest. The new fragments are presumed to be plasmid-chromosome juncture fragments. In the BamHI digest, the probe hybridized to fragments B and weakly hybridized to C and F. In the Bg1II and EcoRI digests, pMC7105 hybridized to fragments C and F, respectively. None of the other fragments of pEX8050 hybridized to pMC7105 probe DNA.

Discussion

The stable integration of pMC7105 into the chromosome in strain LR719 has provided a unique opportunity to develop a genetic system for

Table 1. Approximate molecular weights of *P. syringae* pv. *phaseolicola* plasmids.

Plasmid	Enzyme	Number of fragments	Calculated total molecular weight (Mdal)[a]
pMC7105	BamHI	18	98.02
pMC7105	PstI	34	98.58
pEX8050	BamHI	32	144.27
pEX8050	Bg1II	32	143.90
pEX8050	EcoRI	48	146.54

[a] Molecular weights were calculated by comparison with λ phage DNA fragments produced by HindIII and XhoI (6).
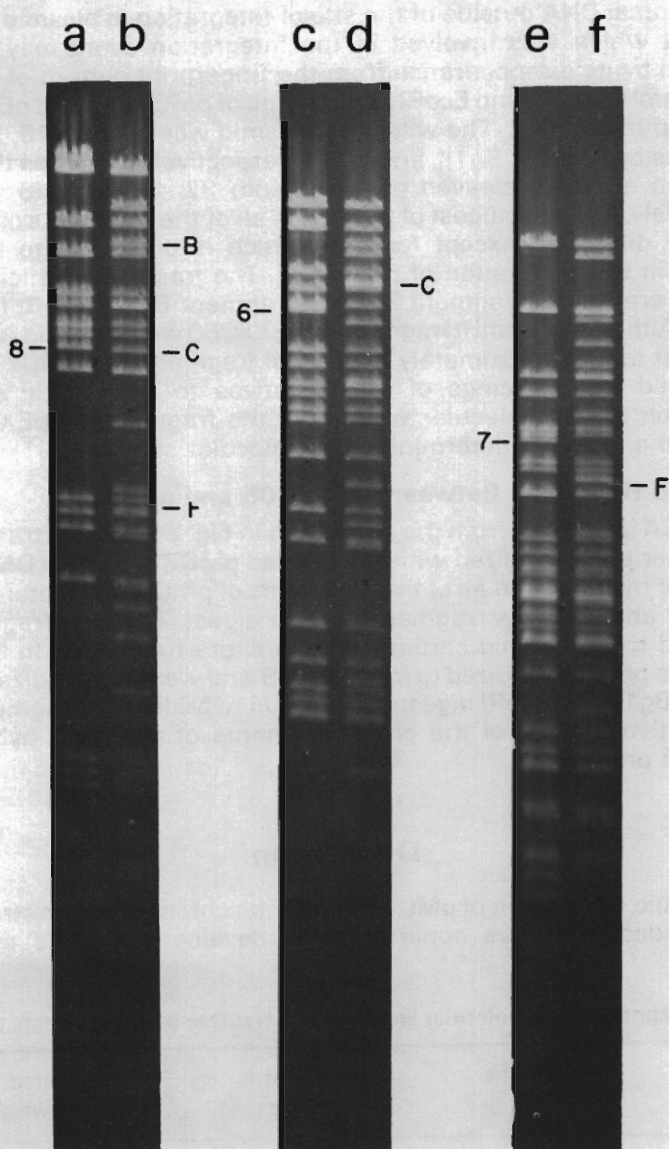
Fig. 2. Fingerprint analysis of *P. syringae* pv. *phaseolicola* plasmid DNA by agarose gel electrophoresis. (a) BamHI digested pMC7105; (b) BamHI digested pEX8050; (c) Bg1 II digested pMC7105; (d) BglII digested pEX8050; (e) EcoRI digested pMC7105; (f) EcoRI digested pEX8050. Numbers indicate pertinent wild-type fragments. Capital letters indicate possible plasmid-chromosome juncture fragments in pEX8050.
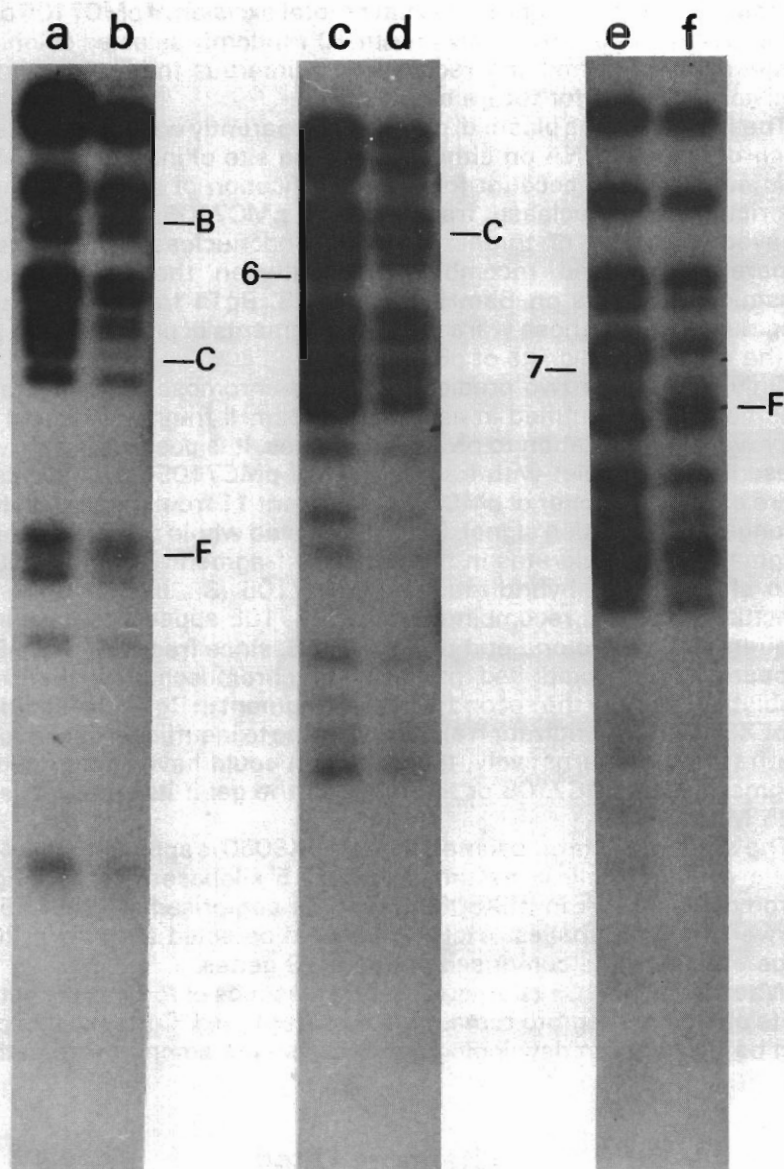
Fig. 3. Autoradiogram of ^{32}P-labeled pMC7105 DNA hybridized to a DNA blot of *P. syringae* pv. *phaseolicola* plasmid DNA digested with restriction endonucleases. pMC7105 a, c, e; pEX8050 b, d, f; BamHI a, b; Bg1II c, d; EcoRI e. f. Numbers indicate pertinent wild-type fragments. Capital letters indicate possible plasmid-chromosome juncture fragments in pEX8050.

analyzing plasmid-encoded gene functions, and also those encoded by certain chromosomal genes. Partial or total excision of pMC7105 occurs at a frequency of approximately one in 10 randomly selected colonies. This frequency will permit the recovery of numerous independently derived excision plasmids for future studies.

The large excision plasmid, pEX8050, apparently was formed by excision of chromosomal DNA on either side of the site of insertion of pMC7105. This model would account for the identification of all except one of the restriction endonuclease fragments of pMC7105 when pEX8050 is cleaved with any of three restriction endonucleases. The integration apparently involved recombinations between the chromosome and plasmid sequences on BamHI fragment 8, Bg1II fragment 6 and EcoRI fragment 7, since these were the only fragments of pMC7105 not present in the respective digests of pEX8050.

Only one of the two predicted plasmid-chromose juncture fragments was positively identified in each digest. BamHI fragments C and F show very weak hybridization to pMC7105 probes. It is possible that F, which is present as a doublet with fragment 11 of pMC7105 may have caused a more efficient transfer of pMC7105 fragment 11 from the gel resulting in a stronger hybridization signal. BamHI-digested whole cell DNA generates a fragment which migrates in the region of fragment C of pEX8050 which also shows weak hybridization to pMC7105 (3). If this is the second juncture fragment, recombination of pMC7105 appears to have involved sequences close to one end of fragment 8, since fragment C of pEX8050 appears to be comprised primarily of chromosomal sequences. Our inability to identify the second juncture fragment in Bg1II and EcoRI digests of pEX8050 by hybridization also may be due to insufficient plasmid DNA in the fragment. Alternatively, this fragment could have comigrated with a fragment from pMC7105 or migrated off the gel if its size was less than 0.25 Mdal.

The amount of chromosomal DNA in pEX8050 is approximately 47 Mdal. If the average gene is assumed to be 1.5 kilobase pairs in length, the chromosomal DNA in pEX8050 would be comprised of about 45 genes. Conversely, the smallest excision plasmid detected thus far is 20 Mdal, large enough to be comprised of about 20 genes.

Whether pMC7105 is unique among plasmids of *P. syringae* pathogens in its ability to integrate remains to be determined. Certainly this property can be exploited in developing genetic systems among these pathovars.

Literature Cited

1. Alwine, J. C., D. J. Kemp, B. A. Parker, J. Reiser, J. Renart, G. R. Stark, and G. M. Wahl. 1979. Detection of specific RNAs or specific fragments of DNA by fractionation in gels and transfer to diazobenzyloxymethyl paper. Pages 220-242 in: R. Wu, Ed. Methods in Enzymology 68. Academic Press. New York, New York.

2. Comai, L., and T. Kosuge. 1980. Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. J. Bacteriol. 143:950-957.

3. Curiale, M. S., 1980. Physical properties and genetic studies of cryptic plasmids discovered in plant pathogenic pseudomonads. 84 pp. Ph D. Thesis, Oregon State University.
4. Curiale, M. S., and D. Mills. 1977. Detection and characterization of plasmids of *Pseudomonas glycinea*. J. Bacteriol. 131:224-228.
5. Currier, T. C., and E. W. Nester. 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 76:431-441.
6. Davis, R. W., D. Botstein, and J. R. Roth. 1980. A manual for genetic engineering. Advanced bacterial genetics. 254 pp. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.
7. González, C., and A. K. Vidaver. 1978. Analysis of plasmids of syringomycin producing strains of *Pseudomonas syringae*. Pp. 31-38 in: Proc. 4th Int. Conf. Plant Path. Bact., Angers, France.
8. González, C., and A. K. Vidaver. 1979. Syringomycin production and holcus spot disease of maize; plasmid-associated properties in *Pseudomonas syringae*. Curr. Microbiol. 2:75-80.
9. Gonzalez, C., and A. K. Vidaver. 1980. Restriction enzyme analysis of plasmids from Syringomycin-producing strains of *Pseudomonas syringae*. Phytopathology 70:223-225.
10. Kado, C. I., and S.-T. Liu. 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. 145:1365-1373.

DNA Homologies Among Plasmids of
Pseudomonas syringae
pv. *phaseolicola*

R. Quant, D. Mills

Oregon State University
Corvallis, OR, U.S.A.

Abstract

Pseudomonas syringae pv. *phaseolicola* is the bacterial agent responsible for halo blight on beans. Five strains of *P. syringae* pv. *phaseolicola*, isolated from regions of the United States as far removed as Idaho and New York, were analyzed for plasmid number, size, and relatedness. EcoRI and BamHI restriction digests of total plasmid DNA suggest that a large degree of homology exists among these plasmids. Strain PP612 harbors two plasmids which are 1.4 and 96 megadaltons in size. Restriction analysis of total plasmid DNA revealed that 36 of 39 EcoRI bands and 16 of 22 BamHI bands were in common with those of a single 97 megadalton plasmid present in strain PP622. The EcoRI fingerprints indicate that the plasmids of these two strains have at least 90% sequence homology. Two other strains, designated PP601 and PP631, contain in addition to a 73 megadalton plasmid, a 29 and a 27 megadalton plasmid, respectively. EcoRI digests indicate that there is at least 93 percent sequence homology between total plasmid DNA of these strains. Strain PP652 contains two plasmids that are 70 and 33 megadaltons in size. EcoRI fingerprints indicate that PP652 has at least 62 percent sequence homology with PP601 and 58% sequence homology with PP631. Thirty-four percent sequence homology is seen in EcoRI digests among all five strains. Solution hybridization has also confirmed a high level of sequence homology among these plasmids.

Introduction

Naturally occurring plasmids are frequently found among isolates of plant pathogenic pseudomonads (1, 3, 4, 7, 8, 9). The plasmids have varied in size from approximately 3 to 109 megadaltons (Mdal). Indeed, one plasmid of *Pseudomonas syringae* pv. *savastanoi* has been shown to carry determinants for disease expression (1) and an association has been suggested of virulence properties with the presence of a 35 Mdal plasmid, pCG131, in *P. syringae* pv. *syringae* (8, 9) and a 22 Mdal plasmid in *P. syringae* pv. *phaseolicola* (7).

The presence of plasmids of similar size in pathovars of *P. syringae* is a relatively common occurrence. However, when restriction endonuclease fingerprints were used as a criterion for establishing homology between pCG131 of *P. syringae* pv. *syringae* and two other similar sized plasmids from strains of this pathovar, little homology was detected (10). Similar

results were obtained when the fingerprints of total plasmid DNA from six strains of *P. syringae* pv. *glycinea* were analyzed (3). Although the fingerprints revealed apparent homology between certain plasmids, the digests lacked a single fragment which was common to total plasmid DNA of these six strains. However, hybridization analyses of the plasmid DNA from these six strains revealed homology that was not apparent from the fingerprints alone.

The purpose of this work was to obtain numerical, physical, and biochemical information about the plasmids carried by five strains of *P. syringae* pv. *phaseolicola*. We have determined by two criteria that the plasmids of these strains share extensive homology.

Materials and Methods

Bacterial Strains and Culture Conditions

The bacterial strains used in this study are listed in Table 1. All strains were cultured on modified MaSNY medium (4) which lacked sucrose, hereafter referred to as MaNY medium. Cultures were stored at room temperature on solid medium (1.5% agar) and shake cultures were grown at room temperatures or 24°C.

Plasmid DNA Isolation

Plasmid DNA was extracted according to the method described by Hansen and Olsen (11) as modified by Curiale (3), or by a modified Currier and Nester technique (5) in which the shearing step was omitted. The DNA was purified by two cesium chloride-ethidium bromide (816.5 mg/ml and

Table 1. *P. syringae* pv. *phaseolicola* strains.

Strain	Genotype[a]	Plasmid	Approximate MW ($\times 10^6$dal)	Previous Strain Designation	Source
PP601	str^r	pPP6010	29	HB-20	A. Anderson[b]
		pPP6015	73		
PP612	rif^r	pPP6120	1.4	R1L	A. Vidaver[c]
		pPP6125	96		
PP622	rif^r	pPP6220	97	R1LD	A. Vidaver
PP631	str^r	pPP6310	27	HB10Y	A. Vidaver
		pPP6315	73		
PP652	rif^r	pPP6520	33	HB-36	A. Anderson
		pPP6525	70		
LR721	ser, rif^r	pMC7105	98	LR700	(3)

[a] str^r, streptomycin resistance; rif^r, rifampicin resistance; ser, serine auxotroph.

[b] Biology Department, Utah State University, Logan, Utah.

[c] Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska.

100 ng/ml, respectively) gradient centrifugations at 50,000 or 55,000 rpm in a Beckman Type VTi65 rotor for 12 to 16 h at 20°C. The plasmid band was removed through the side of the tube with a 22-gauge needle and the ethidium bromide was removed after four extractions with one-half volume of ice-cold isopropanol. The plasmid DNA was either dialyzed against T_{10} E_1 buffer (10 mM Tris, pH 7.2, 1 mM Na_2EDTA) to remove the cesium chloride or diluted with TES buffer (50 mM Tris, pH 8.0, 50 mM NaCl, 5 mM Na_2EDTA) and centrifuged at 50,000 rpm for 12 to 16 h in a Beckman Type SW60 rotor at 20°C to pellet the plasmid DNA. Purified plasmid DNA was stored frozen at -20°C in T_{10} E_1 buffer.

Restriction Endonuclease Digestion

BamHI (Bethesda Research Laboratories, Inc.) and EcoRI (prepared in our laboratory) were used to restrict plasmid DNA. Two micrograms of DNA were digested with a four-fold excess of enzyme for 2 h at 37°C in 20 nl of the appropriate digestion buffer (6). Following the digestion, the sample was heated at 70°C for 5 min to inactivate the enzyme.

Gel Electrophoresis

Gel electrophoresis was carried out on a horizontal slab gel (Model HI; Bethesda Research Laboratories, Inc.), using 0.7% agarose (Sigma) and a running buffer of 40 mM Tris, pH 7.9, 40 mM sodium acetate, 2 mM Na_2EDTA. The buffer was circulated during electrophoresis and the gel was run submersed in buffer. Whole plasmid DNA and restriction fragments were electrophoresed for 10 hours at 3V/cm. Lambda DNA (32 Mdal) and plasmids pMC7114 (50 Mdal), pMC7115 (58 Mdal) (3), RP4 (36 Mdal), pBR322 (2.65 Mdal), and JHC11 (16.6 Mdal) (2) were used as molecular weight standards. Gels were stained in 5.0 ng/ml ethidium bromide for at least 30 min and the DNA bands were visualized on a UV transilluminator (Model C-63B; Ultra-Violet Products, Inc.) The gels were photographed using a Polaroid MP-3 Land camera equipped with an orange filter and using Polaroid Type 55 Land film.

Nick Translation of Plasmid DNA and Solution Hybridization

Plasmid DNA was nick translated with [^{32}P]-dCTP and a nick-translation kit (New England Nuclear) to a specific activity of $2.3 - 16 \times 10^6$ cpm/ng DNA using the protocol outlined for the kit. The proteins were removed from the reaction mixture by a single phenol extraction, which was followed by three extractions with ethyl ether to remove traces of phenol. The plasmid DNA was fractionated from unincorporated nucleotides by Sephadex G-100 (Sigma) column chromatography and precipitated with the addition of three volumes of absolute ethanol after the concentrations of sodium acetate and carrier *Escherichia coli* transfer RNA (Sigma) were adjusted to 0.2 M and 50 ng/ml, respectively. The DNA solution was incubated overnight at -20°C, and the precipitated DNA was collected by centrifugation at 17,400 g and suspended in 0.12 M sodium phosphate.

Driver DNA was shared and precipitated according to White and Nester (15). Labelled probe DNA (1×10^{-3} to 5×10^{-3} ng) and sheared driver DNA (1 to 3.5 ng) were hybridized in 0.12 M sodium phosphate buffer (20 nl total volume) in 500 nl microfuge tubes. DNA was denatured for 10 min, quick

cooled in an ice bath, overlaid with parafin oil, and incubated at 68°C. Five nl aliquots were removed at 0, 24, 48, or 62 h, treated with S1 nuclease (12), and assayed for S1-resistant [^{32}P] -DNA (13).

Results

Plasmid Content of *Pseudomonas syringae* pv. *phaseolicola*

Analysis of five strains of *Pseudomonas syringae* pv. *phaseolicola* by agarose gel electrophoresis revealed that each contains one or two plasmid species (Fig. 1 and Table 1). A sixth strain, LR721, known to contain a single 98 Mdal plasmid, pMC7105 (3), was used as a standard of comparison. A single plasmid with a molecular weight of 97 Mdal was detected in PP622 but all other strains contain two plasmids. Strains PP601 and PP631 each contain a 73 Mdal plasmid and a 29 and 27 Mdal plasmid, respectively. Plasmids of similar size were also detected in PP652. The most striking difference in plasmid size was observed upon analysis of PP612, which contains plasmids of 96 and 1.4 Mdal.

Analysis of Restriction Endonuclease Cleavage Patterns

Total plasmid DNA from each strain was cleaved with EcoRI and BamHI restriction endonucleases to determine the degree of homology among plasmids from the six strains. The fingerprints generated by digestion with EcoRI are presented in Fig. 2. It is apparent upon inspection that these plasmids have many bands in common and that some of the plasmids may be nearly identical. All digests had 15 bands in common, accounting for approximately 28 to 34% homology. The plasmids in PP612 and PP622 have 32 bands in common with the 38 EcoRI bands of pMC7105, representing at least 90% homology.

All of the digests contain a 1.4 Mdal fragment. The small 1.4 Mdal plasmid seen in PP612 apparently contains more than one EcoRI restriction site since an additional fragment of this size was not detected (Fig. 2, lane B). PP612 contains a 4.5 Mdal fragment present as a triplet band which is not present in any of the other digests (Fig. 2, lane B-arrow). Fragment 11 of pMC7105 (lane F-arrow) is not present in the digests of the plasmids in PP612 and PP622 but is present in the digests of total plasmid DNA of the other strains. The plasmids from strain PP601 have 41 bands in common with the 43 bands of PP631 representing approximately 94 percent homology (Fig. 2, lanes A and D). The plasmid species in PP652 have an apparent lower percent homology (60-70%) with all other plasmid species, primarily because the five largest fragments are different in size from any other fragments (Fig. 2, lane E).

The BamHI fingerprints of these plasmids corroborate the homology detected by the EcoRI fingerprints. The plasmids in strains PP612 and PP622 also show a large number of BamHI bands in common with pMC7105 (Fig. 3, lanes B, C, and F). Since fewer bands are generated by BamHI, differences in the number and position of BamHI cut sites have resulted in greater apparent differences in the degree of homology. PP601 and PP631 differ by only two bands, which may have resulted from a minor
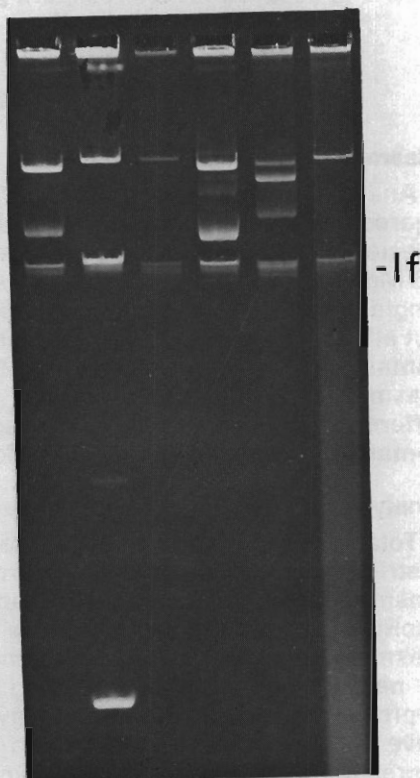
Fig. 1. Agarose gel electrophoresis of purified plasmid DNA from six strains of *P. syringae* pv. *phaseolicola.* Lane (A) PP601, (B) PP612, (C) PP622, (D) PP631, (E) PP652, (F) PP721. It indicates linear fragments.

change in the location of one restriction site. The plasmids in PP652, which appeared most different from the other plasmids upon digestion with EcoRI, also show the greatest differences when digested with BamHI.

Solution Hybridrization of Total Plasmid DNA

Solution hybridizations were carried out to determine more precisely the degree of homology among the plasmids of these strains. The 97 Mdal plasmid, pPP6220, was used as probe DNA because it was present alone in strain PP622 and required no further purification from other plasmids. The plasmids in strains PP601 and PP612 and pMC7105 have greater than 90% homology with pPP6220 (Table 2). The plasmid species in PP652, which showed a lower percent homology with any of these plasmids by fingerprint analysis, also showed a lower percent homology by hybridization. The plasmids in strain PP631 showed a lower percent homology than expected from the fingerprint patterns.

Some of the plasmids in these strains may be identical. However, until it can be shown that they are, each has been assigned a different plasmid designation (Table 1).

416
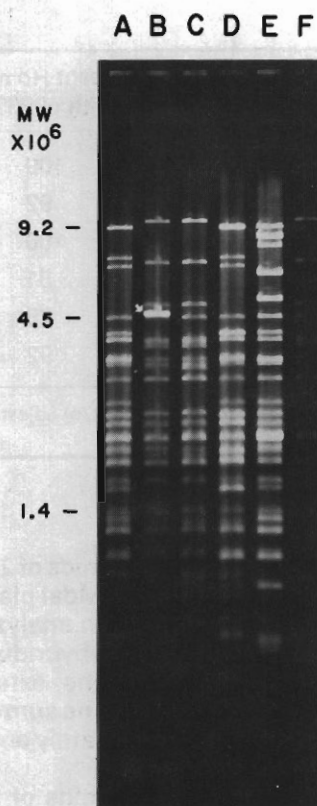
Fig. 2. Fingerprint patterns generated by digestion of total plasmid DNA from six strains of *P. syringae* pv. *phaseolicola* with the restriction enzyme EcoRI. Lane (A) PP601, (B) PP612, (C) PP622, (D) PP631, (E) PP652, (F) PP721. Arrows point to major differences in the banding patterns discussed in text. Molecular weights (Mdal) are listed on the left.
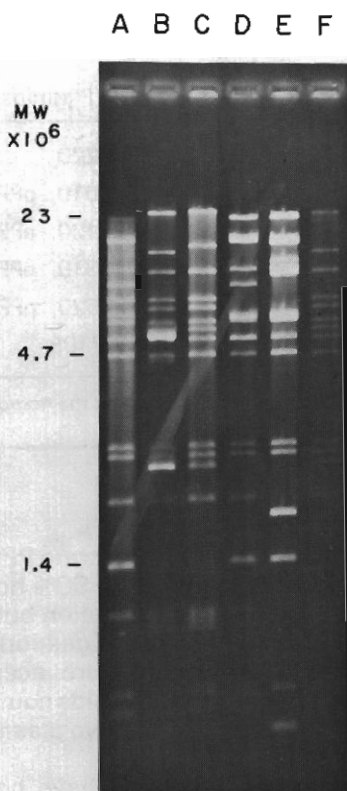
Fig. 3. Fingerprint patterns generated by digestion of total plasmid DNA from six strains of *P. syringae* pv. *phaseolicola* with the restriction enzyme BamHI. Lane (A) PP601, (B) PP612, (C) PP622, (D) PP631, (E) PP652, (F) PP721. Molecular weights (Mdal) are listed on the left.

Discussion

Five isolates of *P. syringae* pv. *phaseolicola* were shown to contain one or two plasmid species ranging in size from 1.4 to 97 Mdal. Site-specific endonuclease analysis of plasmids of similar size from *P. syringae* pv. *syringae* revealed little homology (10). The plasmids analyzed in this study showed extensive homology by fingerprint analysis and DNA solution hybridization.

Table 2. Plasmid homologies.

Strain	Plasmids	Percent Homology with pPP6220[a]
PP622	pPP6220	100
PP601	pPP6010, pPP6015	92
PP612	pPP6120, pPP6125	100
PP631	pPP6310, pPP6315	81
PP652	pPP6520, pPP6525	76
LR721	pMC7105	92

[a] Normalized percentages based on the homologous control hybridization (69%) adjusted to 100 percent.

Total plasmid DNA from strain PP601, which contains plasmids of 29 and 73 Mdal, shows more than 90% homology with a single 97 Mdal plasmid, pPP6220, by DNA hybridization but only 31% homology when analyzed by restriction endonuclease digestion. If one assumes that the hybridization experiments provide a more accurate determination of the extent of homology (14), these plasmids could have a common origin. The sum of the molecular weights of the two plasmids in PP601 is approximately equal to the size of pPP6220.

Presently, no functions have been ascribed to the plasmids of these strains. A 22 Mdal plasmid which is thought to be associated with virulence properties in another strain of *P. syringae* pv. *phaseolicola* (7) was not detected in these six strains. It is of interest, however, that pMC7105, a plasmid which is known to integrate into the bacterial chromosome of this pathovar (3), shows greater than 90% homology with pPP6220 (Table 2). Whether integration, and subsequent excision, is unique to pMC7105 or a property of all the plasmid which show extensive homology with pMC7105, remains to be determined.

Literature Cited

1. Comai, L., and T. Kosuge. 1980. Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. J. Bacteriol. 143:950-957.
2. Cramer, J. H., F. W. Farrelly, J. T. Barnitz, and R. H. Rownd. 1977. Construction and restriction endonuclease mapping of hybrid plasmids containing *Saccharomyces cerevisiae* ribosomal DNA. Molec. gen. Genet. 151:229-244.
3. Curiale, M. S., 1980. Physical properties and genetic studies of cryptic plasmids discovered in plant pathogenic pseudomonads. Ph.D. Thesis, Oregon State University, 84 pp.
4. Curiale, M. S., and D. Mills. 1977. Detection and characterization of plasmids of *Pseudomonas glycinea*. J. Bacteriol. 131:224-228.

5. Currier, T. C., and E. W. Nester. 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 76:431-441.

6. Davis, R. W., D. Botstein, and J. R. Roth. 1980. A Manual for Genetic Engineering. Advanced Bacterial Genetics. 254 pages. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

7. Gantotti, B.V., S. S. Patil, and M. Mandel. 1979. Apparent involvement of a plasmid in phaseotoxin production by *Pseudomonas phaseolicola*. Appl. Environ. Microbiol. 37:511-516.

8. Gonzalez, C., and A. K. Vidaver. 1979. Analysis of plasmids of syringomycin producing strains of *Pseudomonas syringae*. Pp. 31-38 *in* Proc. 4th Int. Conf. Phyt. Bact. 1978, Angers, France.

9. González, C. F., and A. K. Vidaver. 1979. Syringomycin production and holcus spot disease of maize: plasmid-associated properties in *Pseudomonas syringae*. Current Microbiology 2:75-80.

10. Gonzalez, C., and A. K. Vidaver. 1980. Restriction enzyme analysis of plasmids from syringomycin-producing strains of *Pseudomonas syringae*. Phytopathology 70:223-225.

11. Hansen, J. B., and R. H. Olsen. 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group of plasmids pMG1 and pMG5. J. Bacteriol. 135:227-238.

12. Hereford, L. M., and M. Rosbash. 1977. Number and distribution of polyadenylated RNA sequences in yeast. Cell 10:453-462.

13. Salzberg, S., Z. Levi, M. Aboud, and A. Goldberg. 1977. Isolation and characterization of DNA-DNA and DNA-RNA hybrid molecules formed in solution. Biochemistry 16:25-29.

14. Thompson, R., S. G. Hughes, and P. Broda. 1974. Plasmid identification using specific endonucleases. Molec. Gen. Genet. 133:141-149.

15. White, F. F., and E. W. Nester. 1980. Relationship of plasmids responsible for hairy root and crown gall tumorigenicity. J. Bacteriol. 144:710-720.

Plasmids Are Not Associated with Formation of Noncapsulated Variants of *Pseudomonas solanacearum*

T. C. Currier, M. K. Morgan

Kansas State University
Manhattan, KS, U.S.A.

Abstract

Virulent strains of *P. solanacearum* produce large fluidal colonies in complex agar media that contain glucose. Small, compact, nonfluidal colonies are virulent and arise spontaneously from laboratory cultures which are transferred infrequently. In strain K60, the B1 nonfluidal, avirulent strain has been shown to be lacking extracellular polysaccharide produced by the virulent strain. The B1 strain is extremely stable; its reversion to the virulent type has not been reported. Because of the extreme stability of strain K60-B1 it is possible that genetic formation has been lost during its formation. This deletion might occur by the loss of a plasmid. To test this hypothesis we have attempted to isolate plasmids from strain K60, K60(RP4), and K60-B1 by four procedures. Only in the case of K60(RP4) could a plasmid be isolated.

In addition we have attempted to isolate plasmids from 20 strains of *P. solanacearum*, 13 of which were fluidal and produced nonfluidal variants. In only six of these strains were plasmids detected and where fluidal and nonfluidal pairs of strains were available, their plasmid compositions were identical. Taken together these results indicate that nonfluidal strains do not arise as the result of the loss of a plasmid from the fluidal strains used in this study. Therefore, other genetic mechanisms such as site specific recombination might be responsible for the production of nonfluidal isolates of *P. solanacearum*.

Introduction

The vascular wilt caused by *Pseudomonas solanacearum* is one of the most economically important pathogens of solanaceous crops. On appropriate agar media virulent isolates are characteristically non-motile and produce large amounts of extracellular polysaccharide (EPS). By the use of a tetrazolium containing medium, the differences between virulent, fluidal, and the avirulent, nonfluidal type can be accentuated (10). Maintenance of a virulent culture is sometimes a problem because avirulent cells are readily produced. In the case of strain K60, its avirulent B1 derivative is highly motile, non-capsulated, and aerotatic (11). Thus under limiting amounts of oxygen the avirulent form has a selective advantage.

420

The reason for the difference in motility between the virulent and avirulent forms of strain K60 appears to be due to the lack of flagellae on most of the virulent cells (11). Furthermore, the flagellae that are present on a few of the virulent cells are straight and generally shorter than the ones present on the avirulent cells. In contrast, 80 to 85% of the avirulent cells of the strain K60-B1 have one or more long wavy flagellae.

The differences between the virulent and avirulent cell types must represent major differences in gene expression. The mechanism(s) responsible for these changes are not known. However, it is known that the avirulent types are extremely stable (10, 12). This stability might be due to their constant selection in cultures of limiting oxygen concentration. Another possibility is that genetic information might be lost from the virulent types thus preventing the reversion of the avirulent to the virulent cell type. In the present study we have attempted to determine if loss of genetic information, in the form of a plasmid, is associated with formation of avirulent, nonfluidal variants. Several strains from the three races (3) of *P. solanacearum* have been analyzed for the presence of plasmids. Where plasmids were found an attempt was made to isolate nonfluidal variants, which were then analyzed for the presence of plasmids.

Materials and Methods

Bacterial Strains, Media and Culture Conditions

The bacterial strains used in this study are listed in Table 1. These isolates were obtained from W. C. Nesmith, Department of Plant Pathology, University of Kentucky, Lexington, KY 40546. Strains from this collection were streaked from cell suspensions stored in distilled water at room temperature onto TZC medium containing in g/l, glucose, 10; peptone, 10; casamino acids, 1; 2,3,5-triphenyl tetrazolium chloride (TZC) 0.05 and agar, 15. For some of the strains only nonfluidal colony types were recovered (Table 1). Strains K60, K60-B1 and those without an alphabetical prefix were originally from the collection of phytopathogenic bacteria at the Department of Plant Pathology, University of Wisconsin, Madison, WI 53706. Those strains with an S prefix were isolated by L. Sequeira and those KS prefixes were derived in this study.

For plasmid isolation, cells were grown in nutrient broth (Difco). Plasmid isolation from K60 and K60-B1 strains was also attempted with cells grown in the following media: NBY broth (14), L broth (6) and minimal medium (1). Growth on plates or in broth was at 29°C and broth cultures were shaken at 200 rpm.

Isolation of Nonfluidal Strains

For isolation of nonfluidal types, 10 milliliters of TZC medium without agar and TZC were placed in 13 x 150 mm screw cap tubes. Tubes were inoculated with virulent cells and incubated without shaking. At intervals up to 2 weeks, cells were streaked from the surface pellicle to TZC agar for identification of avirulent types.

Table 1. Descriptions and plasmid contents of bacterial strains.

Strain	Race	Colony type[a]	Origin or derivation	Plasmids[b] (mw X 10^6 daltons)	Isolation method[c]
23	1	nfl[d]	Potato, Israel	5, 5.5	I, II
39	1	i	Zebrina, Puerto Rico	ND	I, II
74	1	fl	Potato, Ceylon	ND	I, II
87	1	fl	Tomato, Climax, GA, USA	105, 250	II
199	1	nfl[d]	Tobacco, Philippines	ND	II
203	1	nfl[d]	Tobacco, Georgia, USA	ND	II
267	1	fl	Tomato, Taiwan	ND	II
KS2671	1	nfl	Nonfluidal variant of strain 267	ND	I
K25	1	fl	Tomato, NC, USA	ND	I, II
K60	1	fl	Tomato, NC, USA	ND	I, II, III, IV
K60-B1	1	nfl	Nonfluidal variant of strain K60	ND	I, II, IV
CB-14	1	fl	A K60 strain harboring plasmid RP4, constructed by C. Boucher	38(6)	II
1	2	fl	Banana, Costa Rica	ND	II
16	2	fl	Banana, Honduras	ND	I, II
17	2	fl	Banana, Honduras	ND	I.
KS171	2	nfl	Nonfluidal variant of strain 17	ND	I
53	2	fl	Banana, Honduras	ND	I, II, III
KS531	2	fl	Nonfluidal variant of strain 53	ND	I, II, IV
70	2	fl	Plantain, Colombia	ND	I, II
KS701	2	nfl	Nonfluidal variant of strain 70	ND	I
131	2	fl	Plantain, Peru	105	I, II
KS1311	2	nfl	Nonfluidal variant of strain 131	105	I, II
157	2	nfl[d]	Plantain, Peru	3.5	II
170	2	i	Heliconia, Colombia	ND	II
S135	2	fl	Heliconia, Costa Rica	ND	I, II
S160	2	fl	Heliconia, Costa Rica	105	I, II
KS1601	2	nfl	Nonfluidal variant of S160	105	II
19	3	nfl[c]	Potato, Colombia	5	II

[a] fl, fluidal; nfl, nonfluidal; i, intermediate.
[b] ND, not detected.
[c] Numbers refer to isolation procedures.
  I. Currier and Nester procedure. II. Alkaline lysis procedure. III. Cleared lysate procedure. IV. Hansen and Olsen procedure.
[d] Only non-fluidal isolates were recovered from water stocks.

Plasmid Isolation Procedures

Bacterial cultures were grown in 100 ml of broth in a liter flask at 29°C with shaking at 200 rpm. Cells were harvested by centrifugation at a cell density between 4×10^8 and 6×10^8 cells per milliliter. This was important because cells harvested at higher cell densities were incompletely lysed. The plasmid isolation procedures developed by Currier and Nester (7), Casse et al. (4), Clewel and Helinski, (5) and Hansen and Olsen (8) were used. Hereafter, these will be referred to as methods, I, II, III, and IV, respectively. These procedures utilize different methods of cell lysis and

chromosomal DNA removal. Briefly, procedure I involved cell lysis under non-denaturing conditions followed by an alkaline treatment, a "neutralization" step, and a phenol treatment to remove chromosomal DNA. Procedure II is very similar to Procedure I except that the cells are lysed under denaturing conditions. Procedure III involves a gentle and incomplete cell lysis which allows plasmids but not chromosomal DNA to escape from the cells, and procedure IV involves a lysozyme treatment followed by a detergent lysis and an alkaline treatment. Following neutralization chromosomal DNA is removed by coprecipitation with sodium dodecyl sulfate in the cold.

For routine screening, procedures I and II were used. The latter was preferred because, in general, better cell lysis and higher plasmid yields were obtained. Plasmids were routinely detected by analyzing the DNA prepared in the above procedures in 0.6% agrose gels at 5 V/cm as previously described (6). For K60 and K60-B1 cesium chloride gradients containing ethidium bromide (7) were also used for plasmid detection.

Results

Most of the bacterial strains listed in Table 1 were analyzed for the presence of plasmids by procedure I or II or both I and II. Strains 53, KS531, K60, and K60-B1, were analyzed by additional methods. Where plasmids were detected, all methods used were successful in their isolation. Of the 20 strains from natural sources six contained plasmids (Fig. 1). The molecular weights of these plasmids ranged from 3.5 to 10^6 to 250×10^6 (Table 1). With respect to races, 2 out of 9, 3 out of 10 and 1 out of 1 strains tested from races 1, 2, and 3, respectively, had plasmids.

To determine if loss of a plasmid was associated with production of nonfluidal types an attempt was made to isolate nonfluidal strains from the surface of 1 to 3 week old stagnant tube cultures of three plasmid-containing strains (87, 131, and S160). Non-fluidal variants were isolated from strains 131 and S160. These avirulent variants had plasmid compositions that were the same as their respective virulent parents.

Because strain K60 is used in many studies and can be considered the neotype strain of race 1 organisms (9), special effort has been taken to detect plasmids in this strain and in K60-B1. Agrose gel electrophoresis and cesium chloride gradients containing ethidium bromide were used in various combinations with four different plasmid isolation procedures for this pair of strains. In addition several cell treatments preceding lysis were used. These included a high salt (1 M NaCl) wash, a lysozyme treatment and a brief penicillin treatment. The latter treatment lasted for the time required for one cell doubling to occur and was performed immediately before harvesting the cells. Plasmids were not isolated from K60 or K60-B1 in any of the above procedures. Furthermore, when strain K60 contains the plasmid RP4, it is easily isolated (strain CB-14, Table 1). Therefore, plasmids in K60 are not difficult to isolate because of nuclease problems or some other difficulty intrinsic to the strain. Similarly, nonfluidal variants were isolated from the following plasmidless strains: 267, 17, 53 and 70 (Table 1).
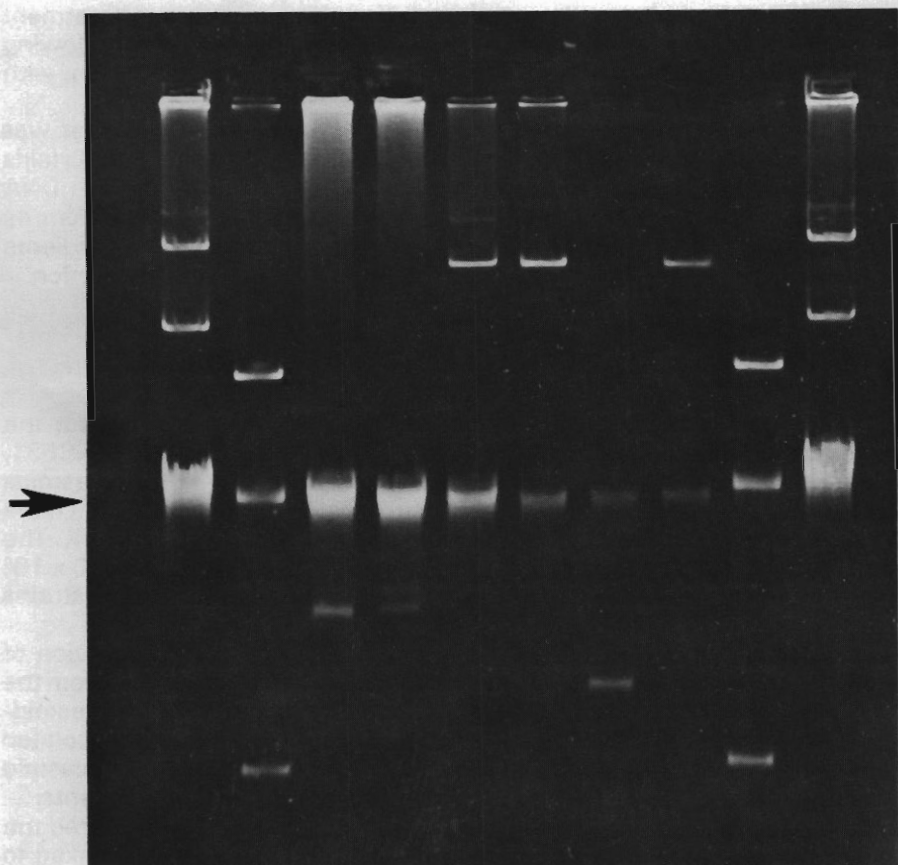
Fig. 1. Agarose gel electrophoresis of plasmid DNA from *P. solanacearum* strains. Lanes A and B contained the same marker plasmids as lanes I and J. DNA from *Agrobacterium tumefaciens* C58 (RP4) was present in lanes A and J. Plasmids of 300×10^6, 120×10^6, and 38×10^6 daltons are present. Lanes B and I contained plasmids R6K, PCR1, and PBR322 whose molecular weights are 24×10^6, 8.7×10^6, and 2.6×10^6, respectively. DNA from *P. solanacearum* strains 19, 23, 87, 131, 157, and S160 were present in lanes C, D, E, F, G, and H, respectively. Chromosomal fragments (arrow) are present in all lanes except B and I.

Discussion

In this study strains from each of the three races of *P. solanacearum* were analyzed for plasmids. At least one strain from each race contained a plasmid. Although this is not a particularly large study it shows that strains from all three races may frequently contain plasmids.

Intensive analyses of strains K60 and K60-B1 indicated that neither of these strains contained a plasmid. This result supports data recently reported (2). Although it is impossible to prove that a strain does not harbor a plasmid, we feel confident that this is true for this virulent and avirulent pair of strains. Similarly other fluidal race 1 (267) and 2 (17, 53, 70) strains did not contain plasmids but still produced nonfluidal variants during the course of this study. Together these results strongly suggest that the fluidal to nonfluidal transition is not plasmid mediated in these strains. The lack of plasmid involvement in this transition is further supported by results with two plasmid-containing strains. Two fluidal race 2 strains, (131, S160), which contained plasmid, gave rise to nonfluidal variants which contained the same plasmids that were present in their respective fluidal parents.

This limited survey suggests that in the *P. solanacearum* strains tested, the conversion from fluidal to nonfluidal cell types is not associated with the loss of a plasmid. However, the possibility exists that in some, as yet undiscovered, strain(s) plasmid loss will result in loss of pathogenicity and/or the loss of EPS production. Because our results suggest that plasmid loss is not associated with the conversion to the nonfluidal form, we feel that other genetic mechanisms need to be investigated as possible sources of this variability. Site specific recombination as exemplified by phase variation in *Salmonella* (13) would be a possible mechanism. Such a mechanism seems attractive because it allows for the alternate expression of sets of genes. Such a mechanism could explain the loss of capsule and the simultaneous gain of flagellae which has occurred during the formation of the avirulent B1 derivative of strain K60. The fact that the conversion back to the virulent form does not seem to occur, does not necessarily argue against such a switch mechanism. It may simply be a reflection of the strong selective advantage of the avirulent forms under microaerophilic conditions and our inability to create conditions that would select the virulent forms out of an avirulent culture.

Addendum

Recent data obtained by C. Boucher (see these proceedings) indicate that K60, K60-B1 and most other strains tested contain a plasmid whose molecular weight is greater than 300×10^6. Because the plasmid content of K60 and K60-B1 is indistinguishable, the conclusions from our study remain unchanged. His success in the isolation of these very large plasmids is probably due to the very gentle isolation method. Since there are very few strains common to both studies, whether or not the very large plasmids are present in most of our strains is not known.

Literature Cited

1. Abo-El-Dahab, M. K., and M. A. El-Gooranhi. 1969 Antagonism among strains of *Pseudomonas solanacearum.* Phytopathology 59:1005-1007.
2. Boucher, C., B. Message, D. Debieu, and C. Zischek. 1981. Use of P-1 incompatibility group plasmids to introduce transposons into *Pseudomonas solanacearum.* Phytopathology 71:639-642.
3. Buddenhagen, I., and A. Kelman. 1964. Biological and physiological aspects of bacterial wilt caused by *Pseudomonas solanacearum.* Ann. Rev. Phytopath. 2:203-230.
4. Casse, F., C. Boucher, J. S. Julliot, M. Michel, and J. Denarie. 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agrose gel electrophoresis. J. Gen. Microbiol. 113:229-242.
5. Clewell, D. B., and D. R. Helinski. 1969. Supercoiled circular DNA-protein complex in *Escherichia coli:* purification and induced conversion to an open circular form. Proc. Natl. Acad. Sci. USA 62:1159-1166.
6. Currier, T. C., and M. K. Morgan. 1981. Restriction endonuclease analyses of the Inc PI plasmids RK2, RP1, RP4, R68, and R68.45. Current Microbiol. 5:323-328.
7. Currier, T. C., and E. W. Nester. 1976. Isolation of covalently closed circular DNA of high molecular weight from bacteria. Anal. Biochem. 76:431-441.
8. Hansen, J. B., and R. H. Olsen. 1978. Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. J. Bacteriol 135:227-238.
9. Hayward, A. C., 1964. Characteristics of *Pseudomonas solanacearum* J. Appl. Bacteriol. 27:265-277.
10. Kelman, A., 1954. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.
11. Kelman, A., J. Hruschka. 1973. The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum.* J. Gen. Microbiol. 76:177-178.
12. Sequeira, L., and T. L. Grahm. 1977. Agglutination of avirulent strains of *Pseudomonas solanacearum* by potato lectin. Phys. Plant Path. 11:43-54.
13. Silverman, M., and M. Simon. 1980. Phase variation: genetic analysis of switching mutants. Cell 19:845-854.
14. Vidaver, A. K., 1967. Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: effect of the carbon source. Appl. Microbiol. 15:1523-1524.

Presence of a Megaplasmid in Strains of *Pseudomonas solanacearum*

C. Zischek, Le Thi Kim Tuyen, P. Boistard, C. Boucher

I. N. R. A. - C.N.R.S., Chemin de Borde-Rouge
Auzeville, Castanet Tolosan, France

Abstract

A megaplasmid of molecular weight clearly over 300 megadaltons was present in 11 out of 12 *Pseudomonas solanacearum* strains tested and chosen because of their variety of geographical and host plant origins. Transposon Tn5 has been selectively inserted in these megaplasmids. The plasmid of one strain was transferred into *Escherichia coli*.

Introduction

Investigators have shown plasmids to be present among various species of plant pathogenic bacteria (16). Their molecular weights (mol. wt) spread over a wide range of values from the very small plasmids of a few megadaltons (Mdal) (21) to plasmids of more than 300 Mdal (5).

In the case of *Pseudomonas solanacearum* the ability of the bacterium to lose its pathogenic properties either spontaneously (14) or after treatment with acridines (19), suggested that these properties could be partly encoded by a plasmid. Several attempts were made to physically demonstrate the presence of a plasmid in this bacterium (3). All the methods used involved an enrichment in supercoiled DNA and a DNA concentration step. Such methods are likely to cause significant damage to very large covalently closed circular (CCC) DNA molecules and lead to negative results.

Here we report that by using procedures where handling of DNA is minimized prior to the plasmid resolution step (either on agarose gels or in CsCl ethidium bromide gradients), it was possible to detect and isolate an extremely large plasmid, of mol. wt clearly higher than 300 Mdal from all but one of the *P. solanacearum* strains tested. Genetic tools were devised to study these plasmids.

Materials and Methods

Bacteria and Culture Media

Bacterial strains used are listed in Table 1. BG and BGT media (2) were used for growth of *P. solanacearum*, *Escherichia coli* and *P. putida*; for *Agrobacterium tumefaciens* and *Rhizobium meliloti*, C broth was used (2).

Table 1. List of bacterial strains used.

Strain	Relevant characters		Reference or source
Pseudomonas solanacearum			
wild type	Isolated from	Geographical origin	
8	*Eupatorium odoratum*	Costa Rica	
19	Potato	Columbia	
40	Banana	Honduras	L. SEQUEIRA
70	Plantain	Columbia	Dept of Plant Pathology
82	Potato	Columbia	University of
85	Tomato	Canada ?	Wisconsin, Madison
145	Potato	Australia	
170	*Heliconia*	Columbia	
199	Tobacco	Philippines	
203	Tobacco	U.S.A.	
K60	Tomato	U.S.A.	
GMI1000	Tomato	Guyana	(5)
mutants			
B1	spontaneous avirulent mutant derived from K60		L. SEQUEIRA
GMI1178	acridine orange induced avirulent mutant derived from		(5)
GMI1224	GMI1000 insertion of Tn5 in the megaplasmid of GMI1000		this paper
GMI2000	spontaneous avirulent mutant derived from GMI1000		(5)
Agrobacterium tumefaciens			
C58	Ti and pAtC58 plasmids		(24)
Rhizobium meliloti			
L5-30	L5-30		(11)
Pseudomonas putida			
PpS1239 (pMG1)	pMG1 plasmid		J. SHAPIRO (Chicago University)
Escherichia coli			
HB101	*rec* A, *r*K⁻, *str* A		(15)
J5-3 (RP4)			J. BERINGER (Jone Innes Institute)
C600 (pACYC184)			(28)

DNA Isolation Procedures

Analytical procedure for detection and characterization of CCC DNA by agarose gel electrophoresis was as described by Eckhardt (10) with a pre-wash of the cells in 0.1% Sarkosyl and use of 2% SDS in both the lysing and the overlay mixtures (23). Preparative extractions of megaplasmids were essentially as described by Schwinghamer (26) except that CCC DNA was further purified by a second run in a dye-buoyant density gradient. The cleared lysate procedure (6) was used for isolation of pACYC184 and RP4 from *E. coli*. Total DNA was prepared according to Roussel and Chabbert (24) except that the proteinase K treatment was omitted.

Transformation Procedures

For *E. coli* the recipient cells were calcium-treated according to Cohen *et al.* (8). For *P. solanacearum* the transformation procedure and the preparation of transforming lysates were described previously (17).

Matings

Matings were performed between 10^8 donor and 10^8 recipient cells on filter membranes placed on BG plates and incubated at 30°C.

In Vitro Recombination

Cloning restriction fragments of *P. solanacearum* plasmids into pACYC184 and RP4 was performed as described by Julliot and Boistard (13). Construction and use of recombinant DNA molecules were done in accordance with the guidelines of the French D.G.R.S.T. commission for the control of recombinant DNA experiments.

Restriction Patterns

These were obtained using an *EcoR1* digest of 2ng of DNA in UREB buffer (25).

Colony Hybridization on Nitrocellulose Filters

This was done according to Grunstein and Hogness (11).

Results

Physical Evidence for the Presence of Plasmids

The analytical method devised by Eckhardt (10), which involves a limited number of steps in the handling of the lysate prior to electrophoresis, is likely to be suitable for the search of plasmids of high molecular weight particularly sensitive to physical manipulations. In addition, resolution of CCC DNA is straightforward for large plasmids in agarose gels because the open circular form cannot enter the gel and CCC DNA migrates much more slowly than the smear of linear DNA. When this method, with slight modifications, was first applied to *P. solanacearum* strains K60 and GMI2000, in each case a molecular species banded in the region of the gel corresponding to CCC DNA. Fig. 1 shows that the electrophoretic mobility of the *P. solanacearum* CCC DNA band is clearly less than that of known large plasmids used as markers; pAtC58 and pMG1 reported to have
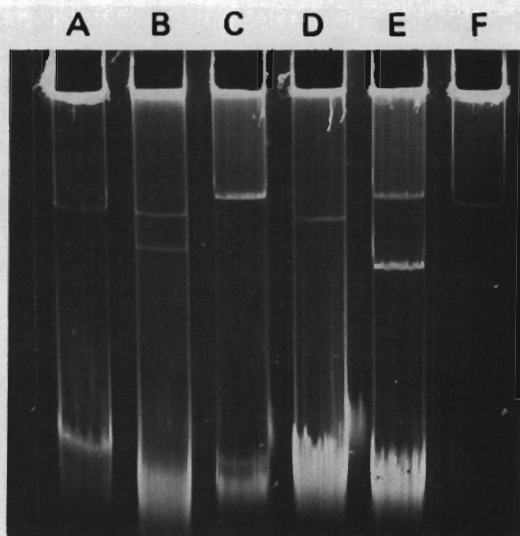
Figure 1. Agarose gel electrophoreis of lysates from: *Paseudomonas solanacearum* strains GM12000 (A) and K60 (F); *Agrobacterium tumefaciens* strain C58 (B); the lower band is the 130 Mdal Ti plasmid and the upper one is the cryptic pAtC58 plasmid of 273 Mdal; *Rhizobium meliloti* strain L5-30 (E); showing a megaplasmid and a smaller one of 90 Mdal, in track (C) is L530 cured of the smaller plasmid; *Pseudomonas putida* strain PpS1239 (D) harbouring the 312 Mdal plasmid pGMI.

respective mol. wts of 273×10^6 (R. Villarroel and M. Van Montagu, personal communication) and 312×10^6 (12). A negative logarithmic correlation has been demonstrated between electrophoretic mobility and mol. wt for CCC DNA up to 180 Mdal (5, 20). As extrapolation of this equation for large molecules gives underestimation of the mol. wt (5), the *P. solanacearum* slow migrating bands must correspond to plasmids much greater than 300 Mdal which we will further refer to as megaplasmids. Those megaplasmids have electrophoretic mobilities which are similar to that of the megaplasmids recently discovered in *Rhizobium meliloti* (Fig. 1 lanes C and G) (9, 15).

The presence of a megaplasmid in strains GMI2000 and B1 (an avirulent derivative of K60) was confirmed when lysates prepared according to Schwinghamer were run on a CsCl-ethidium bromide gradient. After centrifugation, apart from a major band of DNA, a faint denser additional fluorescent band was visible in the gradients. That faint band was shown to consist of CCC DNA since (i) when rerun in a second gradient it dissociated in two bands, one corresponding to the original CCC band, the other having the lower density of the linear DNA and (ii) if prior to the second round of centrifugation the DNA was sheared on a vortex it was completely converted to the light species corresponding to linear DNA. The question

then arose as to whether this CCC DNA corresponded to a plasmid or to the entire chromosome in a CCC form. To solve this problem CCC DNA fractions from several gradients of strain B1 were pooled and submitted to a second round of centrifugation. The purified CCC DNA band of the second gradient was phenol extracted and digested with *EcoR1* and the restriction pattern was compared to the *EcoR1* restriction pattern of the total DNA extracted from the same strain (Fig. 2). The two patterns were clearly distinct, showing that the CCC DNA isolated on a dye-buoyant density gradient did not correspond to the total genome. In addition, the restriction pattern of the plasmid preparation shows a large number of bands; this is in agreement with the presumed large size of the plasmid as determined by agarose gel electrophoresis.

Presence of a Megaplasmid in Other *P. solanacearum* Strains

The question then was whether the presence of such megaplasmids was a common feature among *P. solanacearum* strains. To answer this question, the Eckhardt procedure was used to screen a collection of strains isolated from a variety of host-plant and geographical origins. From the 12 wild type strains studied (Table 1) only one, strain 19, failed to show any plasmid. For this particular strain, no plasmid could be detected in CsCl ethidium bromide gradient. All the other strains tested had a megaplasmid
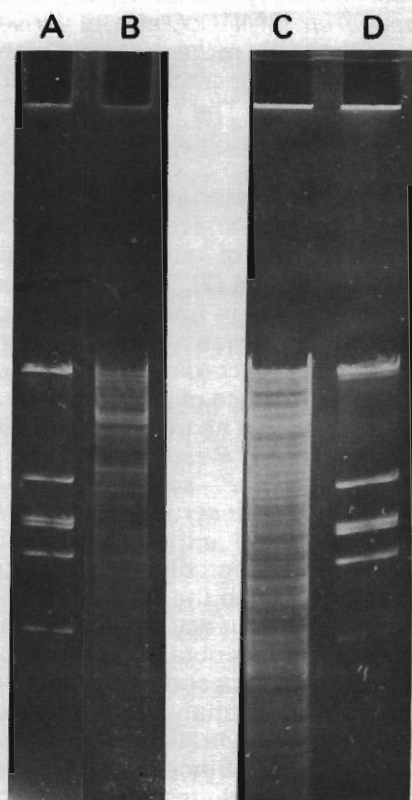
Fig. 2. EcoRI restriction patterns from Phage λ DNA (A and D), strain B1 total DNA (C) and CCC DNA extracted from strain B1 (B).

with roughly the same relative mobility (Fig. 3). The megaplasmid was always the only CCC DNA band which could be seen except in the case of strain 145 where two smaller additional plasmids were present (Fig. 3, lane l). The megaplasmid was also present in the various avirulent mutants studied. Several of the strains showing a megaplasmid were submitted to a dye-buoyant density gradient. In all the cases studied the presence of CCC DNA was confirmed.

To confirm the designation used by Sciaky *et al.* (27) and Casse *et al.* (5) for large cryptic plasmids, we designated the naturally occurring *P. solanacearum* plasmid *pPso* followed by the strain number in which the plasmid was found.

Insertion of Transposon Tn5 into Megaplasmids

To investigate the biological significance of these plasmids, genetic experiments such as curing, transfer, and mutagenesis are required. Since no genetic markers are known on megaplasmids, transposons could be inserted to tag them. In addition, because of the ability of transposons to inactivate the genes in which they insert, they could offer a way to mutagenize the megaplasmids and thus identify plasmid encoded genes.

Transposon Tn5, which encodes resistance to kanamycin (Km) and neomycin, was used in such experiments because of its low specificity for insertion sites (1). Recently, we devised a way to introduce Tn5 into *P. solanacearum* GMI1000 and its derivatives (3) but this procedure does not allow site specific mutagenesis. On the contrary, methods for site specific mutagenesis have recently been used for *R. meliloti* (25) and *A. tumefaciens* (18). The rationale of such methods consists in cloning the fragment to be mutagenized on a vector plasmid, mutagenesis of that specific fragment in *E. coli* and reintroduction of the mutagenized gene in the wild type bacteria through homologous recombination. This was achieved for *P. solanacearum* GMI1000 and K60 by *in vitro* cloning of a *Hind III* fragment of the purified megaplasmid, into the *Hind III* site of plasmid pACYC184 (7). The hybrid plasmids were then used to transform an *E. coli* K12 strain harboring a chromosomal insertion of Tn5.

Selection of derivatives having an insertion of Tn5 in the hybrid plasmid was performed according to Pühler and Klipp (22), using their ability to grow in the presence of high concentration of neomycin. A population of *E. coli* cells harboring an insertion of Tn5 in the hybrid plasmid was lysed and used to transform *P. solanacearum*. Since pACYC184 cannot replicate autonomously in *P. solanacearum*, the Km^r transformants obtained occurred through insertion of the transposon in a resident-replicon. This could be achieved either by recombination between the cloned *pPso* fragment and its homologous region of the resident megaplasmid, or by transposition. Since the frequency of transposition is 100 to 1000 times less than the frequency of homologous recombination, practically all the Km^r transformants obtained corresponded to bacteria which should have acquired the transposon in the megaplasmid. In addition, around 50% of the clones had simultaneously acquired the pACYC184 vector as shown by their ability to hybridize on nitrocellulose sheet with a pACYC184 radioactive probe. Since no CCC DNA other than the megaplasmid was detected in these clones, they probably result from the insertion by one
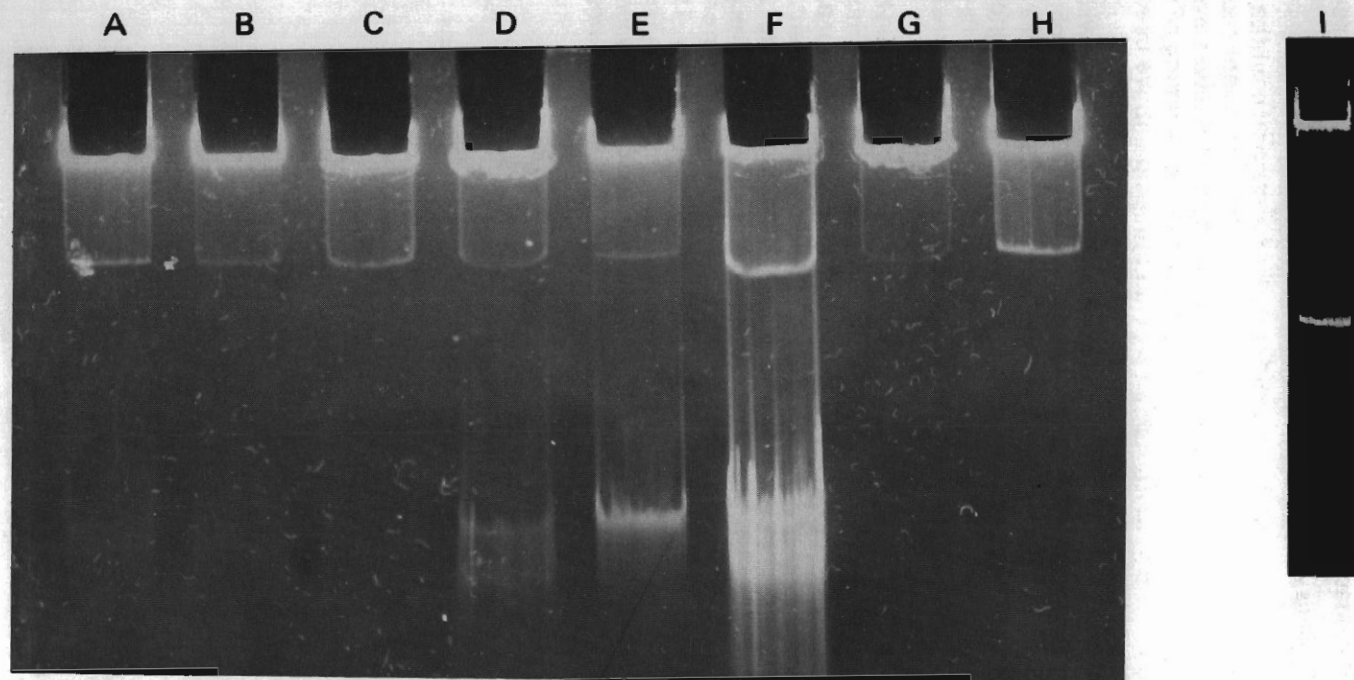
Fig. 3. Agarose gel electrophoresis of lysates from *P. solanacearum* strains: 8 (A); 203 (B); 40 (C); 85 (D); 70 (E); GM12,000 (F); 82 (G); K60 (H); and 145 (I).

single crossing over, of the vector plasmid in between two copies of the cloned *pPso* fragment. It is interesting that in these conditions the pACYC184 encoded chloramphenicol resistance was not expressed.

Mobilization of Megaplasmid

To facilitate the study of the plasmid encoded functions it could be helpful to transfer the plasmid into other strains. A suitable recipient could be a cured derivative of *P. solanacearum*. Since no such strain was available, we used *E. coli* as recipient. To avoid restriction of the incoming *pPso* DNA and possible recombination of *pPso* DNA in the recipient, we used strain HB101, which is deficient for restriction and recombination.

It has been shown that cloning of a piece of a replicon into the wide host-range conjugative plasmid RP4, increases the ability of RP4 to mobilize the genetic markers carried on the rest of the replicon (12). We used a similar strategy to facilitate the mobilization of the megaplasmid. The donor strain was strain GMI1224, a derivative of GMI1000. The sex factor used was RP4'-103 a Km^S hybrid plasmid formed by insertion of *Hind III* restriction fragment of *pPso*GMI2000 into the *Hind III* site of RP4.

When strain GMI1224(RP4'-103) was mated for 24 h with strain HB101, Km^r transconjugants occurred with the frequency of 10^{-6} among the bacteria which had received the RP4 encoded ampicillin resistance. When the sex factor used was a Km^S derivative of RP4 without cloned megaplasmid DNA, the frequency of Km^r bacteria among *E. coli* transconjugants was 10^{-8}, corresponding to bacteria which had received a Tn5 following its transposition on RP4 in *P. solanacearum* prior to or during the mating. The increased frequency of transfer of Tn5 when RP4'-103 was used, suggested that the megaplasmid has been mobilized. This was confirmed by the presence of a DNA band with relative mobility similar to the one found for GMI1224 megaplasmid (Fig. 4) as demonstrated by an
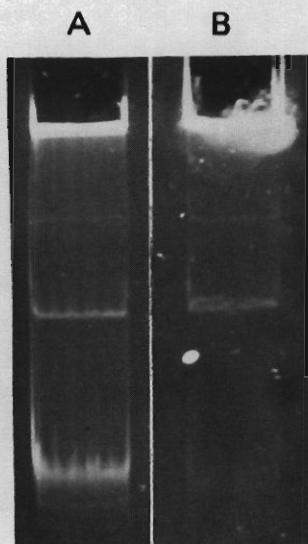
Fig. 4. Agarose gel elec-trophoresis of lysates from *P. solanacearum* strain GMI1224 (RP'4-103) (B) and HB101 kanamycin resistant transconjugant (A).

Eckhardt extraction of the *E. coli* transconjugant clones grown on the selective plates. However, after several subculturings without Km the megaplasmids could not be detected by agarose electrophoresis, suggesting that it is highly unstable in *E. coli*. Incidentally, this is evidence for the insertion of Tn5 in *pPso*GMI1000.

Discussion

It is clear that *P. solanacearum* strains, whatever their geographical origin and their host source, usually harbor a plasmid of uniquely large molecular weight with a similar relative mobility. It is not certain whether the negative results obtained with strain 19 reflect the actual lack of a plasmid or a limit of the extraction procedures for this strain. In all the strains studied but one, the megaplasmid was the only plasmid present in *P. solanacearum*. In this respect, *P. solanacearum* differs from other phytopathogenic *Pseudomonas* where several plasmids are usually present in each strain (16 for references). The question raised now is whether these megaplasmids may be involved in the control of the pathogenic properties of the species. In *Agrobacterium* where, in addition to the Ti plasmid, a larger plasmid is present, it is not known whether this large plasmid controls the relationship between the bacteria and the plant. In the case of *R. meliloti,* where megaplasmids have been found, they have been shown to be involved in the control of symbiotic properties such as formation of nodules and nitrogen fixation (9, 15).

It is interesting that a megaplasmid was detected in the spontaneous avirulent derivatives (B1 and GMI2000) as well as in the acridine orange induced one (19) (data not shown). Thus, in these cases the avirulent phenotype observed is not due to the loss of the entire plasmid. In *R. meliloti* where no strain cured of the megaplasmid has been obtained, it has been shown that spontaneous deletions of the megaplasmid occur, leading to alteration of the symbiotic properties. The question of the involvement of megaplasmid in the control of pathogenic properties in *P. solanacearum* is presently under investigation.

Literature Cited

1. Berg, D., 1977. Insertion and excision of the transposable kanamycin resistance determinant Tn5. In DNA insertions, plasmids and episomes. Bukhari A. I., Shapiro, J.A. and Adhya S. L. eds. Cold Spring Harbor Laboratory, pp. 205-212.
2. Boucher, C., B. Bergeron, M. Barate de Bertalmio and J. Denarie. 1977. Introduction of bacteriophage Mu into *Pseudomonas solanacearum* and *Rhizobium meliloti* using R factor RP4. J. Gen. Microbiol. 98:253-263.
3. Boucher, C., B. Message, D. Debieu, and C. Zischek. 1981. Use of P1-Inc group plasmids to introduce transposons into *Pseudomonas solanacearum*. Phytopathology 71:639-642.
4. Chaang, A. C. Y., and S. N. Cohen. 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from P15A cryptic miniplasmid. J. of Bacteriol. 134:1141-1156.

5. Casse, F., C. Boucher, J. S. Julliot, M. Michel, and J. Denarie. 1979. Identification and characterization of large plasmids in *Rhizobium meliloti* using agarose gel electrophoresis. J. Gen. Microbiol. 113:229-242.

6. Clewell, B. D., and D. R. Hellinski. 1969. Supercoiled circular DNA - protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. natl. acad. sci. U.S.A. 62:1159-1166.

7. Cohen, S. N., A. C. Y. Chaang, H. W. Boyer and R. B. Helling. 1973. Construction of biologically functional bacterial plasmids *in vitro*. Proc. natl. acad. of sci. U.S.A. 70:3240-3244.

8. Cohen, S. N., A. C. Y. Chaang, and L. Hsu. 1972. Non chromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R Factor DNA. Proc. natl. acad. sci. U.S.A. 69:2110-2114.

9. Denarie, J., C. Rosenberg, P. Boistard, G. Truchet, and F. Casse-Delbart. 1981. Plasmid control of symbiotic properties in *Rhizobium meliloti*. In Nitrogen Fixation in Perspective, A. H. Gibson ed., Australian Acad. of Sci. Canberra and North Holland. Elsevier. Amsterdam. 137-141.

10. Eckhardt, T., 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. Plasmid 1, 584-588.

11. Grunstein, M., and D. S. Hogness. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. Proc. nat. acad. of sci. U.S.A. 72:3961-3965.

12. Hansen, J. B., and R. H. Olsen. 1978. Inc P2 group of *Pseudomonas*, a class of uniquely large plasmids. Nature 274:715-717.

13. Julliot, J. S. and P. Boistard. 1979. Use of RP4-prime plasmids constructed *in vitro* to promote a polarized transfer of the chromosome in *Escherichia coli* and *Rhizobium meliloti*. Molecular and General Genetics 173:289-298.

14. Kelman, A., 1954. The relationship of pathogenicity in *Pseudomonas solanacearum* to colony appearance of a tetrazolium medium. Phytopathology 44:693-695.

15. Kondorosi A., Z. Banfalvi, V. Sakanyan, C. Koncz, I. Dusha, and A. Kiss. 1981. Location of nodulation and nitrogen fixation genes on a high molecular weight plasmid of *Rhizobium meliloti*. In Nitrogen Fixation in Perspective. A. H. Gibson ed., Australian Acad. Sci. Canberra and North Holland. Elsevier. Amsterdam 407.

16. Lacy G. H., and J. V. Leary. 1979. Genetic systems in phytopathogenic bacteria. Annual Review of Phytopathology, R. Grogan ed., Palo Alto, California. 17:181-202.

17. Le, T. K. T., D. Leccas, and C. Boucher. 1978. Transformation of *Pseudomonas solanacearum* strain K60. Proc. 4th int. conf. plant path. bact. Angers 819-822.

18. Lemmers, M., M. Holsters, G. Engler, M. Van Montagu, J. Leemans, H. de Greve, J. P. Hernalsteens, L. Willmitzer, L. Otten, J. Schröder and J. Schell. 1981. Le plasmide Ti, vecteur potentiel pour la modification génétique des plantes. Comptes rendus de l'académie d'agriculture de France (in press).

19. Message, B., P. Boistard, M. Pitrat, J. Schmit, and C. Boucher. 1978. A new class of fluidal avirulent mutants of *Pseudomonas solanacearum* unable to induce a hypersensitive reaction. Proc. 4th Int. Conf. Plant Path. Bact. Angers, 823-833.

20. Meyers, J. A., D. Sanchez, L. P. Elwell, and S. Falkow. 1976. Simple agarose gel electrophoretic method for identification and characterization of plasmid deoxyribonucleic acid. J. Bact. 127:1529-1537. (Erratum J. of Bact. 129, 1171).

21. Panopoulos, N. J., B. J. Staskawicz, and D. Sandlin. 1979. Search for plasmid-associated traits and for a cloning vector in *Pseudomonas phaseolicola.* Plasmids of Medical Environmental and commercial importance. K. N. Timmis and A. Pühler ed. Elsevier North-Holland. Biomedical press. Pp. 365-372.

22. Pühler, A., and W. Klipp. 1981. Fine structure analysis of the gene region for N$_2$ fixation of *Klebsiella pneumoniae.* Biology of inorganic nitrogen and sulfur. H. Bothe and A. Trebst eds. Springer Verlag, Berlin-Heidelberg. Pp. 276-286.

23. Rosenberg, C., F. Casse-Delbart, I. Dusha, M. David, and C. Boucher. 1981. Megaplasmids in the plant associated bacteria *Rhizobium meliloti* and *Pseudomonas solanacearum.* Submitted to Journal of Bacteriology.

24. Roussel, A. F., and Y. A. Chabbert. 1978. Taxonomy and Epidemiology of Gram-negative bacterial plasmids studied by DNA-DNA filter hybrization in formamide. J. gen. Microbiol. 104:269-276.

25. Ruvkun, G. B., and F. M. Ausubel. 1981. A general method for site directed mutagenesis in prokaryotes. Nature 298:85-88.

26. Schwinghamer E. A. 1980. A method for improved lysis of some Gram-negative bacteria. F. E. M. S. Microbiology Letters 7. Pp. 157-162.

27. Sciaky D., A. L. Montoya and M. D. Chilton. 1978. Fingerprints of *Agrobacterium* Ti plasmids. Plasmid 1. Pp. 238-253.

28. Tanaka, T., and B. Weisblum. 1975. Construction of colicin El-R factor composite plasmid *in vitro:* Means for amplification of deoxyribonucleic acid. J. Bac. 121:354-362.

29. Van Larebeke N., G. Engler, M. Holsters, S. Van den Elsacker, I. Zaenen, R. A. Schilperoort, and J. Schell. 1974. Large plasmid in *Agrobacterium tumefaciens* essential for crown-gall inducing ability. Nature. London 252:169-170.

Abstracts

CHARACTERIZATION OF PLANT COMPLEXES THAT AGGLUTINATE SAPROPHYTIC PSEUDOMONADS. Anderson, A. J., and P. Walzer. Dept. of Biology, UMC 45, Utah State University, Logan, Utah 84322, U.S.A.

Resistance of plants to bacteria may involve immobilization of the bacteria challenge through a process requiring recognition between bacterial and plant cell surfaces. Extracts were obtained from several species and cultivars of bean and alfalfa agglutinate containing cells of saprophytic pseudomonads. All tissues examined possessed this activity. One agglutinin isolated from red kidney bean leaves was a glycoprotein complex (6:1 carbohydrate: protein) of molecular size greater than 10^8 daltons. Arabinose (18%), galactose (49%), and galacturonic acid (33%) were the major carbohydrate components and 10-15% of the protein was hydrozyproline. Agglutinin activity was destroyed by prolonged treatments with alkali that cause β elimination of sugars from serine and threonine. Linkage of arabinose to hydroxyproline may also be involved since treatments at pH 1 for 1 hour at 100° removed about 50% of this residue from the original complex. Agglutinin activity was also lost by this treatment. Periodate oxidation reduced agglutinin activity and decreased the proportion of galactose and uronic acid. A second lower molecular weight class of agglutinins has been isolated from the same bean extract. These complexes contain neutral sugars and protein although hydroxyproline is absent. The agglutinin activity is distinct from the high molecular weight complexes in that no divalent cation is required.

VIRULENCE OF *Xanthomonas campestris* pv. *manihotis* DETECTED BY TETRAZOLIUM. J. T. Athayde, Laboratorio de Fitopatología - EMCAPA, Vitoria, Espirito Santo, Brazil, and R. S. Romeiro, Universidade Federal de Viçosa, Vicosa, Minas Gerais, Brazil.

Colonies aspects of *Xanthomonas campestris* pv. *manihotis* (Berth & Bondar 1915) Dye 1978, were studied on KADO's medium, containing 0.005% of Tryphenyl Tetrazolium Chloride (TTC) in order to attempt to correlate it with virulence, utilizing cultures stored during 12 months in mineral oil.

Colonies obtained on the TTC medium had variable dimension and variable red/white color proportion, characterizing, principally, two types: one with big colonies, with reddish center and large colorless margins occurring in large percentage and another with small reddish colonies with thin colorless margins. The others were variable from completely white to various red/white color proportion.

Evaluation of colony pathogenicity was accomplished, by three inoculation methods. The big colonies, with reddish center and large

438

colorless margins, showed high virulence; the small reddish colonies, with thin colorless margins showed low or no virulence; while the other types showed little virulence variation related to the high virulence observed. All the colonies were related serologically.

BIOLOGICAL ACTIVITY OF EXTRACELLULAR POLYSACCHARIDE

OF *Xanthomonas campestris* **pv.** *manihotis.* J. T. Athayde, Laboratorio de Fitopatología - EMCAPA, Vitória, Espírito Santo, Brazil, and R. S. Romeiro, Universidade Federal de ' Viçosa, Viçosa, Minas Gerais, Brazil.

The Biological Activity of Extracellular Polysaccharide (EPS) present in *Xanthomonas campestris* pv. *manihotis* (Berthet & Bondar 1915) Dye 1978 was investigated by using the capsule virulent strain UFV-6a. It was obtained from medium containing 0.005% of Triphenyl Tetrazolium chloride and cultivated in KADO's medium. The bacterial suspension obtained in phosphate buffer was precipitated in ethanol, after centrifugation. The extracellular polysaccharide from the precipitate was suspended again in distilled water, and submitted to dialysis. The biological activity of the EPS crude preparation was tested in cassava (cv. chagas) by immersing the bases of young plants sprouting in tubes containing 5 ml of EPS in distilled water, whose concentrations were variable from 8 to 1000 ng/ml equivalent galactose. The wilt duration declined with increase in EPS concentration. At 1.000 ng/ml equivalent galactose, 100% wilt occurred after 30 minutes of immersion.

When 0.5 cm of the wilted sprouting base was removed and the remaining part was immediately immersed in distilled water; turgescence recuperation was observed.

MULTIPLICATION OF *Pseudomonas solanacearum* IN RESISTANT POTATO PLANTS AND THE ESTABLISHMENT OF LATENT INFEC-

TIONS. Ciampi, L., and L. Sequeira. Instituto de Microbiología. Facultad de Ciencias, Universidad Austral de Chile. Valdivia, Chile, and Dept. of Plant Pathology, University of Wisconsin-Madison, Madison 53706, U.S.A., respectively.

When 1 -month- old plants of a wilt-resistant clone of *Solanum phureja* (1386.15) were stem-inoculated with three strains of *Pseudomonas solanacearum* (K60, S123, and S206), the bacteria multiplied rapidly at the point of inoculation and then moved in the vascular system to other parts of the stem. Resistant plants showed a remarkable ability to support relatively high populations of the bacterium in the absence of disease symptoms. Although multiplication in this resistant clone was substantially less than in susceptible Russet Burbank potato plants, large numbers of bacteria (up to 624 x 10⁴ cells of K60 per 5 cm stem segment) reached the base of the stem of plants maintained at high temperature (28°C) for 20 days after stem inoculation. From the base of the stem, the bacteria moved rapidly

into the roots and tubers. Strains of *P. solanacearum* differed in their ability to cause latent tuber infection in different resistant potato clones. When 11 *S. phureja* x *S. tuberosum* hybrids were stem-inoculated, maintained at 28°C for 3 wk and then grown to maturity at 20°C, most of the clones yielded tubers infected by one or more strains. The race 1 strain (K60) was the most infectious; 53,8% of all tubers harvested from all plants inoculated with this isolate carried latent infections. Because one clone (BR 53.1) never yielded infected tubers there appear to be genetic factors which may be useful in breeding programs aimed at eliminating latent tuber infection.

SPECIFIC WATER-SOAKING OF BEAN LEAVES BY *Pseudomonas phaseolicola*, **ITS ROLE IN BACTERIAL MULTIPLICATION AND POSSIBLE MECHANISMS INVOLVED IN SPECIFICITY.** El-Banoby, F.E., and K. Rudolph. Plant Pathology Department, Faculty of Agriculture, Al-Azhr University, Cairo, Egypt and Institut fur Pflanzenpathologie und Pflanzenschutz der Universitat, 3400 Gottingen, Federal Republic of Germany, respectively.

Multiplication of *Pseudomonas phaseolicola* in susceptible and resistant tissues was studied concomitantly with observations on the stages of water-soaking. In susceptible leaves, the first small water-soaked spots appeared 4 days after inoculation, when the bacterial concentration within the leaf was less than $1/10$ of the final one (1.1×10^7 cells/1.5 cm^2 leaf). Seven days after inoculation, the maximum bacterial concentration was nearly reached (1.6×10^8) and the water-soaked spots also reached their final size. Resistant leaves showed no visible water-soaking and the bacterial concentrations were 7×10^5 and 4.7×10^6 at 4 and 7 days after inoculation, respectively. It was concluded, therefore, that water-soaking is a necessity for bacterial multiplication and not a secondary symptom. The hypothesis that bacterial extracellular polysaccharides (EPS) are responsible for specific water-soaking could be further substantiated. When the intercellular washing fluid (IF) of 15 different bean cultivars was tested, a considerable degradation of EPS was observed in the resistant but not in the susceptible cultivars. When IF-preparations of the the bean cultivars, Red Mexican 34 and Opal, which are resistant to race 1 and susceptible to race 2, were tested, EPS from race 1 was much more degraded than EPS from race 2. These experiments indicated that the chemical structures of EPS from race 1 and race 2 differ. Both EPS-preparations could be characterized as levans, that is polyfructosans with

$(2 \quad 6)$-linkages and a MW around 6 to 10^6 daltons. Small differences between the two EPS-preparations were observed, such as a higher degree of branching [$(2 \quad 1)$-linkages in EPS of race 2, as well as quantitative and qualitative differences in content of some common and some rare and partly unidentified amino acids. Further experiments have to show whether these differences are responsible for the different degrees of degradation by the plant extracts.

REACTION OF EXOTIC "RESISTANT" TOMATO CULTIVARS TO BACTERIAL WILT IN NIGERIA. Erinle, I. D. Inst. for Agriculture Research, Ahmadu Bello University, Zaria, Nigeria.

The reaction of tomato cultivars/selections with alleged resistance to bacterial wilt was assessed using local isolates of *P. solanacearum* obtained from infected tomato and potato (*Solanum tuberosum*) in the forest of the southern Guinea and northern Guinea zones of Nigeria. Many of the cultivars/selections were either resistant or moderately so to the tomato strains of *P. solanacearum*. Some of the selections, especially those from the Asian Vegetable Research and Development Center (A.V.R.D.C.) possessed desirable agronomic characteristics in addition to resistance to the tomato strain. All cultivars/selections except NC 1965-51 were susceptible or only moderately resistant to the potato strain of the pathogen.

Erwinias **AFFECTING POTATOES IN THE HUMID TROPICS OF PERU.** E. R. French and Lilliam de Lindo, International Potato Center, Lima, Perú.

At San Ramon, Perú (850 m altitude) *Erwinia carotovora* var. *carotovora* (Ecc) and *E. chrysanthemi* (Ecy) have been determined to cause soft rot of tubers and a soft rotting or blackleg of stems of potato plants grown from either tubers or true potato seed, especially during the period of heaviest rains. The pathogenicity of isolates of Ecc and Ecy were tested on tubers and plants. Tubers of potato varieties Revolucion and Yungay (Tbr x Adg) were tested by infectivity titration with five levels of inoculum. Tubers were injected with 0.01 ml to a depth of 5 mm, avoiding lenticels. They were then incubated anaerobically for 3 days at 26°C. Two isolates caused a significantly greater cylindrical rot than two Ecc isolates. Revolution plants, 10 to 15 cm tall, were inoculated by soil infestation with three isolates of each erwinia, by three methods of inoculation, in a greenhouse at nearly constant 30°C. Stab inoculations at a leaf axil in the top of plants and at the crown gave similar high infection rates; whereas infestation of soil resulted in few plants developing symptoms. Ecy and Eca gave similar symptoms and infection rates.

TRANSFER OF THE DRUG-RESISTANCE TRANSPOSON Tn5 TO *Erwinia herbicola.* B. V. Gantotti, K. L. Kindle, and S. V. Beer. Department of Plant Pathology, Cornell University, Ithaca, NY, U.S.A.

The translocatable drug-resistance element (transposon) Tn5 was transferred from its carrier "suicidal" plasmid pJB4JI, harbored by *Escherichia coli* strain 1830, to *Erwinia herbicola* strain 112Y through conjugation. Transposons are sequences of DNA that code for resistance to antibiotics and have the ability to be translocated from carrier plasmids into other sequences of DNA. As a consequence of insertion, the gene into

which the transposon is inserted is altered or becomes nonfunctional.

Transposon mutagenesis was attempted as part of studies on the inheritance of bacteriocin production in *E. herbicola*, strain 112Y. This strain produces herbicolacin 112Y, a low molecular weight bacteriocin highly specific for *E. amylovora*. An exponential phase culture of the Tn5 donor, *E. coli* 1830, harboring plasmid pJB4JI, was mixed with a similar culture of the recipient, *E. herbicola* 112Y, under conditions that favored conjugation. Mutants of *E. herbicola* were selected based on their acquisition of resistance to the antibiotics kanamycin and neomycin, which is coded for Tn5. The frequencies of transfer of Tn5 to *E. herbicola* 112Y ranged from 0.4×10^{-8} to 26×10^{-8} per recipient cell. Matings carried out on membrane filters yielded more transconjugants than those done directly on plates of permissive agar medium.

Three hundred transconjugants were tested for ability to produce herbicolacin 112Y. Ten of the transconjugants failed to produce the bacteriocin, suggesting that the insertion of Tn5 had destroyed the bacteriocinogenic capacity of *E. herbicola*. The location of Tn5 in the nonbacteriocinogenic mutant genomes was determined by Southern blot followed by hybridization with Tn5 containing ^{32}P-labelled plasmid as probe. Using this technique, the Tn5 insertion site was detected in a 96 megadalton plasmid of *E. herbicola*, indicating that genes on that plasmid may have a role in bacteriocin production by *E. herbicola*.

(A complete report of this work, presented at the 5th International Conference on Plant Pathogenic Bacteria, is being published in *Current Microbiology*.)

CONFUSION IN THE RESEARCH OF BACTERIAL DISEASES IN POTATO CROPS. Maas Geesteranus, H. P. M. Research Institute for Plant Protection (IPO), Wageningen, The Netherlands.

Bacterial wilt, caused by *Pseudomonas solanacearum* E. F. Smith, and blackleg, caused by *Erwinia carotovora* subsp. *atroseptica* (Hellmers & Dowson) Dye have both been known as bacterial diseases of potato since the beginning of the twentieth century. The field symptoms may resemble the original description of the disease, or may slightly differ, presumably due to potato variety or environmental conditions. The symptoms of both diseases are so well known in the areas where they occur that further diagnostic investigations are usually omitted. But even then, diagnosticians try to isolate both pathogens directly on selective media specific for the supposed pathogen and they often find them due to their presence as epiphytes. Only in cases where infestations were considered to be impossible, have investigators discovered another pathogen, *Erwinia chrysanthemi* Burk. *et al.* Only recently, the symptoms developed by this pathogen on potato were described in Japan (Tominaga, 1979). The species was also isolated in the past few years in Australia (Cother, 1979), South America (de Lindo, 1978), Europe and Northern Africa (pers. comm. 1978). Symptom expression may vary with climatic conditions. Under dry

and warm conditions the haulms may wilt, resembling the symptoms of bacterial wilt. Under cool and humid conditions infected plants produced symptoms that may resemble those of both *Erwinia carotovora* subspecies infections. Hyper-colonization of the symptoms by the ever present *Erwinia carotovora* ssp. carotovora (Jones) Bergey *et al.* makes the confusion complete. *E. chrysanthemi* has been overlooked in the past, since this pathogen may grow very poorly on the commonly used nutrient or peptone media. It is easily overgrown on described selective pectate media and does not grow on TZC-media. Better results may be obtained with a double layer pectate medium with yeast and malonate, but since most of the other *Erwinia's*, and other contaminants grow faster, isolation is not always successful. Serological diagnostic techniques might demonstrate that *E. chrysanthemi* is more widespread in the potato crop all over the world than expected at present. There is great need for a description of the specific symptoms caused by the three *Erwinia* pathogens under various conditions in the field in order to be able to distinguish *E. chrysanthemi* infection from that of *Ps. solanacearum* or *E. carotovora*.

GROWTH INHIBITION OF *Xanthomonas campestris* **pv.** *citri* **AND ITS REVERSAL BY AMINO ACIDS FOUND IN THE INTERCELLULAR FLUIDS OF THE DISEASED CITRUS LEAVES.** Goto, M., and K. Yamanaka. Faculty of Agriculture, Shizuoka University, 836 Ohya, Shizuoka 422, Japan.

Twenty-one amino acids were identified in the intercellular fluids taken from healthy citrus leaves. Quantitative analysis was made on changes in the concentration of these amino acids after inoculation of *Xanthomonas campestris* pv. *citri*. The amino acids detectable in significant amount in citrus leaf tissues were tested for their role in bacterial growth. Met was an essential factor needed for growth of the bacterium and the optimum concentration was in the range of 0.05 to 0.1 umol per ml which corresponded with the amount detected in the intercellular fluids. In the presence of met, several amino acids such as asp, asn, glu, val, leu, and pro were utilized by the bacterium as nutrients. Pro was particularly important because it was the major amino acid found in the intercellular fluid of citrus leaves, and it increased rapidly after infection of citrus trees with the bacterium. The concentration several days after inoculation became 10 times higher than that of non-inoculated healthy leaves. On the other hand, ser and hylys were also important because of their ability to inhibit growth of the bacterium. This activity, however, was prevented by the presence of two amino acids, met and pro, at concentrations equivalent to those in the intercellular fluids. Sensitivity to ser and hylys was higher in the virulent isolate than that in the avirulent isolate. Pro prevented more effectively the growth inhibition of the virulent isolate than that of the avirulent isolate. The activity of pro synergistically increased by the coexistence of ala although ala itself did not have such an effect. The concentration of pro in the leaves of *Fortunella japonica*, resistant to citrus canker, was lower than that of susceptible *Natsudiadia* leaves, but the

reverse phenomenon was observed with ser. The higher ratio of ser: pro may be responsible for induction of the strong resistance of this plant to citrus canker.

STUDIES OF BACTERIAL WILT OF STRAWBERRY PLANTS CAUSED BY *Pseudomonas solanacearum.* *II.* β **-D-GLUCOGALLIN, THE ANTIBACTERIAL SUBSTANCE DETECTED IN THE TISSUES OF STRAWBERRY PLANTS.** Kawaguchi, K., K. Ohta, and M. Goto. Fac. of Agriculture of Shizuoka and Nagoya Universities, Shizuoka and Chikusa-Ku, Nagoya, Japan, respectively.

An antibacterial substance inducing growth inhibition of *Pseudomonas solanacearum* on agar plates was isolated from strawberry leaves by Sephadex G-25 and Avicel column chromatography. This substance was identified as β -D-glucogallin (β-1-Ogalloyl-D-glucose) by paper chromatography, UV adsorption spectrum patterns, and acid hydrolysis for determination of the components. The β -D-glucogallin contents of strawberry plants positively correlated with the antibacterial activity in terms of inhibition zone. In assays during the period of one month after inoculation, no detectable change was observed in the content of β-D-glucogallin as well as the antibacterial activity. Extracts taken from younger leaves inhibited the growth of *Ps. solanacearum* most effectively, those from petioles being next. Extracts from stems and roots showed a lower inhibitory activity. Antibacterial activity of the tissues varied depending on the plant age and growing seasons. The crude extracts from strawberry leaves inhibited not only the growth of *Ps. solanacearum* but also that of some other bacteria such as *Ps. meliae, Ps. syrii.. jae* pv. *pisi, Ps. syringae* pv. *lachrymans, Pseudomonas* sp. from *Ficus erecta, Ps. andropogonis* and *Xanthomonas campestris* pv. *oryzae.* No growth inhibition was observed against the other pathovars of *X. campestris,* erwiniae and corynebacteria. However, production of extra-cellular polysaccharides was significantly depressed in most of the pathovars of *X. campestris.*

AN AGGLUTINATION FACTOR PRESENT IN APPLE SEEDS. Romeiro, R. da S. Dept. de Fitopatología, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

An agglutination factor (AF) found in seeds of *Malus sylvestris X.* ranetka was able to agglutinate *in vitro* cells of the avirulent (E8) strain of *Erwinia amylovora* but no cells of other bacterial species. Cells of the virulent (E9) strain are only weakly agglutinated by the AF. The AF is also able to interact in agarose gel with LPS extracted from E8 giving rise to precipitin bands. Some additional properties of the AF are: heat stability, high mobility (0.51 mm/minute) in electrophoretic fields, pH optimum for activity between 2 and 4, apparent molecular weight around 12,000

daltons, activity destroyed by protease and trypsin, activity not inhibited by simple sugars, activity inhibited by E9 EPS, binding to negatively charged polymers. Elution of the AF preparation from a Bio-Gel P-100 column generates two peaks of activity: AF-I and AF-II. AF-I is not retained by the column and it presents poor mobility in agarose gel whereas AF-II is retained by the column and has good motility in this medium. AF-II strongly reacts with LPS prepared from E8 cells. Fractionation of E8 LPS by acid hydrolysis followed by differential centrifugation and gel filtration showed that the AF-II is R-core specific. AF-II possibly interacts with bacterial cells, bacterial extracts, and other negatively charged polymers via a charge-charge interaction. An agglutination factor was also detected in leaves and shoots of apple that appears similar to the seed AF. The AF may play a role in the immobilization of incompatible bacteria by plants *in situ*.

COMPARISON OF METHODS FOR EXTRACTING LIPOPOLYSACCHARIDES FROM *Xanthomonas campestris* pv.

manihotis. Romeiro, R. da S., J. T. Athayde, C. Fukuda, and U. G. Batista. Dept. de Fitopatología, Universidade Federal de Vicosa, Vicosa, Minas Gerais, Brazil.

The efficiencies of five methods of extracting lipopolysaccharides (LPS) from cells of an avirulent strain of *X. campestris* pv. *manihotis* were compared. The methods were: phenol extraction, boiling, boiling followed by phenol extraction, freezing-thawing, and freezing-thawing followed by phenol extraction. After extraction, the samples were dialyzed against distilled water and the dialysates were tested for the amount of LPS, antigenic purity, and contamination with other substances. The amount of LPS was assayed either directly by the KDO method or indirectly by the drop-precipitation technique and by agarose gel immunoelectrophoresis (rochet assay). Antigenic purity was also verified by immunoelectrophoresis. The method of boiling the bacterial cell suspension for 30 minutes followed by phenol extraction was the best for extracting high amounts of LPS; the level of contamination was lower and the antigenic purity was acceptable.

F'*LAC+* PLASMID IN *Salmonella typhimurium* TRANSFERRED FROM

Erwinia amylovora. Wu, W.C., and J. H. Fann. Dept. of Plant Pathology, National Chung Hsing University, Taichung, Taiwan 400, Republic of China.

The F'*lac+* plasmid of *Eschrichia coli* origin harbored in *Erwinia amylovora* was transferred by conjugation into *trp* mutants of *Salmonella typhimurium* LT2 and coexisted autonomously with a resident plasmid. The resulting *S. typhimurium trp* /F' *lac+* heterogenates showed biochemical

and pathological reactions, except lactose fermentation, identical to their female parents. These phenotypic properties showed that the chromosomal genes of *E. amylovora* were not transferred. The F'*lac+* plasmid cured spontaneously from the *S. typhimurium* cells at frequencies of 0.48-1.70% after overnight incubation. Similar frequencies of the F'*lac+* plasmid curing were also observed after 7 years of lyophilized preservation; however, frequencies of the F'*lac+* plasmid curing were increased approximately 10 to 90-fold after 6 years of soft-agar stab preservation and by treatment of acridine orange, respectively. The F'*lac+* plasmid in *S. typhimurium trp* mutants was then transferred by conjugation at the frequencies of $2-4 \times 10^{-1}$ into a wild-type strain of *S. typhimurium* and it mediated mobilization of *hisF+* and *pyrF+* chromosomal genes at the frequencies of 10^{-7} into *hisF* and *cysBpyrF* mutants.

Summary Comments: Advances, Future Needs

K. Rudolph, Chairman

The contributions for session IV were grouped under 3 topics. The large majority (24 papers) fell under the first topic, Pathogenicity. Only 2 papers were presented in the group, Host Responses to Infection, and 7 papers dealt with the third group, Bacterial Genetics. Although some of the papers could not be grouped precisely into one of the 3 topics, the dominant interest of phytobacteriologists in the field of pathogenicity and pathotypes was well documented. This became even more evident in that the contributions under bacterial genetics dealt mainly with studies to understand the genetics of pathogenicity factors.

Traditionally, phytopathologists have always tried to describe and differentiate the pathogenic organisms, and to understand the mechanisms by which diseases are caused. A better understanding of the factors for pathogenicity and virulence, on a genetic and molecular level, will probably only be possible when pathotypes differing only in one defined genetic and pathogenic character are available. In this respect, the contributions on pathogenicity and pathotypes reported here can be regarded as a valuable basis for further studies.

The fast development in the field of bacterial genetics in the past years has also stimulated the interest in the genetics of phytopathogenic bacteria in several laboratories. Pathogenicity could not be easily correlated with the occurrence of certain plasmids in most of the cases studied, but interesting results have been obtained, which may finally lead to a genetical localization of pathogenicity and virulence factors.

Although many contributions dealt with pathogenicity of virulence factors, as extracellular enzymes, toxins, polysaccharides, mobility, DNA and water congestion, interesting observations were also reported for some possible factors involved in the resistant reaction, such as water potential, oxygen content, inhibiting compounds, balance of amino acids, and leaf age. No contributions on agglutination of bacteria were made in this session, although this aspect seems to be of major interest at present.

Compared with pathogenicity factors of bacteria, it seems to be more difficult to analyze resistance mechanisms in the host plant on a physiological or biochemical level. This should be a field of further research in the future, especially where isogenic lines or cultivars, differing only in one gene for resistance, are available.

Further research on host-pathogen interactions in phytobacteriology should, therefore, concentrate on the following species:

1. Explanation of host-specificity.
2. Virulence factors of the bacteria: mode of action and genetical basis
3. Resistant reaction of the host cultivar: physiological and/or
   biochemical mechanisms involved, triggering principle.

Session V

Disease Management of Bacteria and Other Procaryotic Pathogens

Eduardo R. French, Chairman

Disease Management of Bacteria and Other Procaryotic Pathogens

Introductory Remarks
by E. R. French, Chairman, Session V

Managing the diseases that are damaging to a crop requires that the most appropriate combination of control measures be employed. Appropriateness should not be established on the basis of the short term benefit but rather on the basis of ensuring stability of production over decades to come.

Stability is achieved if durable resistance is incorporated into breeding programs rather than "vulnerable" genes. On the other hand, systemic bactericides may give false security, permit an increase of area sown to a crop, and then encounter sudden susceptibility and economic collapse; the sudden spread of a bactericide resistant pathogen strain would only occur, however, with organisms for which a rapid spread mechanism exists, so that there should be instances for which the calculated risk is worth taking.

Good management requires integration of control measures such as use of cultivars incorporating durable resistance genes, biological control, chemical control, control of other organisms that predispose the host or act as vectors, cultural practices that make the environment for the crop less favorable to the pathogens (good drainage, alternate host control), and crop rotation with non-susceptible crops.

The papers presented include the following topics (number for each given in parenthesis): Resistance (10), chemical control (3), use of antibiotics (3), cultural practices (1), inoculum studies (2), biological control (3), production of disease free clones (3), and plant growth promoting rhizobacteria (1).

Ingress of Suspensions of *Erwinia carotovora* subsp. *carotovora* into Tomato Fruit

Jerry A. Bartz

University of Florida
Gainesville, FL, U.S.A.

Abstract

Tomato fruit immersed in suspensions of *Erwinia carotovora* subsp. *carotovora* can be inoculated by a mass flow (infiltration) of suspension through the stem scar. Fruit inoculated in this manner typically have a very short shelf-life. Lesions originate inside the fruit; bacterial ooze may emanate from the stem scar before water-soaked areas are visible externally. Intuitively, two physical phenomena are responsible for the infiltration of submersed tomatoes, reduced internal gas pressures associated with cooling and water-head forces from immersion. Three other physical phenomena, fruit temperature, liquid surface tension, and water temperature influence the potential for infiltration. Fruit at 40°C immersed to 122 cm for 5 min in water at 20, 30, or 40°C increased in weight six times more than did 20°C fruit. Fruits inmmersed in 0.1% solutions of the nonionic surfactant Tergitol- NPX increased in weight over five times more than those treated in water only. The effect of fruit temperature and liquid surface tension appear additive. Fruit stem scars exposed to surfactant-amended suspensions may absorb the fluid spontaneously rather than from external-internal pressure imbalances.

Introduction

An infiltration of contaminated dump tank water into tomato fruit was implicated in the loss of a shipment to bacterial soft rot (1). Sixty percent of the fruit developed bacterial soft rot within 4 days of harvest. Most lesions seemed to originate inside rather than at wounds on the surface of the diseased fruit. Although six different plant pathogenic organisms were isolated, three pectolytic *Erwinia* spp. [*Erwinia carotovora* subsp. *carotovora* (Jones, 1901) Bergey *et al.* 1923 (Ecc.), *E. carotovora* subsp. *atroseptica* (van Hall, 1902), Dye 1969, and *E. chrysanthemi* Burkholder, McFadden, and Dimock, 1953] were responsible for most of the lesions.

Two different phenomena were associated with infiltration of fruits immersed in water. If fruit are cooled while submersed in suspensions of Ecc, they may increase in weight and subsequently decay (4). If fruits increase in weight by at least 0.1 g, they invariably decay. The portal for flow of water and microbes into fruit was the stem scar. This region of the

fruit surface was previously shown to be the major pathway for gas exchange (5, 6). Hydrostatic forces which accompany immersion also caused infiltration (2, 4). With both phenomena, gas pressures inside the fruit are lower than the fluid pressures on the surface of the stem scar. If a sufficient external-internal pressure imbalance exists, fluid is forced into the pores. Fortunately, tomato stem scar tissues appear to be hydrophobic, not only at the surface but below the surface as well (2). The net movement of water and microbes into fruit did not accompany immersion unless depths were excessive (greater than 122 cm). At moderate depths, infiltration did not occur unless exposure was longer than from 2 to 15 min, depending on the actual depth used and the fruit lot. Thus, tomatoes are safely handled in water in most circumstances. However, under certain environmental conditions infiltration occurs with only moderate pressure imbalances and relatively short exposures. In the following, conditions that lead to infiltration of submersed tomatoes are described and illustrated.

Materials and Methods

Tomato Fruit

Tomato fruit of various ripeness were hand-harvested or were purchased from commercial packinghouses in Florida. Fruit used for each test were of the same cultivar, those in each treatment of the same ripeness. Often fruit were harvested by clipping the pedicle with a pruning shears so that part of the stem remained on the fruit. The stems were removed just prior to treatment because fresh stem scars are more porous that old ones (4).

Inocula

Bacterial suspensions used were prepared from 24-h nutrient broth shake cultures of Ecc, Florida isolate SR-12, as described previously (3). Two inoculum concentrations, 1×10^6 and 1×10^7 colony forming units (CFU) per mililiter were used. Temperatures of bacterial suspension, water, and fruit were measured to within $0.5^\circ C$.

Treatments

In the immersion treatment individual fruit were weighed, treated, rinsed in 50 ppm NaOCl, wiped free of surface moisture with a cotton towel, reweighed, and stored at 20 to $30^\circ C$ depending on the test. In some tests the NaOCl rinse was omitted. Fruit weights were measured to the nearest 0.01 g but are expressed here as a percentage of the final fruit weight.

Temperatures of fruit and water or suspensions are those occurring prior to immersion. For temperature differential tests, fruit were held just under the surface of the test liquid for the indicated time interval. Immersion depths were simulated in a 22 qt pressure cooker-canner ("Presto" Aluminum Pressure Cooker, National Presto Industries, Inc. Eau Claire, WI 54791). Tomatoes were placed in the test liquid in the canner, a slotted bottom-plate was placed on the fruit and weighted down so that the fruit

were completely submerged (1-4 cm). The unit was sealed and compressed air was applied to create the desired water-head. Air pressure in the chamber was monitored continuously with a manometer. Treatment pressures were expressed as cm of water. The air pressure was released and the fruit immediately removed from the liquid when the desired exposure was achieved.

In some tests a nonionic surfactant, Tergitol-NPX (TNPX) (Union Carbide Corp.) was added to the fluid and expressed as a percentage, w/v, of the final liquid. The air-water interface surface tension of these surfactant solutions was measured with a Model 21, Fisher Surface Tensiomat (Fisher Scientific C., 711 Forbes Ave, Pittsburgh, PA 15219). The apparent surface tension was expressed as dynes/cm.

During storage the treated fruit were observed daily. Diseased fruit were discarded immediately in order to reduce the likelihood of fruit-to-fruit spread of disease. Disease resulting from such spread or from contamined surface wounds were omitted from the tabular data on the percentage of fruit with disease. As a result, the latter accurately represented fruit that were inoculated during the treatments.

Statistical comparisons of treatments were made by the one-way analysis of variance, linear regression, correlation coefficient, and multiway factorial analysis of variance tests. The latter were done using the SAS system at the facilities of the Northeast Regional Data Center at the State University System of Florida, Gainesville.

Results

As reported by Bartz and Showalter (4), warm fruit immersed in cool suspensions of Ecc for at least 10 min not only increased in weight but also decayed during storage (Table 1). However, warm fruit immersed in warm

Table 1. Weight increase and bacterial soft rot that resulted from immersion of mature green tomatoes at 37° C in suspensions of *Erwinia carotovora* subsp. *carotovora* at two temperatures C.[a-d]

Time	Weight increase (%) at temp. (°C)		% Decay[e] at temp. (°C)	
	20	37	20	37
10	0.25	0.00	95	15
5	0.01	0.00	60	15
2	0.00	0.00	15	5

[a] Weight increase of fruit = (final weight — initial weight) x (final weight)$^{-1}$ x 100.

[b] Average of 20 fruit −10 fruit for each of two replicates.

[c] Fruit held just under the surface of suspension, rinsed in 50 ppm NaOCl, dried with a towel, weighed to nearest 10 mg, and then stored at 26 C and over 75% RH.

[d] Florida SR-12 at 1 x 10^7 CFU/ml.

[e] Percentage of fruit with bacterial soft rot after 8 days storage.

suspensions for the same time interval did not increase in weight and most did not decay during storage. Most of the lesions began inside the fruit; water-soaking was often first observed on the shoulder of diseased fruits. An appreciable weight increase led to early appearance of bacterial soft rot. The incidence of decay among fruits that increased in weight by at least 0.1 g was 24, 52, and 66% at 48, 72, and 96 h after treatment, respectively. All of the fruit that gained 0.1 g or more were diseased by the end of the 8-day storage.

With some fruit, inoculation occurred in the absence of a detectable weight increase. However, only 12% of these fruit were diseased; symptom development was nearly identical with that which occurred among the fruit that gained at least 0.1 g. At the end of the storage period, the fruits were cut open to observe possible lesions that were not yet visible externally. Some discoloration and dry necrosis was found in over 50% of the fruits in the 37°C fruit, 20°C suspension treatment. None of fruit in the 37°C fruit, 37°C suspension treatment were affected in that manner. The dry necrosis and discoloration may be regarded as incipient lesions, evidence that some inoculum ingress had occurred. This illustrates the time-dependent nature of infiltration caused by a negative temperature differential. Vacuums do not develop inside fruit until they cool.

In commercial practice, infiltration by temperature differential can be prevented by either warming the water or reducing the length of exposure. The effect of warm water on the second physical phenomenon that can cause mass flow inoculation, hydrostatic forces, was examined. This phenomenon is nearly time independent (2). The exposure of fruit to water was limited to 5 min to minimize forces arising from temperature differentials.

Fruit at 40°C when immersed increased in weight approximately six-fold more than did those at 20°C (Table 2). This result appeared

Table 2. Weight increase that resulted from immersion of mature green tomatoes in water to a depth of 122 cm for 5 min. I. Fruit stored at 20° C were warmed to 30 or 40° C.[a-c]

Water temp. (C)	Fruit temp. (C)			
	20	30	40	Avg.[e]
20	0.3	0.9	1.8	1.0
30	0.2	0.8	2.0	1.0
40	0.3	0.6	1.3	0.7
Avg.[d]	0.3	0.8	1.7	

[a] Weight increase of fruit $=$ (final weight $-$ initial weight) x (final weight)$^{-1}$ x 100.

[b] Values are the average of 10 fruits.

[c] Five fruits at each temperature were treated in the pressure chamber containing water at indicated temperature.

[d] Effect of fruit temp., F $=$ 70.0, Probability of greater F $=$ 0.0001

[e] Effect of water temperature, F $=$ 6.01, Probability of greater F $=$ 0.0164.

independent of water temperature. However, increased water temperature significantly reduced the average weight increase of the immersed fruits. Increased fluid uptake associated with warmer fruit temperatures was a reversable phenomenon. Fruit stored at 40°C and then cooled to 20 or 30°C before immersion gained less weight than did those held at 40°C (Table 3). Once again water temperature had a significant impact of infiltration although of less importance than that of fruit temperature.

In these immersion depth tests, fruit were infiltrated by water because of hydrostatic forces that accompanied immersion. The average amount of water absorbed was considerably greater than that absorbed in the temperature differential test. This was probably due to greater fluid forces on the fruit. For example, in the first experiment on temperature differentials, if the fruit were cooled the full 17°C temperature difference, the resulting force imbalance on the fruit would be equivalent to approximately half of that existing in the immersion depth treatments.

One other physical phenomenon that might affect ingress of water into tomato fruit is the surface tension of the fluid in which the fruit were immersed. Hartman and Kelman (7) found that a reduced surface tension resulted in increased penetration of bacterial inoculum in corn stem tissues after whorl inoculations. Bartz (2) reported that a wax used commercially for treating tomato fruits was readily absorbed by stem scar tissues.

When a surfactant was added to the test fluid in an immersion depth treatment, the average uptake for each fruit in two separate tests was correlated, $r = 0.96$ and 0.99, respectively, with the concentration of surfactant, TNPX, unless the concentration was increased to about 1.0% (Table 4). The TNPX in the 1.0% treatment was not completely dissolved;

Table 3. Weight increase that resulted from immersion of mature green tomatoes in water to a depth of 152 cm for 5 min. II. Fruit stored at 40°C were cooled to 20 or 30°C. [a-c]

Water temp. (C)	Fruit temp. (C)			
	20	30	40	Avg.[e]
20	0.6	1.5	1.5	1.2
30	0.3	0.9	1.8	1.0
40	0.1	0.3	1.1	0.5
avg.[d]	0.3	0.9	1.5	

[a] Weight increase of fruit $= $ (final weight $-$ initial weight) $\times$ (final weight)$^{-1} \times 100$.

[b] Values are the average of 10 fruits.

[c] Five fruits at each temperature were treated in a pressure chamber containing water at indicated temperature.

[d] Effect of fruit temperature, $F = 25.4$, Probability of greater $F = 0.001$.

[e] Effect of water temperature, $F = 8.6$, Probability of greater $F = 0.0044$.

Table 4. Weight increase and disease that resulted from immersion of mature green tomatoes in various concentrations of Tergital-NPX (TNPX). a-d.

o/o (w/v) TNPX	Weight increase (o/o)		Decay (o/o)	
	Test 1	Test 2	Test 1	Test 2
water	0.23	NTe	0	NT
0.001	0.46	0.23	0	13
0.01	0.53	0.23	0	21
0.1	1.31	0.73	90	29
1.0	0.85	0.50	100	100

[a]Weight increase of fruit = (final weight − initial weight) $\times$ (final weight)$^{-1}$ $\times$ 100.
[b]Test 1 — Average of 10 fruit held 4 ft. below surface of solution for 10 min.
 Test 2 — Average of 24 fruit; eight fruit per treatment held 4 ft. below surface of solution for 5 min. Each treatment repeated 3 times.
[c]Apparent surface tensions of increasing concentrations of TNPX were 76, 59, 36, and 36 dynes/cm, respectively, that of water was 77.
[d]Incidence of fruit decay resulting from naturally occuring inocula at 3 days after treatments.
[e]NT = not tested.

the undissolved particles may have clogged some of the pores in the stem scar. This would result in partial anaerobiosis which would explain the increased decay but reduced uptake associated with the 1.0% TNPX treatment. Bacterial inoculum was not added to the water used in these treatments. The decay resulted from naturally occurring inoculum. The disease was mostly sour rot caused by *Geotrichum candidum* Link ex Pers. emend Carmichael. Lesion contents were quite acidic, pH 4.0 to 4.4, a characteristic of sour rot (1).

The difference in uptake between the control and the 0.001% TNPX treatment was not statistically significant when analyzed by the one way analysis of variance test. The lack of a significant difference may have been due to fruit-to-fruit variation within the treatments. For example, the range of weight increases in the control treatment was no increase (0.00 g) to 0.8 g. In the 0.001% TPNX treatment the range was 0.00 g to 1.8 g. Such variation was observed in nearly every test.

When a surfactant was added to suspensions of Ecc, a weight increase and inoculation occurred within seconds even though relatively shallow depth treatment was used (Table 5). Most bacterial soft rot appeared within 48 h after treatment; by that storage interval 100, 100, 90, and 40% of the fruit in the 91 cm 60 sec, 91 cm 30 sec, 61 cm 60 sec, and 61 cm 30 sec treatments, respectively, were diseased. The 10 sec treatment represented the time required to apply the indicated pressure and then release it. With that exposure, fruit were inoculated but did not increase in weight when the depth was equivalent to 91 cm.

In a subsequent test, warm (40°C) fruits were exposed to surfactant solutions. Fruit at 20°C immersed in water without surfactant to a depth of

Depth of immersion (cm)	Length of exposure (sec)					
	10		30		60	
	wt. inc.	% decay	wt. inc.	% decay	wt. inc.	% decay
2	0.0	0	0.0	0	0.0	0
31	0.0	0	0.0	0	0.0	0
61	0.0	0	0.2	80	0.3	100
91	0.0	40	0.5	100	0.6	100

[a] Incidence of bacterial soft rot among treated fruit after 6 days storage at 20° C.

[b] Weight increase of fruit = (final weight - initial weight) x (final weight)$^{-1}$ x 100.

[c] Florida isolate SR-12 at 1×10^8 CFU/ml buffered saline plus 0.1% Tergitol-NPX.

61 cm for 4 min did not increase in weight, whereas, those similarly treated with surfactant increased by an average of over 0.2 g per fruit. Fruit at 40°C treated without surfactant increased by about 0.3 g; those treated with surfactant increased in weight by over 1.3 g per fruit. The presence of a surfactant increased the potential for infiltration more than did elevated fruit temperatures.

The surface tension of the fluid from bacterial soft rot lesions in tomato fruits was 37 dyne/cm; juice from a mechanically macerated (blended) healthy fruit was 49 and that of a 0.1% solution of TNPX was 36. Some tomatoes at equilibrium with room temperature spontaneously (no external force) absorbed 0.1% TNPX-India ink suspensions within a 10 min exposure. Positive absorption was confirmed by the presence of the ink in a large portion of the central core of the fruit. Other tomatoes were observed to spontaneously absorb the fluid that oozed from rotted fruit. Infiltration can occur in the absence of negative temperature differentials or substantial hydrostatic forces.

Discussion

The potential for infiltration or the mass flow of bacterial suspensions into tomatoes was demonstrated. Infiltration was caused by two physical phenomena. Both are predictable from the general gas law as has been discussed previously (2, 4). The potential for infiltration is greatly affected by two other physical phenomena, the surface tension of the fluid outside immersed fruit and the temperature of the fruit. When either the surface tension of the fluid is low or the fruit are warm, infiltration may occur spontaneously. Decreased surface tensions and increased fruit temperatures appear additive. Warm fruit exposed to suspensions

containing surfactants are very likely to be infiltrated, inoculated, and later decay.

An infiltration of even small percentages of the total volume of tomatoes that move through a commercial packinghouse is likely to lead to substantial post-harvest losses. The infiltration may involve a mass flow of literally milliliter volumes of inocula into fruit. The inocula are deposited in an enviroment that seems ideal for an infection court. Bacterial soft rot resulting from mass flow inoculation develops and spreads quickly. A sign of disease, bacterial ooze, often emanates from diseased fruit before external symptoms are evident. This ooze has a low surface tension and consequently, may be quickly absorbed by the tissues of adjacent fruit. A truck load of tomatoes may be destroyed by bacterial soft rot within days, especially at temperatures ideal for the pathogen (25-35°C).

Infiltration with the potential for mass flow inoculation is not just a problem for commercial handlers of fresh market tomatoes. For example, bell pepper fruit (*Capsicum annuum* L.) are readily inoculated in stem and calyx tissues when fruit are immersed in suspensions of Ecc (Bartz, unpublished).Furthermore, mass flow inoculations are not unique to post-harvest handling. Tomato fruit with attached stems are infiltrated almost as readily as those without stems (Bartz, unpublished). Thus, tomatoes may be inoculated with Ecc or other microbes by mass flow around the sepals of the fruit prior to harvest. Samish and Etinger- Tulczynka (8) found various types of bacteria distributed throughout the interior of freshly harvested, healthy tomato fruit. The greatest concentrations were located in the tissues beneath the stem scar.

Certain steps can be taken to reduce the potential for infiltration during postharvest handling. Produce should be kept shaded during the interval between harvest and packing; exposure to direct sunlight leads to increased fruit temperatures, especially among those on the surface of the load. Dump tanks and fruit washers should be designed to keep fruit from being immersed too deeply (below ca. 3 1 mm) or in the water too long (more than 5 min). Soaps, detergents or other surfactant materials should not be added to wash or dump tank water. Water in washers or dump tanks should be changed whenever foaming occurs as the latter is an indication of a buildup of surfactants in the water. Finally, fruits that are wetted by the ooze from diseased fruits should either be discarded or at least handled separately from non-contaminated fruits.

Literature Cited

. Bartz, J. A. 1980. Causes of postharvest losses in a Florida tomato shipment. Plant Dis. 64:934-937.

. Bartz, J. A. 1981. Infiltration of tomatoes immersed at different temperatures to different depths in suspensions of *Erwinia carotovora* subsp *carotovora*. Plant Dis. 65: (In press).

. Bartz, J. A., J. P. Crill, and C. A. John. 1975. Inheritance of tolerance to *Erwinia carotovora* var *carotovora* in Florida MH-1 tomato. Phytopathology 65:1146-1150.

. Bartz, J. A., and R. K. Showalter. 1981. Infiltration of tomatoes by aqueous bacterial suspensions. Phytopathology 71:515-518.

5. Brooks, C. 1937. Some effects of waxing tomatoes. Proc. Am. Soc. Hortic. Sci. 35:720.

6. Clendenning, K. A. 1941. Studies of the tomato in relation to its storage. II. The effects of altered internal atmosphere upon the respiratory and ripening behavior of tomato fruits stored at 12.5°C. Can J. Res. 19:500-518.

7. Hartman, J. R., and A. Kelman. 1973. An improved method for the inoculation of corn with *Erwinia* spp. Phytopathology 63:658-663.

8. Samish, Z., and R. Etinger-Tulczynska. 1963. Distribution of bacteria within the tissue of healthy tomatoes. Appl. Microbiol. 11:10-17.

Non-toxic Chemical Control of Soft Rot Disease

Shinji Tsuyumu

Shizuoka University,
Shizuoka, Japan

Abstract

A broad variety of carboxylic acids was found to be a new group of pectinase inhibitors. The inhibition of this group apparently depends on the carboxyl group, and the remaining structure affects the efficiency and the specificity of inhibition of each pectinase. Most of the acids tested could also inhibit both the maceration and the disease development (soft rot) caused by *Erwinia carotovora* subsp. *carotovora*. Especially, the acids like sodium malonate and gluconic acid δ lactone were shown to inhibit the maceration and soft rot without any inhibition of the growth of the pathogen. Therefore, the pectinase was confirmed to be one of the important factors causing maceration and soft rot. This finding immediately suggests the possibility that the specific pectinase inhibitors may be used as non-toxic control of other diseases, especially post-harvest diseases.

Introduction

Pectic substances are composed primarily of linear polymeric chains of D-galacturonic acid linked by α -1, 4 glycosidic bonds and containing carboxyl groups either not esterified (pectic acid) or esterified to different degrees with methanol (pectin). These pectic substances are regarded generally as the main constituents of the "inter-cellular cement" or middle lamellar structure in plant tissues (5).

Pectinase is the general term of the enzymes which degrade these pectic substances. The involvement of pectinase in the degradation of plant tissues has been reported for such diverse types of diseases as soft rots, dry rots, wilts, blights, and leaf spots and for such diverse types of pathogenic agents as fungi, bacteria, and nematodes.

Thus, pectinase has been considered to play an essential role in the establishment of pathogenicity (5, 26). Therefore, if the specific inhibition of the pectinase is possible, the cellular toxicity may not be required for the purpose of the control of these plant diseases. In fact, Grossman showed experimentally that rufianic acid, a pectinase inhibitor, was an effective control agent of Fusarium wilt (14). However, there have not been many reports on this subject since then. I report here that carboxylic acids are a new group of pectinase inhibitors and that many of them can prevent the soft rot diseases of the vegetables caused by *Erwinia carotovora* subsp. *carotovora* without depending on cellular toxicity.

Materials and Methods

Enzymes

Endo-pectic acid lyase (EC. 4.2.2.2., endo-PALY) from sonicated extract of *E.c.* subsp. *carotovora* (strain EC-1) was partially purified by ammonium sulfate fractionation, dialysis, and chromatography on diethyl aminoethyl - and phospho-cellulose. Purified endo-polygalacturonase (EC. 4.2.3.15, endo-PG) which had been shown to be homogenous upon ultracentrifugation and disc electrophoreses was supplied from Dr. S. Ishii [16]. Endo-PALY activity was assayed mostly by the measurement of changes in extinction at 235 nm in reaction mixture [15]. The reaction mixture consisted of enzyme, 0.08% sodium pectate, 1 mM $CaCl_2$, 0.04 M Tris-HCl buffer, pH 8.7, and test compound in a total volume of 2.5 ml.[2]

The periodate-thiobarbiturate method [20] was employed when UV absorption method was not applicable. Endo-PG activity was measured by the loss in viscosity of 0.43% (w./Vol.) sodium pectate in Oswald-Fenske viscometers (Hario No. 3) at pH 5.6 [16]. Other components in the reaction mixture were 0.02 M Na_2-HPO_4-KH_2PO_4 buffer (pH 5.6), purified endo-PG, and test compound.

Chemicals

Most of the carboxylic acids tested were obtained from commercial source in the form of sodium - or potassium-salt. The pH of free acids was adjusted to 7.0. The galacturonides were synthesized by specific oxidation of carbonyl group of various galactose derivatives using platinum black as a catalyst [2].

Maceration Test

The maceration test was performed by immersing the radish disks (4 mm diameter, 2 mm thick) in a test tube where either purified enzyme solution of endo-PALY (0.15 units/ml) or of endo-PG (0.53 units/ml), or the suspension of EC-1 strain which has been grown in minimal + 2% glycerol medium was mixed with the same volume of test solution (final 50 mM). Next day, the maceration was scored after vigorous agitation by Vortex for 20 seconds. Minimal medium contains 0.1% $(NH_4)_2SO_4$, 0.01% $MgSO_4$.7 H_2O, 0.7% K_2HPO_4 and 0.3% KH_2PO_4.

Inhibition of Soft Rot

Scoring of the soft rot was performed by placing filter paper immersed in a suspension of EC-1 strain onto a plant disk in a petri dish. The plant disk had been placed on the filter paper which absorbed 1 ml of test compound solution (50mM). The plant disks were reversed on the filter paper 30 minutes before inoculation of EC-1 strain. After 1 day incubation at 28°C, the results were scored.

Growth Inhibition

Overnight culture of EC-1 strain in YP broth (1% peptone, 0.5% yeast extract, pH 6.8) was washed twice by centrifugation and resuspended in minimal medium with or without carbon source either in the presence or absence of the test compounds. The growth was recorded by bio-photorecorder (Toyo Co.) at 28°C.

462

Results

Inhibition of Pectinase

α -D-galacturonic acid, the main building block of pectic substance, inhibited the activities of both endo-PALY and endo-PG (Table 1). Thus, it was suspected that analogues of α -D-galacturonic acid would have an inhibitory effect on these enzymes. Among the tested analogues, sugar acids other than galacturonic acid could inhibit these enzymes while α-D-galactose and its derivatives did not inhibit these enzymes (Table 1). These data seem to indicate that the carboxyl group may be the most important structure required for inhibition of this group. To test this interpretation and to study the constituent required for inhibition, broad variety of carboxylic acids other than sugar acids were tested for their inhibitory activities. Salts, ester, and lactones of many carboxylic acids tested inhibited both endo-PALY and endo-PG or at least one of the enzymes (Table 2). Aliphatic monocarboxylic acids inhibited endo-PALY strongly but did not affect endo-PG. However, sodium pyruvate did inhibit both activities.

Aliphatic dicarboxylic acids and the class of carbocyclic compounds showed a diverse inhibitory effect on each enzyme. Sodium citrate, an aliphatic tricarboxylic acid, showed the highest level of inhibition of endo-PALY activity among the tested carboxylic acids, but it inhibited endo-PG activity poorly. However, D, L-isocitrate lactone showed high inhibition of endo-PG only. These data seem to indicate presence of the carboxyl group is the minimum required for the inhibition and that the remaining structure determines the specificity of the inhibition.

Comparison of the inhibition by sodium salts of oxalate, citrate, galactarate, gluconate, and D-saccharate to the inhibition by their methyl esters or lactones indicates that methylation or lactonization of the carboxyl group influences the specificity of inhibition. Since the mode of inhibition by these acids was shown to be non-competitive, allosteric sites of these enzymes may be distinct (Tsuyumu and Endo, unpublished). Also, since the non-competitive type of inhibition is not affected by the concentration of substrate, these acids should be effective inhibitors even in the enviroments such as plants where fairly high concentration of the substrate (pectic substance) exists.

Inhibition of Maceration and of Soft Rot

The pectinase of plant pathogens is best known in relation to soft rot diseases during which a relatively large mass of parenchyma is rapidly invaded to give a mass of watersoaked tissue generally soft in texture and with little coherence (i. e. maceration) (26). The most common and the most destructive soft rots are caused by bacteria in the genus *Erwinia*. Of these, *Erwinia carotovora* subsp. *carotovora* seems to be most prevalent (1). Also, since main pectinase excreted by *E. c.* subs. *carotovora* are endo-PALY and endo-PG, the regulations of which have been well characterized (7, 8, 23, 24), the effects of the pectinase inhibition by these acids on the pathogenesis of *E. c.* subsp. *carotovora* were tested.

This was done by the observation of the maceration and of the soft rot symptom in the presence and absence of these acids. When plant tissue discs were immersed either in the proper concentration of purified enzyme

463

Table 1. Inhibition of endo-PALY and endo-PG by the analogues of galacturonic acid.

Compound	endo-PALY		endo-PG	
	Concentration	Inhibition[a]	Concentration	Inhibition
α -D-Galacturonate	11.0 (mM)	26 (%)	22.0 (mM)	67 (%)
α -D-Galactose	4.4	0	22	6
p-Nitrophenyl- α-D-Galacturonate	0.6	45	4.5	37
p-Nitrophenyl- α -D-Galactose	2.0	0	22	5
D-Galactone- γ -lactone	4.4	0	22	20
	87.0	88		
γ -D-Galactonolactone	4.4	0	22	95
	87.0	89		
Galactarate (Na)	2.5	56	22	0
	25.0	100		
Heated, Galactarate	2.5	29	9.1	83
Galactarate (methyl ester)	ND		22	33
D-Saccharate (K)	4.4	65	22	10
D-Saccharic acid-1,4-lactone	4.4	9	22	56
	20	90		
Gluconate (Na)	4.4	69	22	33
Gluconic acid- δ -lactone	4.4	25	22	60
L-Ascorbate	1.8	68	22	40
	2.3	100		

[a] The inhibition at various concentrations of the test compound was expressed in terms of per cent of the uninhibited control. The pH of the free acids was adjusted to 7 with NaOH. Symbol: ND, not done.

Table 2. Inhibition of endo-PALY and endo-PG by carboxylic acids.

Compound	endo-PALY		endo-PG
	Concentration (mM)	Inhibition (%)	Inhibition at 22 mM (%)
I. Aliphatic Monocarboxylic acids:			
Formate (Na)	4.4	64	20
Acetate (Na)	4.4	66	20
Dehydroacetate (Na)	ND		20
n-Butyrate (Na)	4.4	71	0
n-Capronate (Na)	4.4	66	20
n-Caprylate (Na)	4.4	65	0
Peralgonate (Na)	4.4	67	20
Glycolate (Na)	3.3	5	20
Thioglycolate (Na)	2.2	58	20
Methyl Lactate	3.3	63	20
Pyruvate (Na)	4.4	68	60
II. Aliphatic Dicarboxylic acids:			
Oxalate (Na)	4.4	45	60
Methyl Oxalate	4.4	13	82
Succinate (Na)	4.4	71	50
Fumarate (Na)	.3.3	32	20
Maleate (Na)	3.3	13	56
D, L-Malate (Na)	3.3	58	43
Malonate (Na)	4.4	70	79
L-(+) - Tartarate (Na)	4.4	70	79
meso-Tartarate	3.3	69	85
2-keto Glutarate	3.3	34	83
cis-Aconitate (anhydryde)	3.3	34	0
III. Aliphatic tricarboxylic acids:			
Citrate (Na)	0.65	78	20
D,L-Isocitrate lactone	4.4	0	69
IV. Carbocyclic Carboxylic acids:			
Cinnamate (Na)	3.3	66	0
Benzoate (Na)	3.3	8	20
Sulfo Salicylate (Na)	3.3	71	73
Hippurate (Na)	3.3	0	43

solution of endo-PALY or of endo-PG, or in the suspension of *E. c.* subsp. *carotovora,* the maceration of the tissues could be observed within a day. When tissue discs of radish or carrot were inoculated with a suspension of *E. c.* subsp. *carotovora,* soft rot symptoms appeared in a day or so. Most of the acids tested inhibited the maceration induced by purified enzyme (Table 3, Fig. 1). Though the inhibitors of the maceration induced by endo-PALY usually prevented the maceration induced by pathogen, the inhibitors of the maceration induced by endo-PG did not always show this result. Therefore, though both enzymes have the capabilities of causing maceration of plant tissues, endo-PALY seems to have the primary role in this particular combination of host-pathogen.

The inhibitory efficiencies against the maceration induced by pathogen and the ones against the soft rot induced by pathogen were almost parallel

Fig. 1. Photograph of soft rots in the presence and absence of carboxylic acids. Top left petri dish is the treatment with Na-thioglycollate; top right, Na-n-caprylate bottom left, dehydroacetic acid; bottom right, control. Two carrot discs (top) and two radish discs (bottom) were placed in each petri dish and were inoculated with 5×10^5 (left) or 5×10^8 (right) (cells/ml) of *E. c.* subs. *carotovora.*

Table 3. The effects of carboxylic acids on the maceration and soft rot caused by enzyme solution of endo-PALY and endo-PG or by the suspension of *Erwinia carotovora* subsp. *carotovora*.

Carboxylic acids	Maceration by purified pectinase		Maceration by *E.c. subsp. carotovora*	Soft rot by *E.c. subsp. carotovora*	
	endo-PALY	endo-PG		Radish	Carrot
Control	+a	++	+++	+b	++
α-D-Galacturonate	±	+	+	−	−
p-Nitrophenyl-α-D-Galacturonate	−	−	−		
D-Galactone-γ-lactone	−	−	−	−	−
γ-D-Galactonolactone	−	−	−	−	−
Galactarate	ND	ND	±	−	−
D-Saccharate	ND	ND	+	+	+
D-Saccharic acid 1-4-lactone	−	++	−	−	−
Gluconate (Na)	+	++	±	+	++
Gluconic acid-α-lactone	−	−	−	−	−
L-Ascorbate (Na)	+	−	±	+	++
Formate (Na)	−	+	−	+	++
Acetate (Na)	−	−	±	+	++
Dehydroacetate (Na)	−		−	−	−
n-Butyrate (Na)	−	−	−	−	−
2-Hydroxy-iso-Butyrate	−	−	−	−	−
n-Capronate (Na)	−	−	−	−	−
n-Caprylate (Na)	−	−	−	−	−
Pelargonate (Na)	−	−	−	−	−
Thioglycolate (Na)	−	±	−	−	−
Glycolate (Na)	+	+	−	+	+
Pyruvate (Na)	±	−	−	−	−
Oxalate (Na)	−		±	++	++
Methyl Oxalate	−	++	−	−	−
Succinate (Na)	−	−	±	+	++
Fumarate (Na)	−	−	±	+	+
Maleate (Na)	−	−	++	+	+
D,L-Malate (Na)	−	−	−	++	+++
Malonate (Na)	−	−	−		−
Tartarate (Na)	±	++	±	+	+
meso-Tartarate	++	±	±	−	−
2-keto Glutarate	ND	ND	±	−	−
Citrate (Na)	−	−	++	+	++
(H)	−	−	−	−	−
D,L-Isocitric acid-lactone	ND	ND			
Cis-Aconitate (anhydryde)	ND	ND	−	−	−
Cinnamate (Na)	−	−	−	−	−
Benzoate (Na)	−	−	−	−	−
Sulfosalicylate (Na)	−	−	+	−	+
Hippurate (Na)	−	−			

aThe results of maceration test +++, complete maceration. ++, severe maceration but remaining tissue blocks; +, moderate maceration; ± slight maceration; −, no maceration. ND, not done.
bThe results of soft rot test: +++, soft rot symptom of whole disk; ++, soft rot around the filter paper; ±, very slight soft rot underneath of the filter paper; −, no symptom.

467

Thus, it seems that maceration is the mandatory step which eventually leads to soft rot. However, sodium salts of several acids, especially acids of tricarboxylic acid cycle, failed to prevent the maceration and the soft rot induced by pathogen while they could prevent the maceration induced by purified enzyme. The reason for this may be rapid metabolism of these acids during respiration of the pathogen and/or plant. An interpretation was seen in the experiment that the acids in the tricarboxylic acid cycle such as citric acid could serve as a sole source of carbon for the growth of *E. c.* subsp. *carotovora* (Table 4). In these cases the devices such as chemical modification of these acids should be required to minimize the disappearance of these acids. In the case of bactericidal acids, as observed for sodium carprylate and sodium n-butyrate (Table 4), their disappearance may not need be considered. However, one should be aware that the preventing effects observed in this case may be due not only to the pectinase inhibition but also to their bactericidal activities.

Table 4. Effects of carboxylic acids on the growth of *E. c.* subsp. *carotovora*.

Carboxylic acid	Min.	Min. + Glycerol[a]
Control	$-$[b]	$+$
Gluconate (Na)[c]	$\underline{+}$	$+$
Gluconic acid- δ -lactone	$\pm$	$+$
n-Butyrate (Na)	$-$	$\pm$
Caprylate (Na)	$-$	$-$
Methyl Oxalate	$-$	$+$
Maleate (Na)	$+$	$+$
D, L-Malate (Na)	$+$	$+$
Malonate (Na)	$-$	$+$
Citrate (Na)	$+$	$+$
Tricarballylic acid	$-$	$+$

[a] The components in minimal medium are described in text. Glycerol was added after separate autoclave (final 2%)

[b] $+$, normal growth; $\pm$, growth at inhibited rate; $-$, no growth.

[c] The concentrations of the acids were 20 mg/ml (final).

Discussion

It was shown here that the maceration and soft rot could be prevented by the application of pectinase-inhibitor without depending on cellular toxicity on the pathogen. Since many plant diseases, especially postharvest diseases, show the maceration at their infected site, they may be controlled by the same mechanism. Postharvest diseases cause serious and rapid

loss of crops, but they are controlled with difficulty by fungicide treatment because of toxic residue on the crops (1, 5). Therefore, the possibility of non-toxic control of the postharvest diseases shown in this study may be especially noteworthy. In fact, some of these acids were shown to be effective for the control of the diseases caused by *Botrytis cinerea* and by *Rhizopus* spp. (Tsuyumu and Endo, unpublished).

The choice of the acids to be used for the control should be done after careful consideration of the efficiency of pectinase inhibition and of the stability, especially the loss due to the metabolism of the microorganisms and/or of the plant. Mixing these acids should be an effective way, because the selection of the acids which show the highest inhibition for each type of pectinase is possible. And, there will be more chance that some acids in the mixture remain intact.

Some acids are safe enough to humans yet retain the bactericidal activities against the pathogens. These acids may be added either singly or in the mixture. For example, although short and medium-chain fatty acids can inhibit the growth of microorganisms, it has been known that they are ingested in significant quantities but do not grossly interfere with human organ function as judged by toxicity test (12, 13). In fact, their bacterial activities have been suggested even as one of important host-resistant factors of human milk for the infants, and they have been permitted as antimicrobial food additives (17). The carbocyclic acids such as dehydroacetic acid, sodium benzoate, and sodium sulfosalicylate belong to another group of bactericidal agents, and they also have been permitted as antimicrobial food additives (18). In this case, however, the total intake should be carefully considered due to toxicity to humans at high concentration.

Since the pectinase has been known to distribute in plants and play essential roles in many plant physiological aspects such as ripening, releasing seed, and defoliation (19), these acids may also be used for the control of these responses. Thus, if these acids or their derivatives are used for the control of the diseases in field, the effects on the physiological response by inhibiting plant-pectinase should be considered.

The cells of higher plants often contain one or a combination of these acids at a concentration higher than the one required for the inhibition of these enzymes (21, 25). Therefore, it became conceivable that a high concentration of a particular combination of these acids could inhibit the pectinase of pathogen. Defense mechanisms of the plants against the pathogen due to the resistance of pectinase either by changing their substrate or by the appearance of their inhibitors (mostly oxidized phenolic compounds) have been suggested (2, 3, 4, 9, 10, 11). These carboxylic acids may also contribute to such defense mechanisms. Since they are concentrated mostly in cytoplasm, particularly in the vacuole (21, 25), this type of defense mechanisms may be effective after the cells are disrupted either by the initial attack of the pathogen or by other means such as mechanical injury. Thus, inoculation of the pathogen onto the plant should be done after consideration of the concentration and kind of the acids at the infection site when pectinase is suspected to be involved in their pathogenicity.

The broad variety of inhibitors includes the acids the effects of which are immediately obvious in the experimental system of many pectinase studies. For example, some of these acids, like citrate and acetate, have been commonly used for the preparation of the buffers to study these enzymes. One should be aware of the inhibition.

Literature Cited

1. Agrios, G. N. 1969. Plant Pathology. Academic Press, New York. 629 pp.
2. Albersheim, P., and A. J. Anderson. 1971. Proteins from plant cell walls inhibit polygalacturonases secreted by plant pathogens. Proc. Nat. Aca. Sci. USA. 68:1815-1819.
3. Albersheim, P., T. M. Jones, and P. D. English. 1969. Biochemistry of the cell wall in relation to infective processes. Annu. Rev. Phytopathol. 7:171-193.
4. Bateman, D. F., and R. D. Lumsden. 1965. Relation of calcium content and nature of the pectic substances in bean hypocotyls of different ages to susceptibility to an isolate of *Rhizoctonia solani*. Phytopathology 55:734-738.
5. Bateman, D. F., and R. L. Millar. 1966. Pectic enzymes in tissue degradation Annu. Rev. Phytopathol. 4:119-146.
6. Chesson, A. J. 1980. Maceration in relation to the post-harvest handling and processing of plant material. J. Appl. Bacteriol. 48:1-45.
7. Cooper, R. M., and R. K. S. Wood. 1973. Induction of synthesis of extracellular cell-wall degrading enzymes in vascular wilt fungi. Nature 246:309-311.
8. Cooper, R. M., and R. K. S. Wood. 1975. Regulation of synthesis of cell wall degrading enzymes by *Verticillium albo-atrum* and *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. 5:135-156.
9. Corden, M. E., and L. V. Edington. A calcium requirement for growth regulator-induced resistance to *Fusarium wilt* of tomato. Phytopathology 50:625-626.
10. Deverall, B. J., and R. K. S. Wood. 1961. Chocolate spot of beans (*Vicia feba* L.) interactions between phenolase of host and pectic enzymes of pathogen. Ann. Appl. Biol. 49:473-487.
11. Edington, L. V., M. E. Corden, and A. E. Dimond. 1961. The role of pectic substances in chemically induced resistance to Fusarium wilt of tomato. Phytopathology 51:179-182.
12. Fay, J. P., and R. N. Farias. 1975. The inhibitory action of fatty acids on the growth of *Escherichia coli*. J. Gene. Microbiol. 91:233-240.
13. Freese, E., C. W. Shen, and E. Galliers. 1973. Function of lipophyllic acids on antimicrobial food additives. Nature 241:321-325.
14. Grossman, F. 1962. Untersuchungen über die Hemmung pektolytisher enzyme von *Fusarium orysporum* f. *lycopersici*. III. Wirking einiger Hemmstoffe *in vivo*. Phytopath. Z. 45:139-159.
15. Hasegawa, S., and C. W. Nagel. 1962. The characterization of and α unsaturated digalacturonic acid. J. Biol. Chem. 237:619-621.
16. Ishii, S., and T. Yokotsuka. 1972. Purification and properties of endopolygalacturonase from Aspergillus japonicus. Agric. Biol. Chem. 36:1885-1893.
17. Kabara, J. J. 1980. Lipids as host-resistance factors of human milk. Nutr. Rev. 38:65-73.

18. Kawashiro, I., and S. Fujii. 1969. Food additives. Koseido, Tokyo. 261 pp.
19. Kertestz, Z. I. 1951. The pectic substances. Interscience, New York. 628 pp.
20. Preiss, J., and G. Ashwell. 1963. Polygalacturonic acid metabolism in bacteria. I. Enzymatic formation of 4-deoxy-L-threo-5-Hexoseulose uronic acid. J. Biol. Chem. 238:1571-1576.
21. Ranson, S. L. 1965. The plant acids. pages 493-525 in J. Bonner and J. E. Varner (eds) Plant Biochemistry. Academic Press, New York, 1054 pp.
22. Srivastava, R. M., R. Hudson, F. Seymour, and B. Weissman. 1978. Preparation of (Aryl α-L-Idopryranosid) uronic acids. Carbohydr. Res. 60:315-326.
23. Tsuyumu, S. 1977. Inducer of pectic acid lyase in *Erwinia carotovora*. Nature 269:237-238.
24. Tsuyumu, S. 1979. "Self-catabolite repression" of pectate lyase in *Erwinia carotovora*. J. Bacteriol. 137:1035-1036.
25. Ulrich, R. 1970. Organic acids. Pages 89-118 in A. C. Hulme (ed.) The Biochemistry of fruits and their products. Vol. 1. Academic Press, New York, 620 pp.
26. Wood, R. K. S. 1960. Pectic acid cellulolytic enzymes in plant disease. Ann. Rev. Plant. Physiol. 11:292-322.

Acknowledgment

Resistance to *Erwinia chrysanthemi* in Tomato

J. I. Victoria
J. Jaramillo

Instituto Colombiano Agropecuario
Palmira, Colombia

Abstract

Germplasm of *Lycopersicon esculentum, L. peruvianum, L. glandulosum,* and *L. pimpinellifolium* was evaluated for its level of resistance to bacterial soft rot (*Erwinia chrysanthemi*). Most of the varieties of *L. esculentum* were found highly susceptible to *E. chrysanthemi* except for 'Ronita', 'Luisiana', and 'VF 105-2', which graded as moderately resistant; accessions of *L. pimpinelli folium* and *L. hirsutum* were graded as moderately susceptible. Highly resistant accessions were found in *L. peruvianum* (PI 126431 and PI 128653) and *L. glandulosum* (PI 126440 and PI 126448). F_3 progeny of *L. esculentum* x *L. peruvianum* supplied by Dr. N. G. Hogenboom (IVT) was similarly found highly resistant. A breeding program has been started with this material to introduce this resistance into commercial tomato varieties.

Introduction

Tomato crop (*Lycopersicon esculentum* Mill.) is affected by several diseases in the Cauca Valley (Colombia). One of those is called huequera or bacterial rot, induced by *Erwinia chrysanthemi* pv. *zeae* [1] which showed up for the first time in 1977. Since then this bacterial disease has increased its incidence and severity, occasionally causing up to 60% losses. It is mainly observed under moderate temperature (20°C) and high precipitation.

Main symptoms are characterized by soft rot of petioles, branches, and stems, with desintegration of pith, followed by wilting, premature yellowing and death of affected plants. This bacterial disease appears in general during the flowering stage of tomato plants.

Since this bacterial disease has become very important and most common commercial varieties are susceptible, it was considered necessary to determine levels of resistance of commercial varieties and start a breeding program to develop resistance in susceptible ones.

Materials and Methods

Inoculation Method

Tomato plants (*L. esculentum*) cv. chonto, were inoculated with a bacterial suspension (5 x 10^8 cells/ml) by injection into the stem; by depositing a drop of bacterial suspension on the petiole base of third leaf

and wounding the stem through the drop; and by depositing bacterial growth and wounding the stem at 5 cm from the soil level. Once the inoculation system was determined, effect of relative humidity was studied in the disease development; inoculated plants were incubated under 72 or 100% relative humidity environments for 48 h. Thereafter, tomato plants were inoculated with several bacterial concentrations (5×10^6 - 5×10^8 cells/ml) and finally optimum inoculation age was evaluated by testing tomato plants 15, 25, 35, 45, 55, and 65 days old. To compare each of the previous treatments, 10 cultivars (KT 278, L 274, 8029-4-9, Chonto D, Bon Set, Indian River, Chonto Mejorado, Bonny Best, Chonto Mataverde, and Floradel) were evaluated. Five plants per variety were inoculated each time.

Evaluation of Cultivars

The following 56 cultivars of *L. esculentum* were evaluated: KT 278, L 274, 8029-4-9, Chonto D, Bon Set, Indian River, Chonto Mejorado, Bonny Best, RT 046, Chonto Mataverde, Floradel ARO 149, Lucy TMV, Gevas, Gloriana, Hebros, Córdoba 2 SM-3, VF 145-B 8, Panaset, C 5, Potomac, Walter, Australia 2, Napoli VF, Mecano VF, Luisiana, Linda VF, Ronita, VF 105-2, Perlita 73, Homestead Elite, ES 58-FR, Droplet, Roma VF, Heinz 1439 VF, Step 413, Platense, Brazil SM-2, Glamour, Marette-4, VE-1, Cavalier, Marmande, Nuova-Super Roma, Orot 47, VE-3, CVF-27, Marette, L 3000-R, 8021-2-2, 8020-4-1, 8047-1-10, 8029-4-9, Chonto Buga, PI 92863, PI 99782, and PI 127802. Seed of *L. esculentum* different from seed obtained from Plant Introduction was taken from the tomato germplasm of the vegetable program of the Instituto Colombiano Agropecuario.

Several Plant Introductions also were evaluated: PI 126445 and PI 127826 of *L. hirsutum;* PI 126440 and PI 126448 of *L. glandulosum;* PI 79532, PI 112215, and PI 126932 of *L. pimpinellifolium* and PI 126431, PI 128653 and 128657 of *L. peruvianum.* Seed from Plant Introduction (PI) was supplied by the Regional Plant Introduction Station in Ames, Iowa.

Six plants per variety were inoculated each time.

Results

Inoculation Method

Among the three inoculation systems, the most variable results were found with injection of bacterial suspension into the stem. Depositing bacterial growth and wounding the stem induced a very severe reaction; probably the best and most consistent results were found by depositing a drop of bacterial suspension on the petiole base of the third leaf and wounding the stem through it.

Inoculation of tomato plants by depositing a drop of bacterial suspension on the stem and wounding the stem through it and incubating the plants at 72% R. H. induced very little infection in comparison to incubation for 48 h at 100% R. H. Combination of the best previous results in evaluation of 12 cultivars by using different inoculum concentrations produced the average

reaction given in Table 1. Based on the previous results, incubation of 6 plants of 10 varieties and six different ages produced reactions given in Table 2.

Table 1. Reaction of *L. esculentum* cultivars to different concentrations of *Erwinia chrysanthemi*.

Concentration	Reaction[a,b]
5×10^6 cells/ml	3.6 ± 0.98
5×10^8 cells/ml	4.0 ± 1.12

[a] Average of 12 cultivars

[b] Scale 1 to 5

Table 2. Reaction of *L. esculentum* cultivars of different ages to *Erwinia chrysanthemi*.

Age (Days)	Reaction[a,b]
15	4.5 ± 0.57
25	4.7 ± 0.40
35	3.9 ± 0.61
45	4.4 ± 0.48
55	4.4 ± 0.63
65	4.1 ± 0.57

[a] Average of 10 cultivars

[b] Scale 1 to 5

From these results, the inoculation method developed to evaluate levels of resistance consisted of depositing a drop of bacterial suspension (5×10^6 cells/ml) on the axile of third leaf and wounding the stem through it, on plants 15 days old. Once inoculated they were incubated at 100% R.H. for 48 h.

Evaluation of Cultivars

Six plants from different cultivars of *Lycopersicon* species were evaluated by using the system developed previously. Results obtained are given in Table 3. Most of the *L. esculentum* cultivars were higly susceptible to *E. chrysanthemi* but Ronita, Luisiana, and VF 105-2 were moderately resistant. Cultivars from *L. pimpinellifolium* and *L. hirsutum* were moderately susceptible.

Highly resistant cultivars were found in *L. peruvianum* (PI 126431 and PI 128653) and *L. glandulosum* (PI 126440 and PI 126448). *L. peruvianum* cultivars produced high numbers of flowers although it was not possible to

Table 3. Reaction of *Lycopersicon* species to *Erwinia chrysanthemi.*

Species	Reaction[a]
L. esculentum	3.40 ± 0.87
56 accessions	
L. peruvianum	1.33 ± 0.34
4 accessions	
L. glandulosum	1.20 ± 0
2 accessions	
L. pimpinellifolium	2.53 ± 0.64
3 accessions	
L. hirsutum	2.70 ± 0.71
2 accessions	

[a] Scale 1 to 5

set down an interspecific crossing with *L. escolentum* cv. Chonto mejorado. *L. glandulosum* plants did not produce flowers under our conditions.

F_3 progeny of *L. esculentum* x *L. peruvianum* was kindly supplied by Dr. N. G. Hogenboom (IVT). These plants showed similar results of high resistance, but crossing to *L. esculentum* cv. Chonto L. 21 only induced production of parthenocarpic fruits.

Discussion

The evaluation method that showed the most consistent results in determination of levels of resistance in *Lycopersicon* cultivars consisted of depositing a drop of bacterial suspension (5×10^6 cells/ml) on the third axile and wounding the stem through it in plants 15 days old which were incubated at 100% R. H. for 48 h.

The best sources of resistance to *E. chrysanthemi* pv. *zeae* were found in PI 126431 and PI 128653 of *L. peruvianum* and PI 126440 and PI 126448 of *L. glandulosum*. Progeny F_3 of interspecific cross between *L. esculentum* x *L. peruvianum* produced plants that were highly resistant to infection by the bacteria. At present a breeding program is underway with this material to produce commercial tomato varieties that are resistant to the bacterial soft rot of tomato.

Literature Cited

1. Victoria, J. I., and G. A. Granada. 1981. A bacterial soft rot of tomatoes induced by *Erwinia chrysanthemi* in the Cauca Valley (Colombia). *In* Proc. 5th Int. Cong. on Plant Path. Bact. Cali, Colombia.

Resistance to Bacterial Common Blight *(Xanthomonas campestris pv. phaseoli)* in Beans

J. I. Victoria, G. Bastidas, O. Agudelo

Instituto Colombiano Agropecuario
Palmira, Colombia

Abstract

In 1969 several bean varieties were evaluated for their level of resistance to artificial inoculation of *Xanthomonas campestris* pv. *phaseoli*, bean common mosaic virus (BCMV), and *Uromyces phaseoli* var. *typica*, main diseases of dry-beans in the Cauca Valley (Colombia). Sources of resistance were selected and included in a bean breeding program of commercial varieties Diacol Calima, Diacol Nima, ICA Guali, ICA Duva, ICA Tui, and ICA Huasanó. In 1974 several progenies were evaluated for agronomic characteristics and level of resistance to bacterial common blight, BCMV, bean rust, and some other constraints of beans. Since 1977, selected lines have been included in yield experiments in comparison to commercial varieties in different sites of the Cauca Valley, Colombia, and Caribbean countries. Finally a line (ICA L-23) has been selected and will soon be released as a new commercial variety. Level of resistance of L-23 has recently been increased by crossing to Jules variety and backcrossing to the original line.

Introduction

Dry bean is one of the main legumes grown in the Cauca Valley (Colombia), but it has lost importance in this area in recent years. One of the main reasons for the decrease has been the incidence of several insects and diseases, such as bacterial common blight *(Xanthomonas campestris* pv. *phaseoli)*, rust *(Uromyces phaseoli* var. *typica)*, bean common mosaic virus (BCMV), and *Empoasca kraemeri*.

Main commercial varieties grown in the area are susceptible to these constraints. This work was carried out to select good sources of resistance to be included in a breeding program to produce improved varieties with resistance to these limiting factors.

Materials and Methods

Method of inoculation

Evaluation of levels of resistance of bean plants was done by using two inoculation procedures: 1. The scissor method, developed by Correa *et al.*

(1), which consists in dipping the scissor in a bacterial suspension (5×10^7 cells/ml) and cutting twice, the three leaflets per bean plant. 2. High pressure spraying method developed by Coyne and Schuster (2), which consists of spraying a bacterial suspension (5×10^7 cells/ml) at high pressure (120 pounds/sq. in.). The scissor method was used mainly in inoculations in the greenhouse and the pressure spraying method, in the field evaluations.

Evaluation of Cultivars

In 1969, an initial study was conducted to select from the bean germplasm of the Instituto Colombiano Agropecuario, those cultivars with reaction to natural infection by *X. campestris* pv. *phaseoli* lower than 3.0, in a scale 1 to 5. Selected cultivars were sown in the field in four replicates, row per replicate, and inoculated by spraying a bacterial suspension (5×10^7 cells/ml) at high pressure. Cultivars with reaction lower than 3.0 were sown and evaluated again in a similar way. The same materials were sown in another experiment to be evaluated under field conditions for resistance to bean rust (*U. phaseoli* var. *typica*). Plants were powdered with uredospores of the fungus. A third group of the same cultivars was grown in the greenhouse and inoculated with sap from infected plants by bean common mosaic virus (BCMV).

Breeding Program

Cultivars selected as resistant to the three diseases mentioned above were included in a crossing program with the commercial varieties present at that time. Once crosses were made, they were left to segregate for five generations, selecting each time for agronomic characteristics, such as plant architecture, number of pods, number of grains per pod, size, and color of seed.

Table 1. Reaction of bean cultivars to different diseases.

Cultivar	Añublo bacterial (*X. phaseoli*)	Roya (*Uromyces phaseoli* var. *typica*)	Virus (CBMV)
Diacol Calima	MS[a]	MS	S
ICA Guali	S	MR	S
ICA Nima	MS	MR	S
ICA Tui	S	HS	S
ICA Duva	HS		S
50987 - C	MR	HS	S
Nicaragua 90	MR	HS	S
Pintado	MR	MS	S
Red Kote	R	MR	R

[a] R = resistant; MR = moderately resistant; MS = moderately susceptible; S = susceptible; HS = highly susceptible.

Starting with the sixth generation, selected materials were evaluated for resistance under field conditions by artificially inoculating plants with *X. campestris* pv. *phaseoli* and *U. phaseoli* var. *typica* and under greenhouse conditions with *X. campestris* pv. *phaseoli* and BCMV. Materials found as resistant to the three diseases were evaluated in similar way in the three following generations.

Regional Trials

Materials in the ninth generation that were resistant to the three diseases, *X. campestris* pv. *phaseoli*, *U. phaseoli* var. *typica*, and BCMV, were included in yield trials in comparison to commercial varieties in experimental plots at the Experimental Center of ICA at Palmira and in regional trials with farmers.

Results

Method of Inoculation

Both methods of inoculation were found reliable in differentiating levels of resistance to the bacterial common blight (*X. campestris* pv. *phaseoli*).

Evaluation of Cultivars

In the bean germplasm of the Instituto Colombiano Agropecuario, which includes 870 entries, only 25 cultivars showed a reaction lower than 3.0 to bacterial blight. From these, only 11 cultivars were selected to be evaluated in level of resistance to bean rust (*U. phaseoli* var. *typica*) and BCMV; some of the results are given in Table 2. The best materials were Pintado and Red Kote.

Table 2.   Reaction of advanced bean lines to bacterial blight *(Xanthomonas campestris* pv. *phaseoli).*

Lines (77-B)	Experiments	
	Field	Greenhouse
10204	3.5[a]	1.0
10211	2.0	3.2
10203	2.5	4.1
10219	2.0	2.6
L 22	2.0	1.8
L 23	2.0	1.9
L 24	2.0	2.8
Diacol Calima	3.5	3.4
ICA Palmar	3.5	4.6
ICA Duva	5.0	4.8

[a] Scale 1 to 5

Breeding Program

Pintado and Red Kote were   in a breeding program with commercial varieties Diacol Calima, Diacol Nima, ICA Guali, ICA Duva, and others. In the fifth generation of segregation, 16 lines were selected as having suitable agronomic characteristics, to be evaluated for level of resistance to bean bacterial blight, rust, and BCMV under field and greenhouse conditions. Some of the results are shown in Tables 2 and 3. The best resistance was obtained with bean lines L 22, L 23, and L 24 from the cross Diacol NINA x Red Kote. Some selected cultivars were evaluated for level of resistance to several isolates of *X. campestris* pv. *phaseoli,* from different places with the results shown in Table 4.

Table 3. Reaction of advanced bean lines to different diseases.

Cultivar	Bacterial Blight (*Xanthomonas campestris* pv. *phaseoli*)		Rust (*Uromyces phaseoli* var. *typica*)		Virus (CBMV)
	Foliage	Pods	Number of pustules	Type of pustules	
L 22	1.9[a]	3.0	1.0	2.0	0
L 23	1.4	3.0	1.0	1.0	0
L 24	2.5	3.0	1.0	1.3	0
ICA Duva	4.9	4.3			5
ICA Bunsi			4.2	4.8	5
Diacol Calima			3.3	3.0	5

[a] Scale 0 to 5

Table 4. Reaction of bean cultivars to different isolates of *Xanthomonas campestris* pv. *phaseoli.*

Cultivars	Isolates[a]				
	Xp 5 (ICA Colombia)	Xp U2 Uganda	Xp 123 (CIAT Colombia)	Xp S (Nebraska)	Xp Br (Brasil)
ICA Palmar	5.0	2.7	4.1	2.4	3.8
L 22	3.1	1.9	2.7	1.5	2.2
L 23	3.1	1.9	3.0	2.3	2.6
L 24	2.7	2.1	2.5	2.4	2.6
Jules	1.1	1.4	1.6	1.1	2.0

[a] Scale 1 to 5

Regional Trials

Bean lines L 22, L 23, and L 24, selected for their resistance to bacterial blight (*X. campestris* pv. *phaseoli*), rust (*U. phaseoli* var. *typica*) and BCMV, were included in a program of yield trials in comparison to the commercial varieties Diacol Calima and ICA Palmar. Trials were planted at the Experimental Center of ICA-Palmira and in regional trials with several farmers. Some results are shown in Table 5, where the best results were obtained with the lines L 22, L 23, and L 24, which out-yielded commercial varieties Diacol Calima and ICA Palmar by 13.5 to 16.2% in experimental plots and 32.0 to 38 % in farmers fields.

Table 5. Yield of commercial varieties and promisory lines of bean.

| | Centro Experimental ICA-Palmira | | Farmers | |
	kg/ha	Percent	kg/ha	Percent
Line 22	1653.7[a] ± 307	95.0	1910.0[b] ± 212	98.1
Line 23	1741.2 ± 286	100.0	1946.3 ± 440	100.0
Line 24	1676.2 ± 273	96.3	1769.4 ± 431	90.9
Diacol Calima	1458.9 ± 242	83.8	1207.3 ± 664	62.0
ICA Palmar	1505.4 ± 234	86.5	1324.3 ± 424	68.0

[a] Average of nine semesters of plantings

[b] Average of crops in five different places in Colombia: Huila, Candelaria (Valle), Fresno (Tolima), Miranda (Cauca).

Discussion

Methods of inoculation developed by several authors to detect levels of resistance to *X. campestris* pv. *phaseoli* were found reliable in selection of resistant materials. Some materials were eliminated during evaluation of resistance to bean rust (*U. phaseoli* var. *typica*) and BCMV. Only Pintado and Red Kote presented an acceptable reaction to the three diseases.

These two varieties, once included in a breeding program with commercial varieties, produced the bean lines L 22, L 23, and L 24 with excellent level of resistance to *X. campestris* pv. *phaseoli, U. phaseoli* var. *typica*, and *BCMV*, as well as against *Empoasca kraemeri* (CIAT, 1979 VEF). They out-yielded Diacol Calima, the main commercial variety of this area, by 16.2% in experimental plots and 38 % at farmer levels. Most outstanding in agronomic characteristics, resistance to diseases, and yield of the three was L 23. It has been suggested that this line be distributed as a new commercial variety for the Cauca Valley (Colombia). Line L 23 resulted from crossing Diacol Nina x Red Kote.

In spite of the good performance of the three lines, a new program has been started to increase level of resistance to bacterial blight (*X. campestris* pv. *phaseoli*) and keep the other good characteristics by crossing to Jules variety and backcrossing to original lines. At present, material is in the fourth backcrossing, with high level of resistance to the three diseases. This material will enable selection of still better materials in the near future.

Literature Cited

1. Correa, F., J. I. Victoria, and D. Webster. 1978. Desarrollo de un Sistema de Evaluación y Pruebas de Resistencia al Añublo Común del Fríjol. (*Xanthomonas phaseoli* Dawson). *In* Resúmenes de Trabajo 3er. Congreso ASCOLFI, Manizales, Oct. 25/27-1978, p 2.

2. Coyne, D P., and M. L. Schuster. 1968. Tara-A New Great Northern Dry Bean Variety Tolerant to Common Blight Bacterial Disease. Nebraska Agr. Exp. Sta. Bull. 506-1-10.

Protection of Tobacco Plants from Bacterial Wilt With Avirulent Bacteriocin-Producing Strains of *Pseudomonas solanacearum*

W. Chen, E. Echandi
North Carolina State University
Raleigh, NC, USA

H. W. Spurr, Jr.
Agricultural Research Service USDA
Oxford Tobacco Research Laboratory
Oxford, NC, USA.

Abstract

Tobacco seedlings were protected from bacterial wilt caused by *Pseudomonas solanacearum* with avirulent bacteriocin-producing strains (ABPS) of *P. solanacearum* in the greenhouse and in an infested field. One hundred forty-nine strains of *P. solanacearum* from 9 different hosts were assayed for bacteriocin activity. All strains were assayed as bacteriocin producers against 22 bacteriocin indicator strains. Strains with the most bacteriocin activity were selected and avirulent strains obtained from each. Four ABPS inhibited over 90% of the 149 strains tested. Disease control was investigated in the greenhouse and field by first dipping the roots of tobacco seedlings in water suspensions of ABPS containing 2×10^9 colony forming units (CFU)/ml for 30 min prior to transplanting to infested soil. Selected ABPS protected tobacco seedlings from bacterial wilt in the greenhouse; however, the level of protection was altered by the population level of the virulent strain. Most disease control resulted when the population of the virulent strain in soil was at or below 10^6 CFU/g of oven dried soil. Three ABPS significantly protected a susceptible tobacco cultivar for 80 days in a naturally infested field in which the inoculum potential was artificially augmented with a virulent strain of *P. solanacearum*.

Introduction

Bacterial wilt, caused by *Pseudomonas solanacearum* E. F. Smith, is a serious disease of tobacco. In North Carolina, losses to bacterial wilt were 3.5 and 8.0 million dollars in 1979 and 1980, respectively (1).

Bacterial diseases of plants have been controlled by the application of avirulent bacteriocin-producing strains (ABPS) of bacteria. Crown gall (*Agrobacterium tumefaciens*) of apples and stone fruits was controlled by dipping the root system of seedlings in water suspension of the avirulent bacterium-producing strains 84 of *A. radiobacter* before planting them in

infested soil (9). Bacterial canker of tomato was also controlled in the greenhouse by spraying tomato plants with an ABPS of *Corynebacterium michiganense* (3).

Bacteriocin-like activity in *P. solanacearum* was first reported by Okabe in 1954 (10). In 1976, Cuppels (2) reported that 48 strains of *P. solanacearum* from 11 different hosts and 9 different regions of the world produced bacteriocins that selectively inhibited other strains of the same species; these strains were bacteriocinogenic for at least one of the 53 indicator strains tested.

This work reports on selection and isolation of avirulent bacteriocin-producing strains of *P. solanacearum* and their application to tobacco seedlings to control bacterial wilt.

Materials and Methods

Bacterial Strains

One hundred and forty-nine strains of *P. solanacearum* (tobacco, 61; tomato, 29; banana, 39; potato, 8; pepper, 5; eggplant, 4; peanut, 3) from a collection maintained in the Department of Plant Pathology at North Carolina State University were used. When needed, strains were grown on tetrazolium chloride (TZC) agar (6) plates at 30°C for 48 h.

Selection of Bacteriocin-Producing Strains

Bacteriocin-producing strains of P. solanacearum were selected by a modification of the procedure of Echandi (4). Twenty-two strains of *P. solanacearum* were used as indicators (Table 1). A 20 ml layer of casamino acid-peptone-glucose (CPG) agar (2) in a 9-cm petri dish was spot-seeded on the surface with 12 different test strains and incubated at 30°C for 40 h. Colonies were transferred from these master plates using a multipoint replicator with 4-mm diameter aluminum rods to fresh CPG plates previously dried for 3 to 5 days at room temperature. After incubation at 30°C for 40 h, bacterial cells were killed by inverting the plates and exposing them for 60 min to the vapor from 3 ml of chloroform in a Microvoid transfer chamber with continuous air circulation.

Two-tenths ml of indicator strain (10^7 cells/ml) was added to 4 ml of 0.7% melted water agar and poured over the bottom layer of agar. After the agar solidified, the plates were inverted and reincubated at 30°C for 24 h. Inhibition zones were measured. Strains with activity against the greatest number of *P. solanacearum* strains were selected. These were tested for lysogenicity by the procedure of Gratia (8). Twenty-four hour CPG broth cultures with bacteriocin-like activity were subjected to a ten-fold serial dilution and spotted (0.02 ml) on plates previously seeded with an indicator strain. Plates were incubated at 30°C for 24 h and observed for plaque formation.

Isolation of Avirulent Strains from Bacteriocin-producing Strains

Avirulent bacteriocin-producing strains of *P. solanacearum* were obtained by the procedure of Kelman and Hruschka (7). Selected bacteriocin-producing strains were cultured in glucose-proteose peptone-

Table 1. Strains of *Pseudomonas solanacearum* used to detect bacteriocinogenic strains.

Strain	Origin	
	Host	Location
121	Potato	North Carolina
124	Tobacco	North Carolina
128	Tomato	Georgia
129	Peanut	North Carolina
137	Potato	North Carolina
139	Tomato	North Carolina
155	Marigold	North Carolina
156	Eggplant	North Carolina
166	Tobacco	North Carolina
169	Tobacco	Florida
180	Banana	Costa Rica
183	Tobacco	North Carolina
197	Banana	Costa Rica
212	*Heliconia*	Costa Rica
245	Banana	La Lima, Perú
249	*Heliconia*	Colombia
286	Sunflower	North Carolina
C 12	Tobacco	Taiwan
C 34	Tobacco	Taiwan
C 61	Tomato	Taiwan
C 75	Tomato	Taiwan
C 107	Tobacco	Taiwan

yeast extract broth at 25°C for 5 to 10 days, streaked on TZC plates and incubated at 30°C for 48 h. Avirulent strains appeared as small butyrous colonies. A single butyrous colony was transferred to sterile water, shaken thoroughly and streaked on TZC plates. This procedure was repeated at least three times with each strain.

Strains were then inoculated in 4 to 6-week-old tobacco (*Nicotiana tabacum* L., cv. Hicks). tomato (*Lycopersicon esculentum* Mill., cv. Homestead-24), pepper (*Capsicum annuum* L., cv. Bull nose), eggplant (*Solanum melongena* L., cv. Duksy), and potato (*Solanum tuberosum* L., cv. Red Pontiac); root systems of tobacco, tomato, eggplant, and pepper seedlings were dipped for 30 min in a water suspension of each strain containing 5×10^8 colony forming units (CFU)/ml and planted in a mixture

of soil and sand (2:1, v/v) contained in 10-cm clay pots. Potato seedlings were inoculated by the method of Winstead and Kelman (11) using a bacterial suspension containing 5×10^8 CFU/ml. Inoculated plants were placed in a greenhouse at 28 to 40°C and observed for 4 weeks. If avirulent, the strains were retested in vitro against the original 149 strains of *P. solanacearum* to confirm their bacteriocinogenic activity range.

Application of ABPS for Disease Control

Avirulent bacteriocin-producing strain 121 and the virulent strains K-60 were grown separately in CPG broth on a shaker at 28°C for 48 h. A water suspension from each strain was prepared by centrifuging the culture at $16,000 \times g$ for 15 min, resuspensing the pellet in sterile distilled water and adjusting the suspension to 2×10^9 CFU/ml.

Treatments consisted of: 1) dipping the root systems of tobacco seedlings in a suspension of ABPS 121 containing 2×10^9 CFU/ml for 30 min and transplanting them to pots containing soil mixture incorporated with the virulent strain K-60 at a concentration of 1.1×10^6 CFU/g oven dried soil, 2) dipping the root systems of tobacco seedlings in sterile water for 30 min and transplanting them to pots containing soil mixture incorporated with the virulent strain K-60 at a concentration of 1.3×10^6 CFU/g of oven dried soil, respectively, 3) the same as 2, but the soil mixture was incorporated with ABPS 121 at a concentration of 1.3 to 10^8 CFU/g of oven dried soil instead of 1.3 to 10^6 CFU/g of oven dried soil; 4) dipping the root systems of tobacco seedlings in a suspension of ABPS 121 containing 2×10^9 CFU/ml for 30 min and transplanting them to pots containing soil mixture incorporated with the virulent strain K-60 and the ABPS 121 at concentrations of 1.1 to 10^6 and 1.3 to 10^6 CFU/g of oven dried soil, respectively; 5) the same as 4 but the soil mixture was incorporated with the ABPS 121 at a concentration of 1.3 to 10^8 CFU/g of oven dried soil, instead of 1.3 to 10^6 CFU/g oven dried soil; 6) dipping the root systems of tobacco seedlings in sterile water for 30 min and transplanting them to pots containing soil mixture incorporated with the virulent strain K-60 at a concentration of 1.1×10^6 CFU/g of oven dried soil.

Sixteen 5-week-old tobacco seedlings of the cultivar Hicks were used in each treatment. All plants were placed in a greenhouse at 28 to 40°C. Disease development was recorded at 5-day intervals for 30 days using the following index: 0 = no visible wilt; 1 = 1-15% leaves wilted; 2 = 16-30% leaves wilted; 3 = 31-60% leaves wilted; 4 = more than 60% leaves wilted; 5 = all leaves wilted or plant dead.

Protection Efficacy of ABPS for Granville Wilt

An aqueous suspension of virulent strain K-60 was incorporated into the soil mixture to provide inoculum levels of 2×10^8 to 2×10^3 CFU/g of oven dried soil at ten-fold intervals. Also, water suspensions of ABPS 121 and ABPS 237 containing 2×10^9 CFU/ml were prepared as described above. The root systems of eight 5-week-old tobacco plants of cultivar Hicks were dipped in each suspension of ABPS 121 and ABPS 237 for 30 min and planted in 10-cm clay pots containing soil mixture incorporated with K-60. Untreated control plants were dipped in sterile distilled water. Disease development was recorded as described above.

Greenhouse Evaluation of ABPS for Disease Control

Seven ABPS and the avirulent K-60 BI strain were evaluated for protection of tobacco seedlings against bacterial wilt in the greenhouse. The root systems of twelve 5-week-old Hicks tobacco plants were dipped in one of the suspensions of ABPS or K-60 BI (2×10^9 CFU/ml) for 30 min, and transplanted into a sandy loam soil from Oxford, NC, in 10-cm clay pots infested with the virulent strains K-60 at a concentration of 2×10^6 CFU/g of oven dried soil. Disease development was recorded for 30 days as described above.

Field Evaluation of ABPS for Disease Control

A field experiment was conducted in a sandy loam at Oxford, NC, infested 3 years previously with the virulent strain K-60. To assure sufficient inoculum, K-60 was grown in shake-culture in a semi-synthetic medium (5) at 28°C for 72 h, diluted 1:40 with sterile distilled water (1×10^8 CFU/ml) and sprayed (3200 ml/100 feet) in a band 20 cm wide on an open furrow. The furrow was closed immediately after spraying. One week later, the root system of 50-day-old tobacco seedlings of the cultivar NC 2326 were dipped as described above in ABPS 121, 146, 149, 153, 175, and K-60B1. The experiment was designed as a completely randomized block with 4 replications. Each replication was a single row of 22 tobacco plants. Disease development was recorded at 15-day intervals for 80 days using the scale described above.

Results

Selection of ABPS

One hundred twenty-one of 149 strains of *P. solanacearum* tested had bacteriocin activity against the 22 indicator strains of *P. solanacearum*. Ten bacteriocin-producing strains with a wide spectrum of activity and 6 bacteriocin-producing strains isolated from North Carolina and Georgia were selected for activity against the virulent strain K-60. When serial dilutions of bacteriocin-containing supernatants were spotted onto plates previously seeded with the indicator strain, no individual plaques were observed. Selections were obtained from the 16 strains and proved avirulent on tobacco, tomato, eggplant, pepper, and potato. The selected ABPS were retested for bacteriocin activity against the149 strains of *P. solanacearum* and ABPS 199, 237, 238, and 274 retained a wide spectrum of activity (Table 2 .

Application of ABPS for Disease Control

ABPS protected tobacco seedlings from bacterial wilt when applied as a root dip or when incorporated in the soil. Dipping the root systems of tobacco seedlings in a suspension of ABPS 121 containing 2×10^9 CFU/ml protected tobacco seedlings from bacterial wilt for 20 days when planted in soil incorporated with the virulent strain K-60 at a concentration of 1.1 to 10^6 CFU/g of oven dried soil; incorporating the ABPS 121 in soil at a concentration of 1.3 to 10^6 did not increase protection from bacterial wilt.

Table 2. Sensitivity of virulent *Pseudomonas solanacearum* strains to avirulent bacteriocinogenic strains of the same bacterium.

Bacteriocin producing strains	No. of bacteriocin-sensitive strains in indicator group						
	Tobacco (61)[z]	Tomato (29)	Banana (39)	Potato (8)	Pepper (5)	Eggplant (4)	Peanut (3)
121	48	21	27	7	4	4	3
146	14	7	22	5	1	0	0
149	48	21	32	6	3	4	3
175	42	14	29	7	3	1	1
180	54	22	10	7	3	4	3
199	57	23	15	7	3	4	3
237	57	28	38	7	4	4	3
238	59	29	38	7	4	4	3
274	56	28	38	7	3	4	3

[z]Numbers in parentheses indicate the number of strains in each indicator group tested for bacteriocin sensitivity.

When the concentration of ABPS 121 was increased to 1.3×10^8, complete protection of tobacco seedlings was obtained for the duration of the experiment. Similar protection from bacterial wilt, resulted when tobacco seedlings were dipped in water and planted in soil incorporated with ABPS 121 at 1.3×10^8 CFU/g of oven dried soil (Table 3). Although complete protection of tobacco seedlings from bacterial wilt resulted when ABPS 121 was incorporated in soil at a concentration of 1.3×10^8, root dip was used in most other experiments, because it was easy to perform and required less inoculum.

Protection Efficacy by ABPS

Protection of tobacco seedlings from bacterial wilt with ABPS was influenced by the inoculum density of pathogen in soil. When roots of tobacco seedlings were dipped in ABPS 121 or ABPS 237 and transplanted into soil infested with the pathogen at various densities (10^3 - 10^8 CFU/g of oven dried soil of virulent strains of *P. solanacearum*), symptom expression was delayed and the infection was reduced except with 10^8 CFU/g of oven dried soil (Fig. 1). Infection was related to pathogen concentration in soil. The smaller the pathogen population, the smaller the disease incidence and severity. However, more protection from bacterial wilt resulted when the pathogen population in soil was at or below 10^6 CFU/g of oven dried soil (Fig. 1).

Table 3. Comparison of root dip and soil incorporation of avirulent bacteriocin-producing strain (ABPS) 121 of *Pseudomonas solanacearum* for protection of tobacco from bacterial wilt.[x]

Treatment	Concentration of ABPS 121 at		Disease index[z]
	Root dip[y] (CFU/ml)	Soil incorporation (CFU/g of oven dried soil)	
Root dip	2×10^9	—	0.9
Soil incorporation	water	1.3×10^6	2.2
Soil incorporation	water	1.3×10^8	0.0
Root dip + soil incorporation	2×10^9	1.3×10^6	0.9
Root dip + soil incorporation	2×10^9	1.3×10^8	0.0
Untreated control	water	—	3.5
LSD (P = 0.05)			1.0

[x] Soil infested with virulent strain K-60 at the concentration of 1.1×10^6 CFU/g of oven dried soil.

[y] Root systems of seedlings dipped in a suspension of ABPS or water for 30 min before transplanting into soil-sand mix with K-60 or K-60 plus ABPS 121.

[z] Disease index ranged from 0 = no visible symptom to 5 = completely wilted or dead. Figures listed are the average of 16 plants 20 days after transplanting.
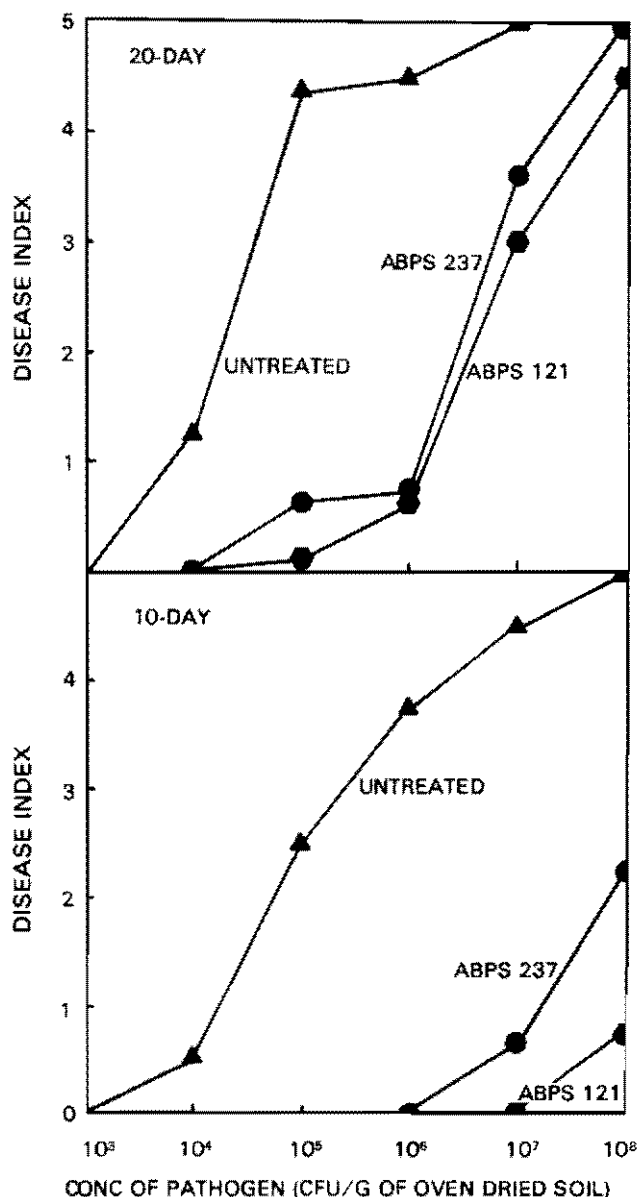
Fig. 1. Efficacy of protection of tobacco from bacterial wilt by avirulent bacteriocin-producing strain (ABPS) in relation to inoculum density of pathogen at 10 and 20 days after transplanting. Index ranged from 0 = no visible wilt to 5 = entire plant wilted or dead.

Evaluation of ABPS for Disease Control in Greenhouse

Avirulent bacteriocin-producing strains 121, 146, 175, 180, 237 and 274 protected tobacco plants from bacterial wilt; symptom expression was delayed and disease incidence was reduced as compared to untreated seedlings planted in either non-autoclaved soil or autocalved soil. Plants treated with K-60 BI were as affected as the untreated control. None of the ABPS treatments gave complete protection from bacterial wilt (Table 4).

Table 4. Effect of avirulent bacteriocin-producing strains (ABPS) of *Pseudomonas solanacearum* on bacterial wilt of tobacco in the greenhouse.[x]

Avirulent bacteriocin-producing strains (ABPS)[y]	Disease index[z] Days after treatment	
	20	30
121	0.3	1.7
146	0.2	1.9
149	0.5	3.3
175	0.4	2.8
180	0.0	0.9
237	0.0	1.6
274	0.7	1.3
K-60 B1	1.7	3.8
Nonautoclaved soil, untreated	2.2	3.9
Autoclaved soil, untreated	3.8	5.0
LSD (P = 0.05)	1.2	1.7

[x] Natural sandy loam soil was infested with pathogenic strain K-60 at the conc of 1.6×10^6 CFU/g of oven dried soil. All treatments except one of untreated were nonautoclaved soil.

[y] 40-day-old Hicks tobacco plants were dipped in a suspension of 2×10^9 CFU/ml of ABPS for 30 min prior to transplanting. Untreated plants were dipped in water.

[z] Disease index ranged from 0 = no visible wilt to 5 = completely wilted or dead. Figures listed are the average of 12 plants.

Field Evaluation of ABPS for Disease Control

In a field experiment in which the root systems of tobacco seedlings were dipped in ABPS, most ABPS protected tobacco plants from bacterial wilt. Water-treated control plants had wilt symptoms 20 days after transplanting. At 80 days K-60 BI and all ABPS treatments except ABPS 149 showed significant protection of tobacco plants from bacterial wilt (Table 5).

Table 5. Effect of avirulent bacteriocin-producing strains of *Pseudomonas solanacearum* on bacterial wilt of tobacco in the field.

Avirulent bacteriocin-producing strains	Disease index[z]
121	0.9
146	0.9
149	2.8
153	1.7
175	0.8
K-60 B1	1.5
Untreated control	3.3
LSD (P = 0.05)	1.5

[z] Disease index ranged from 0 = no visible wilt to 5 = completely wilted. Figures listed are the average of 88 plants at 80 days after transplanting.

Discussion

Bacterial wilt was controlled with ABPS of *P. solanacearum* in the greenhouse and in one field experiment. Avirulent bacteriocin-producing strains gave better control than the avirulent strain K-60 BI of *P. solanacearum;* however, the level of control with ABPS varied with individual strains. None gave complete control. It is possible that the ABPS did not colonize the new roots in sufficient numbers or that they did not produce enough bacteriocins to protect them from infection by the pathogen.

Although the mechanism of protection of ABPS has not been elucidated, it seems unlikely that competition for root infection sites will be the only mechanism involved in protection of tobacco plants from bacterial wilt. It is possible that ABPS may not only attach to root infection sites; but may also produce bacteriocins that will kill the pathogen and grant additional protection to the plants.

Avirulent bacteriocin-producing strains to be effective in biological control should have a wide spectrum within *P. solanacearum*, multiply profusely in the root system of the treated plants, and produce sufficient bacteriocins to protect the roots from infection by the pathogen. We have obtained some information on the effectiveness and possibilties of ABPS as biological control agents for bacterial wilt, but many questions must be answered before ABPS can be used for control of bacterial wilt.

Literature Cited

1. Collins, W. K., G. F. Peedin, F. A. Todd, R. Watkins, C. R. Pugh, and S. Southern. 1981. Tobacco Information. N.C. Agri. Ext. Service. AG-187.

2.   Cuppels, D. A. 1976. Bacteriocin production by *Pseudomonas solanacearum*. P. 120 in: L. Sequeira and A. Kelman, Eds., Proc. Ist Int. Plan. Conf. and Workshop on the Ecology and Control of Bacterial Wilt caused by *Pseudomonas solanacearum*. 18-23 July 1976, Raleigh, N. C. 166 pp.

3.   Echandi, E. 1975. Biological control of bacterial canker of tomato with bacteriocins from *Corynebacterium michiganense*. (Abstr.) Ann. Meeting Am. Phytopath. Soc., Aug. 10-14, No. 154.

4.   Echandi, E. 1976. Bacteriocin production by *Corynebacterium michiganense*. Phytopathology 66:430-432.

5.   Husain, A., and A. Kelman. 1958. Relation of slime production to mechanism of wilting and pathogenicity of *Pseudomonas solanacearum*. Photypathology 48:155-156.

6.   Kelman, A. 1954. The relationship of pathogenicity of *Pseudomonas solanacearum* to colony appearance on a tetrazolium medium. Phytopathology 44:693-695.

7.   Kelman, A., and J. Hruschka. 1973. The role of motility and aerotaxis in the selective increase of avirulent bacteria in still broth cultures of *Pseudomonas solanacearum*. J. Gen. Microbiology 76:177-188.

8.   Mayr-Harting, A., A. J. Hedges, and R. C. W. Berkeley. 1972. Methods for studying bacteriocins. Pp. 315-422, in: Norris, Jr. R. and D. W. Ribbons (eds.). Methods of Microbiology Vol. 7A. Academic Press, London and New York.

9.   Moore, L. W. 1979. *Agrobacterium radiobacter* strain 84 and biological control of crown gall. Ann. Rev. Phytopathol. 17:163-179.

10. Okabe, N. 1954. Studies on *Pseudomonas solanacearum*. V. Antagonism among the strains of *P. solanacearum* Reports of the Fac. Agri. Shizuoka University 4:37-40.

11. Winstead, N. N., and A. Kelman. 1952. Inoculation techniques for evaluating resistance to *Pseudomonas solanacearum*. Phytopathology 42:628-634.

Comparing 2 Inoculation Techniques for Evaluating Resistance in Beans to *Xanthomonas campestris* pv. *phaseoli*

M. A. Pastor-Corrales, S. E. Beebe, F. J. Correa

CIAT, Cali, Colombia

Abstract

Two inoculation techniques were compared for efficacy in screening resistance to common bacterial blight (CBB) caused by *Xanthomonas campestris* pv. *phaseoli* (Xp). Bean cultivars Jules, BAT 93, ICA L24, BAT 47, BAT 76, and Porrillo Sintetico, with known reactions to the blight in Colombia were inoculated in the field and in the greenhouse with Colombian isolate Xp-123. A razor blade procedure and a modification of it using surgical blades were compared. In all cases plants were inoculated in the first trifoliate 31 days after planting and evaluated 8 and 12 days after inoculation. In the field and greenhouse, the ranking of the cultivars based on their CBB reactions was the same with both inoculation techniques. However, the surgical blade was less effective in eliciting the expected CBB reaction from cultivars ranked as intermediate. The uniformity of CBB reaction was greater with the surgical blade procedure. It was also easier to use and faster. Both procedures are excellent methods for the evaluation of CBB resistance in beans in the field and both allow the simultaneous evaluations of other characters such as adaptation, architecture, and resistance to other diseases.

Introduction

Common bacterial blight (CBB) caused by *Xanthomonas campestris* pv. *phaseoli* (Smith 1897) Dye and Wilkie 1978b (ISPP List 1980) (*Xanthomonas phaseoli* (Smith) Dowson) is a major disease of dry beans. The disease is widely distributed in both temperate and tropical bean growing regions where it can cause considerable reductions in yield and in seed quality (13). The pathogen is seed transmitted and can attack leaves, stems, pods, and seeds. In the U.S.A., the use of clean seed produced in the semiarid regions of the west and other cultural practices and chemical controls are important components of the disease management strategy (13, 14). In the tropics, particularly where subsistence agriculture may prevail, such practices are difficult to utilize, thus the use of resistant cultivars provides the most adequate and practical method of CBB control (10).

An important initial step in the generation of disease resistant cultivars is the development of a reliable procedure for inoculating plants with pathogens artificially. Some of the criteria for choosing the inoculation procedure should be effectiveness in inducing distinct responses in resistant and susceptible cultivars, repeatability, and ease of using in the field where large numbers of plants may be evaluated. In addition, the procedure should be rapid, uniform, and easy to learn and implement.

In the past, several procedures have been utilized, including: A, the pricking of the stem with a needle (4) or a scapel (2) previously immersed in a bacterial suspension; (B) rubbing leaves with a bacterial suspension mixed with carborundum (3); C, spraying entire leaves with a bacterium suspension at high pressure (2, 8); D, vacuum leaf infiltration (9); E, perforation of leaves with a multiple needle inoculator (1, 7); and F, clipping of leaves with contaminated scissors (6, 10).

At CIAT, a modification of the scissors leaf-clipping procedure as reported by Webster (10) was used initially. Webster inoculated half primary or trifoliate leaves by cutting about 1.5 cm into the leaf with scissors previously dipped in inoculate. A second cut was also made about 2 cm toward the tip of the leaf (10).

In a modified procedure, two razor blades were mounted 2 cm apart and parallel to each other on a wooden handle. Trifoliate leaves were then placed on a sponge soaked in a bacterial cell suspension and cut in two areas as shown in Fig. 1. A modification of this "razor blade method" called "the surgical blade" procedure, was developed later at CIAT.

The objectives of the present study were: To report the surgical blade method as a new inoculation procedure; and to compare the efficacy of the razor blade and surgical blade procedures, the two inoculation techniques utilized at CIAT for the evaluation of common bacterial blight resistance in beans.

Materials and Methods

A randomized block design with five replications was employed. The field experiment was conducted at the CIAT experimental fields near Palmira in 1981. Six bean cultivars, representing a cross section of the different host reactions to the CBB pathogen were used (Table 1). The six included 2 resistant materials, Jules (P698) and BAT 93; 2 intermediates, ICA Line 24 and BAT 47; and 2 susceptibles, Porrillo Sintético (P566) and BAT 76. Twenty seeds were planted per 2 m row plots with rows spaced 60 cm apart. The field was isolated from other bean plots.

The experiment was also conducted in the greenhouse and in a growth room. In the last two locations 2 plants per pot constituted a plot. Field plots were planted January 13, 1981 and greenhouse and growth room planting was conducted the next day. Inoculation was done on February 6.

Inoculation with the razor blade has been described previously (5). In the surgical blade procedure, a bacterial cell suspension was placed in a plastic bottle equipped with a rubber stopper with two small round perforations in which pieces of sponge were placed to allow the inoculum to pass slowly.

Fig. 1. Razor blade equipment used to inoculate bean plants with the common bacterial blight pathogen.

Two steel surgical blades were placed in the rubber stopper next to the perforations. Both the rubber stopper and blades were enveloped in a piece of cheese cloth to hold them in place. Leaves were placed on a sponge in a tray and stabbed with the blades; inoculum flowed through the holes in the

Table 1. Known reaction of selected bean cultivars to *Xanthomonas campestris* pv. *phaseoli.*

Cultivar	Origin	Reaction[a]
Jules (P 698)	U.S.A.	R
BAT 93	CIAT	R
ICA Linea 24	ICA-Colombia	R - I
BAT 47	CIAT	I
Porrillo Sintetico	El Salvador	S
BAT 76	CIAT	S

Disease (Common bacterial blight) reaction; R = resistant; I = intermediate; S = susceptible; based on 1-5 scale, 1-2 = resistant; 3 = intermediate; 4 and 5 = susceptible.

stopper, saturating the cheesecloth and blades (Fig. 2). In all cases, the inoculum potential consisted of 5 x 10^7 bacterial cells/ml of water. One trifoliate leaf was inoculated per plant. All plants in the row were inoculated. Rows of control plants were inoculated separately with both procedures using sterile distilled water. Isolate XP 123 collected in Palmira was used.

The maximum and minimum temperatures were: field, 30° and 19°; greenhouse, 30.8 and 19.9°; growth room, 22.3 and 18.2°. The maximum and minimum relative humidities were: field, 91.5 and 46°; greenhouse, 98 and 46°; growth room, 93 and 44.2°. In another treatment, plants were placed in a greenhouse bench until inoculation time; subsequently, they were placed in a humidity chamber with 100% RH for two days. Plants were evaluated 8 and 12 days later in the field and growth room. Only one evaluation, 10 days after the inoculation, was possible in the greenhouse, due to early senescence of some of the trifoliates. Bacterial blight severity was rated using a 1:5 scale (Fig. 2 and Fig. 3).

Fig. 2. Surgical blade inoculation equipment. Leaves are placed on a sponge saturated with inoculum. The plastic bottle also contains inoculum. The leaf is punctured with two blades. Inoculum flows through two holes in the stopper next to the blades. The holes are fitted with sponges. Flow from the bottle keeps the blades and cheese cloth holding sponges in place saturated with inoculum.
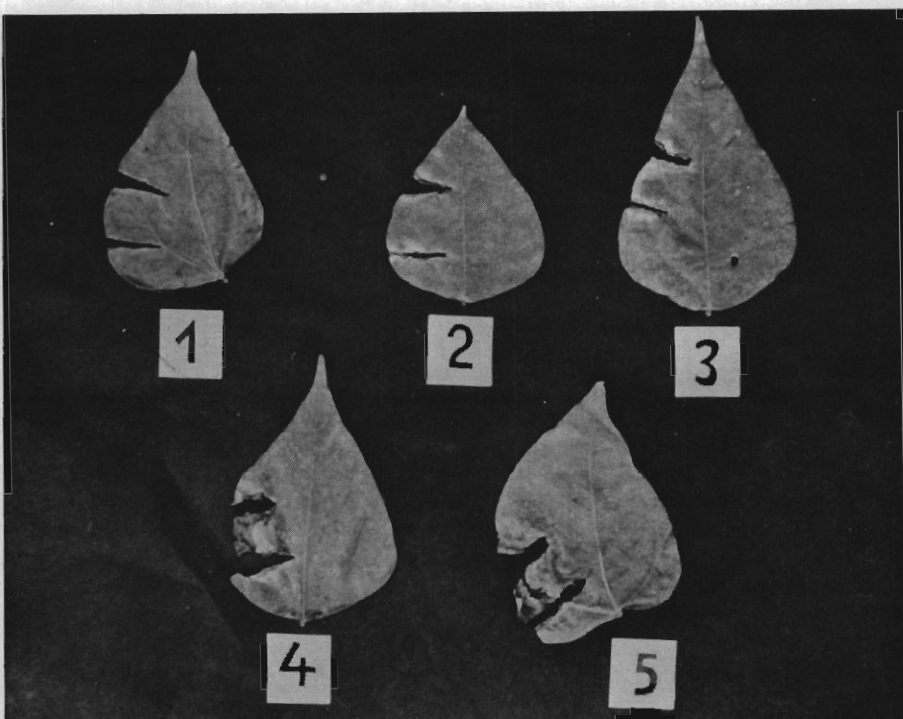
Fig. 3. Common bacterial blight severity scale used for razor blade inoculation procedure.

Results and Discussion

The objective was to compare two inoculation procedures for the evaluation of CBB resistance in beans. One criteria was that the procedure should eliminate the possibility of selecting scapes as resistant. In addition, the procedure should be rapid, to allow the inoculation and evaluation of large populations in the field. It should be easy to use, uniform, and should allow simultaneous evaluation in the field for CBB resistance and other characters such as pod load, architecture, and reaction to other diseases such as rust and bean common mosaic virus.

During the first field evaluation, ranking of the cultivars based on their CBB reaction was the same with both inoculation methods (Fig. 4); however, the CBB reaction was lower than the expected disease reaction from these cultivars under local conditions for both inoculation methods. Differences in CBB scores were not significant during the first evaluation between the two procedures. During the second field evaluation, however, there were significant differences in CBB scores between the razor blade and surgical blade procedures. The razor blade plus bacteria treatment induced a wider range of reactions and distinguished better between intermediate BAT 47 and resistant cultivars (Fig. 5). The surgical blade procedure was less effective in eliciting the expected reaction from cultivars ranked as intermediate.

497
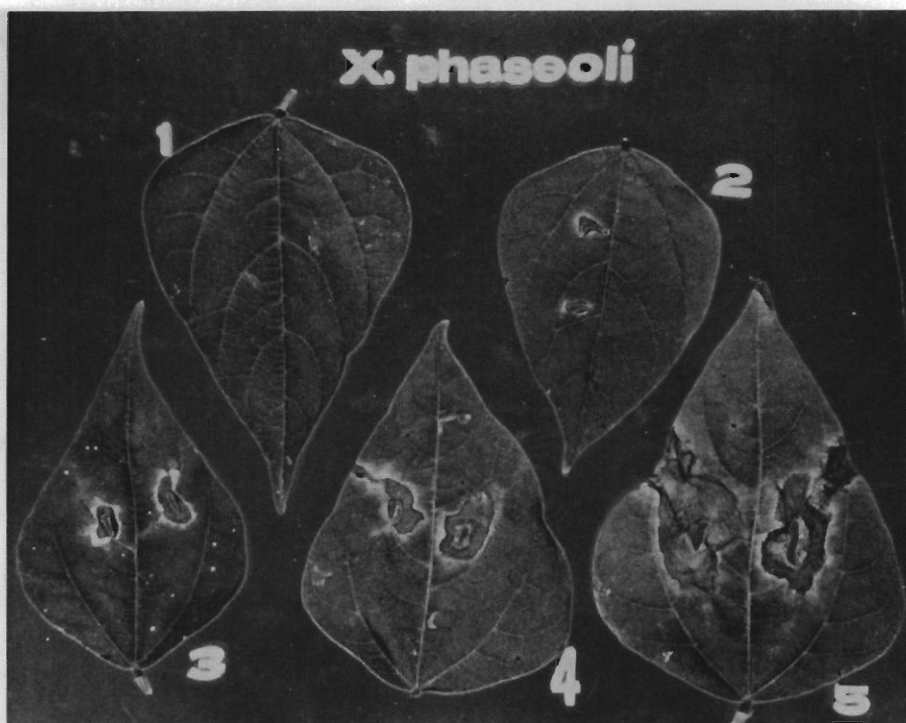
Fig. 4. Common bacterial blight severity scale used for the surgical blade inoculation procedure.

The separation of the cultivars into resistant, intermediate, and susceptible categories was more definite and clear with the razor blade procedure. A second evaluation 13 days after inoculation, was necessary before the known reaction to the bacteria by the cultivars used could be observed. The controls, where water rather than a bacterial cell suspension was used as the inoculum, did not cause any reaction.

The evaluation in the greenhouse yielded results similar to those observed during the second evaluation in the field. There were significant differences in CBB scores between the two procedures (Fig. 6). Even though the ranking of the cultivars based on their CBB reaction was similar with both procedures, the segregation of the cultivars into their known resistant, intermediate, and susceptible field reaction groups was much wider with the razor blade procedure than with the surgical blade. ICA Line 24 generally has an intermediate to resistant CBB reaction in the field; however, under greenhouse conditions, when inoculated with either procedure, its reaction is relatively more susceptible (Fig. 5 and 6). We have observed that many determinate cultivars (CIAT Type I) have similar CBB field reaction to that of ICA L 24, possibly due to the foliage type. The foliage, characterized by larger and thicker leaves, may be altered under greenhouse conditions, causing the difference in CBB reaction between the field and greenhouse.
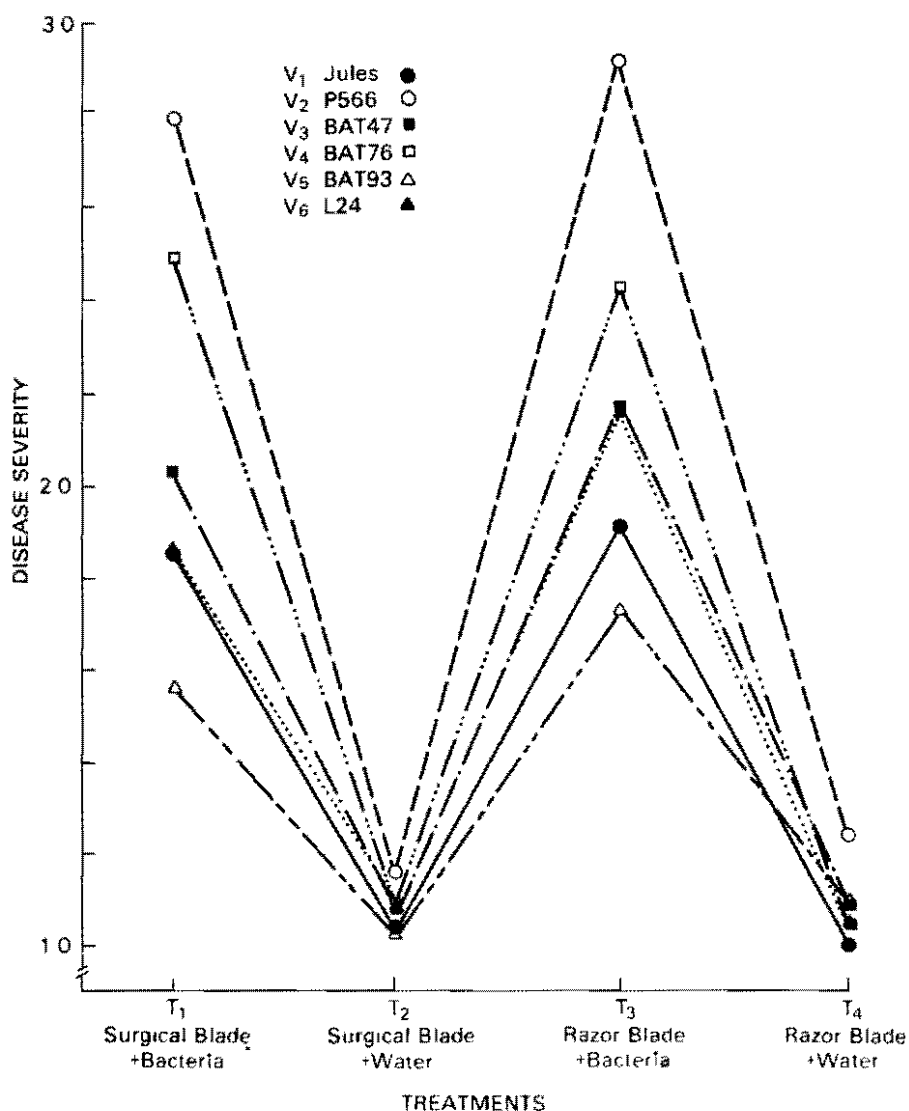
Fig. 5. Common bacterial blight reaction of six bean cultivars inoculated in the field with the razor blade and surgical blade procedures and evaluated eight days after inoculation.
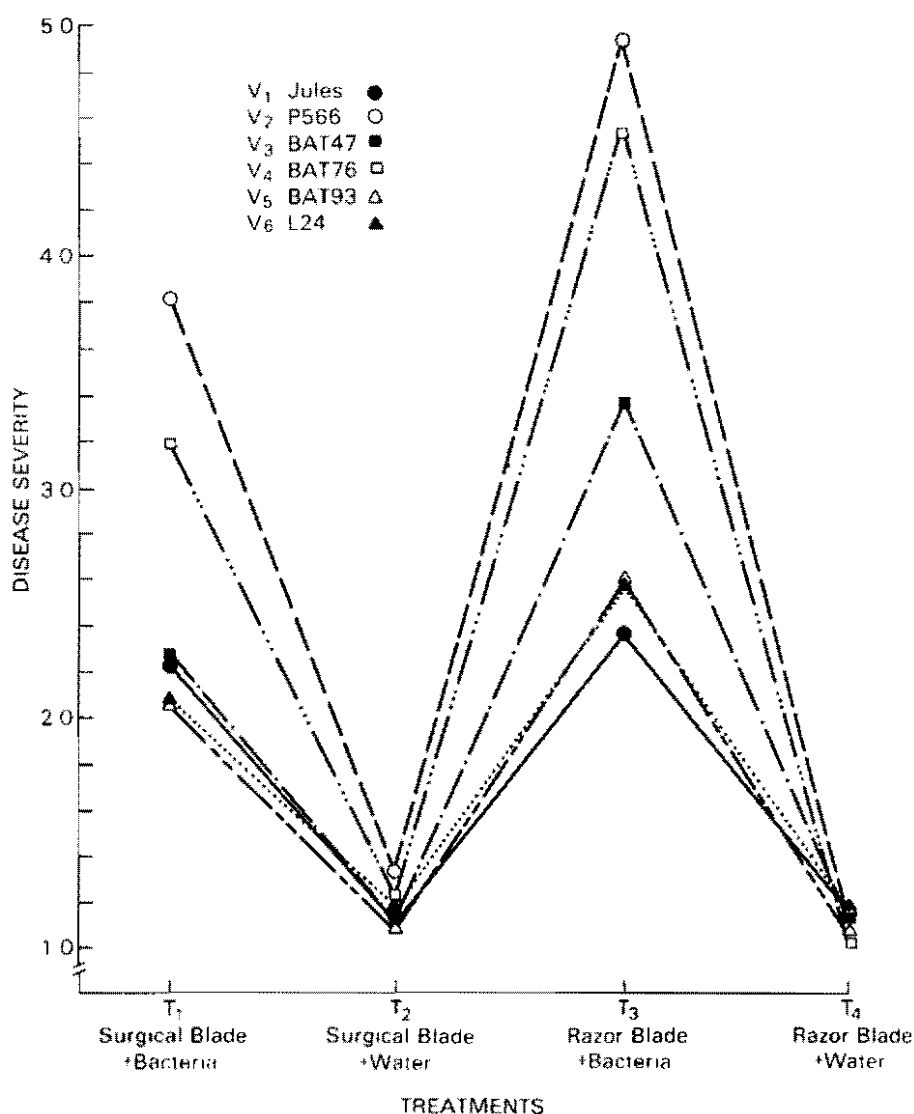
Fig. 6. Common bacterial blight reaction of six bean cultivars inoculated in the field with the razor blade and surgical blade procedures and evaluated eight days after inoculation.
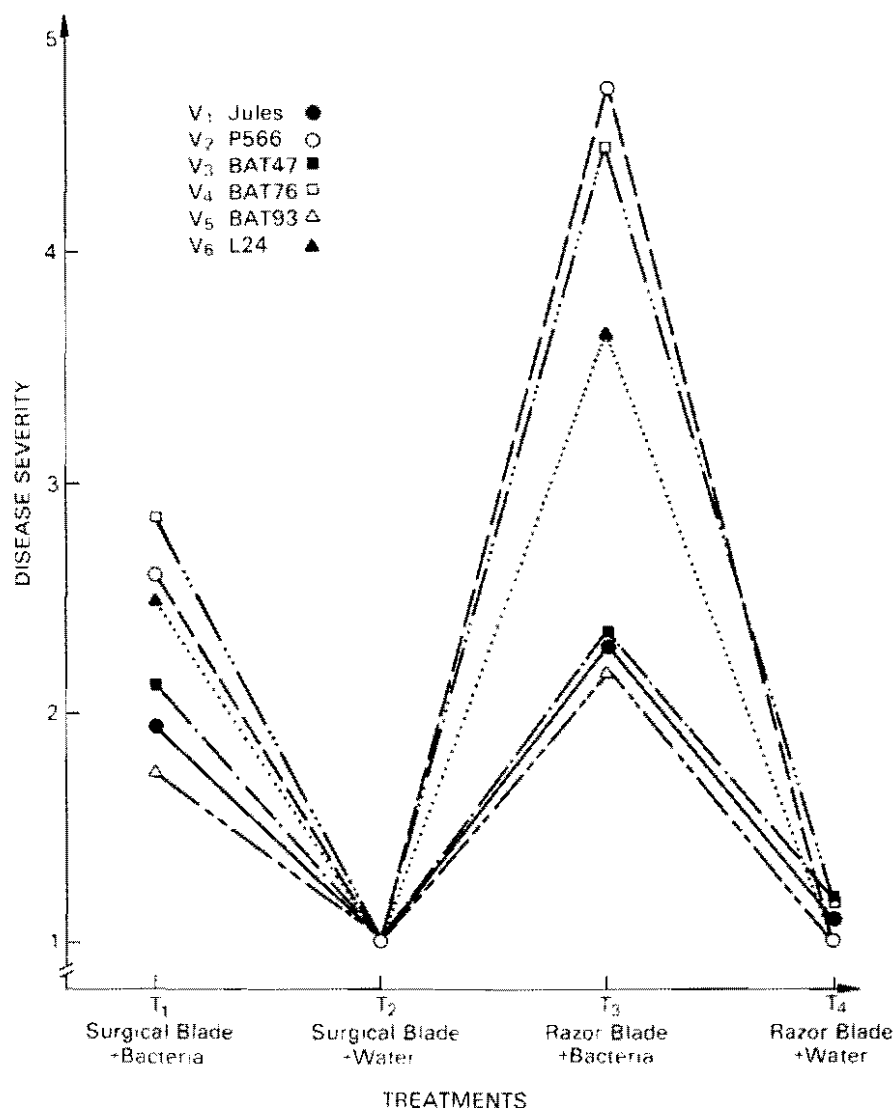
Fig. 7. Common bacterial blight reaction of six bean cultivars inoculated in the greenhouse with the razor blade and surgical blade procedures and evaluated ten days after inoculation.

The treatment in which cultivars maintained in a greenhouse were submitted to a 48 h period of high humidity in a humidity chamber after inoculation yielded similar results to those in which the cultivars were maintained continually in the greenhouse. In the humidity chamber treatment ICA Line 24 had a susceptible reaction, contrary to results found in the field. In general, the disease severity in this treatment was lower for all cultivars due, perhaps, to the lower temperature of the humidity chamber. For methods of inoculation which introduce the pathogen into a wound, the pathogen need not survive externally on the host, hence a humidity treatment is probably not necessary.

In the growth room disease symptoms were very slow to develop. Here the temperature during the entire period was lower than in the greenhouse and field. The ranking of the cultivars based on their CBB reaction was similar to that of the field and greenhouse. The growth room and humidity chamber treatments offered conditions less adequate than the field and greenhouse for CBB screenings.

In general, the razor blade method of CBB inoculation elicited higher disease severity in the susceptible cultivars than the surgical blade procedure. With the surgical blade procedure, the discrimination between the resistant and intermediate cultivars was lower than with the razor blade procedure. It may be possible to increase severity and discrimination of the surgical blade procedure by increasing inoculum concentration. However, uniformity of CBB reaction on a given cultivar was greater with the surgical blade method, as evidenced by lower standard deviations calculated on ratings of individual plants within a genetically uniform cultivar. Applications also were more uniform between field workers when they used the surgical blade procedure.

The razor blade and surgical blade procedures are both excellent methods for the evaluation of resistance in bean to CBB. Both methods are easy to learn and utilize in the field when screening large populations; however, with the surgical blade it is possible to inoculate a larger number of plants in the same time period. Both procedures permit the evaluation in the field of other characters such as other diseases and architecture. This is an important factor for bean improvement programs that handle large populations.

Literature Cited

1. Andrus, C. F. 1948. A method of testing beans for resistance to bacterial blights. Phytopathology 38:757-759.
2. Arp, G., D. P. Coyne and M. L. Schuster. 1971. Disease reaction of bean varieties to *Xanthomonas phaseoli* and *Xanthomonas phaseoli* var *fuscans* using two inoculation methods. Plant Dis. Rep. 55:577-579.
3. Bohn, G. W. and J. C. Maloit. 1947. The effects of carborundum in inoculating bean plants with bacteria. Phytopathology 37:196-198.
4. Burkholder, W. H. and E. T. Bullard. 1946. Varietal susceptibility of beans to *Xanthomonas phaseoli* var. *fuscans*. Plant Dis. Rep. 30:446-448.
5. CIAT, 1979. Bean Program, Annual Report. Centro Internacional de Agricultura Tropical, Cali, Colombia.

6. Epko, E. J. A. and A. W. Saettler. 1976. Pathogenic variation in *Xanthomonas phaseoli* and *X. phaseoli* var. *fuscans*. Plant Disease Reporter 60:80-83.

7. Pompeu, A. S. and L. V. Crowder. 1973. Methods of inoculation and bacterial concentrations of *Xanthomonas phaseoli* Dows. for the inheritance of disease reaction in *Phaseolus vulgaris* L. crosses (dry beans), under growth chamber conditions. Ciencia Cult. (Sao Paulo) 25:1078-1081.

8. Schuster, M. L. 1955. A method for testing resistance of beans to bacterial blights. Phytopathology 45:519-520.

9. Venette, J. R. and J. B. Nayes. 1978. A seed test to detect internally-borne bacterial pathogens of beans. Phytopathology News 12:92. (Abstr.).

10. Webster, D. M. 1978. Evaluation of resistance in beans (*Phaseolus vulgaris* L.) to *Xanthomonas phaseoli*. PhD. Dissert. Univ. of Wisconsin, Madison. 117 pp.

11. Webster, D. M., S. R. Temple and H. F. Schwartz. 1980. Selection for resistance to *Xanthomonas phaseoli* in dry beans. Crop Science 20:519-522.

12. Weller, D. M. and A. W. Saettler. 1976. Chemical control of common and fuscous bacterial blights in Michigan navy (pea) beans. Plant Dis. Rep. 60:793-797.

13. Yoshi, K. 1980. Common and Fuscuous blights. Pg. 155-165 In: H. F. Schwartz and G. E. Galvez, eds. Bean production problems: Disease, insect, soil and climatic constrains of *Phaseolus vulgaris* L. Centro Internacional de Agricultura Tropical, Cali, Colombia, 424 pp.

14. Zaumeyer, W. J. and H. R. Thomas. 1957. A monographic study of bean diseases and methods for their control. U.S.D.A. Agr. Tech. Bull. No. 868, p. 65-74.

Breeding for Bacterial Blight Resistance in Rice

T. W. Mew and G. S. Khush

International Rice Research Institute (IRRI)
Los Baños, Philippines

Abstract

Bacterial blight of rice caused by *Xanthomonas campestris* pv. *oryzae* is one of the major rice diseases in Asia. Major epidemics were reported in the 1960s, after the introduction of improved high yielding varieties such as Taichung Native 1, IR8, and Jaya that do not have adequate resistance to disease. Since then, breeding for resistance to bacterial blight has become a major component of varietal improvement in many national programs. In Japan, effort to breed resistant varieties has increased since the variety Asakase became susceptible to a new strain of the pathogens. The 1980 bacterial blight epidemic in Punjab, India showed the threatening nature of the disease to rice production. Disease control measures should follow an integrated approach, where resistance serves as a base for alternatives to fit into the developed disease control and management system. This appears to be the most logical approach when we consider the climate and the diverse cropping systems of rice culture in Asia. Other national programs and the International Rice Research Institute have identified resistance sources and utilized them in hybridization. These efforts may provide a foundation to manage and control the rice bacterial blight in Asia.

Introduction

Bacterial blight has occurred throughout Asia, in northern Australia, and Malagasy. The disease was reported in Latin America in 1977 (7) and in mainland Africa in 1979, first in Mali and then in Cameroon (Notteghem, personal communication).

In Asian regions where resistant varieties are grown, the disease damage has been minimal with no major economic importance. In other areas, however, the disease continues to be a threat to rice production.

In the Punjab, India, a severe outbreak of bacterial blight occurred in 1980. Three widely grown varieties, IR8, Jaya, and PR 106, were susceptible and affected by the disease. Several thousand hectares of severely blighted fields were plowed under by farmers (9).

This epidemic demonstrates the potential danger from bacterial blight in Asia. The deployment of resistant cultivars appears to be the most effective and economical method of controlling the disease. Recent findings of pathogen specialization for rice varieties with specific genes for resistance (8, 10), show there is need not only for resistant cultivars but also for cultivars differing in resistance.

IRRI continues to identify new resistance sources from its vast germplasm collection and to utilize them in a crossing program. IRRI's progress and the prospect in breeding for bacterial blight resistance are reported and discussed in this paper.

Identification and Evaluation of Resistance Sources

Since 1975, more than 40,000 accessions from IRRI's germplasm bank have been evaluated in the field for resistance to bacterial blight using a common strain of *Xanthomonas campestris* pv. *oryzae*. The strain, PX061, is virulent on varieties without genes for resistance. Varieties and breeding lines with resistance carry the *Xa-7* gene (Mew, unpublished data). About 3,000 entries have been identified and confirmed as resistant in the Bacterial Blight General Screening Nursery (BBSN) in the field (scored 1 to 3 on a 1 to 9 system based on % leaf area infected). These were then tested against different bacterial strains in the greenhouse or in the field by artificial inoculation. Table 1 shows the result of this screening. Varieties were evaluated for adult plant resistance (resistance at reproductive stage) and for overall resistance (tillering, maximum tillering, and reproductive stages). Selected entries would be further screened for inheritance study to identify the genes controlling resistance. Figure 1 shows the flow of the materials and the general operation of the program.

In the breeding program, hybrid populations in the segregating generations are screened in the field using the clipping method of inoculation. Bacterial strain PX061 at an inoculum density ca. 10^9 cells/ml is used. The susceptible F_2 and F_3 plants are rogued. Selections will continue to be screened until they are homozygous and resistance to bacterial blight is fixed (4).

Utilization of Resistance Sources

More than 100 varieties are used as sources of resistance to bacterial blight. Table 2 shows the seven known genes controlling resistance to bacterial blight; one (*Xa-3*) was identified in Japan (11) and the other six at IRRI (5). All have been utilized in the crosses (Table 2). Thirty-six of the donors are known to carry the *Xa-4* gene for resistance, which is the most

Table 1. Evaluation of rice germplasm accession for resistance to bacterial blight, 1975 to 1980, at IRRI.

Disease score[a]	Entry (no.)[b]
1 to 3	2,274
5	198
7 to 9	46,390
Total	48,862

[a] Scale 0-9 SES.

[b] There were repetitions in the total, but those with 1 to 3 and 5 were sorted out individually and filed.

widely used source, contributing to about 55% of the total 33,898 crosses made. There are two allelic genes at the $Xa-4$ locus, $Xa-4^a$ and $Xa-4^b$. The resistance expression conditioned by $Xa-4^a$ is effective against bacterial strain PX061 and others belonging to the group I of the Philippines at both vegetative and reproductive stages, while $Xa-4^b$ is effective against PX061 only at the reproductive stage. The resistance conditioned by these genes is not, however, effective against strain PX063 of group II and PX079 of group III (Mew, unpublished data). Among the 36 varieties having $Xa-4$, for resistance, there are 10 that carry $Xa-4^a$ and 26 $Xa-4^b$ (Table 3).
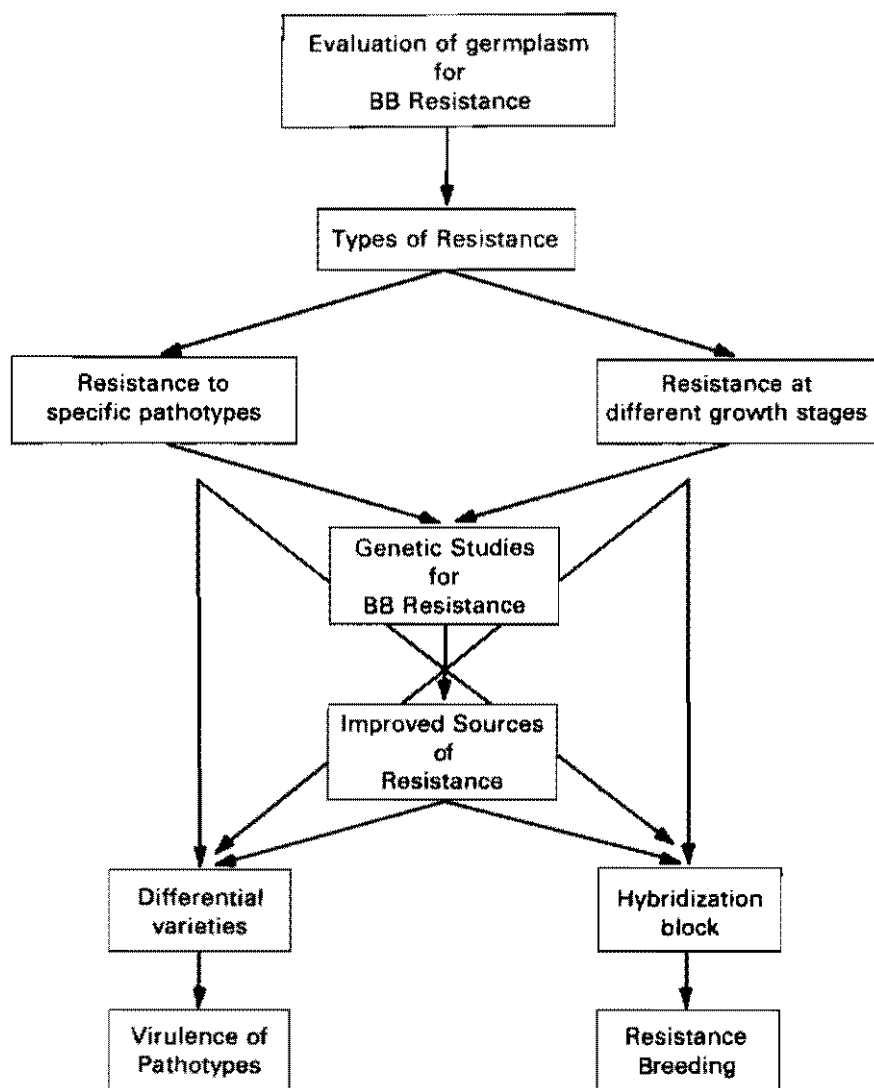
Fig. 1. Systematic utilization of genetic resistance to bacterial blight (BB) of rice.

Table 2. Sources of different genes for resistance to bacterial blight of rice used in the IRRI crossing program, 1980.

Genes for resistance	Donor variety (no.)	No. of crosses	Frequency[a] (%)
Xa-3	1	49	0.14
Xa-4	36	18,576	54.80
xa-5	19	3,099	9.16
Xa-6	1	883	2.60
Xa-7	1	95	0.28
xa-8	1	3	0.09
xa-9	1	1	0.03
Unknown	54	1,688	4.97
Total	114	24,394	71.96

[a] Based on a total of 33,898 crosses.

The second large group of 17 resistance donors involved in IRRI's crossing program carries the recessive gene at the *Xa-5* locus (Table 3). These varieties are involved in 9% of the total crosses. The other genetic sources of bacterial blight resistance include *Xa-6*, *Xa-7*, *xa-8* and *xa-9*, but they are used less often. The recessive gene *xa-9* in the variety Sateng, recently identified from Laos, has been used only once in the crossing program. The origin and IRRI accession number donor varities and the gene for resistance they carry are listed in Table 3.

Potential Combination of Genetic Sources for Resistance

Sources of resistance channeled into the hybridization program are utilized in single as well as complex, crosses. The mass crossing and screening work at IRRI allows recombination of different genes for resistance in the progenies (Table 4). Of the 33,898 crosses performed by 1980 at IRRI, 24,307 (or 67%) involved known genes of bacterial blight resistance. Many crosses have been performed involving more than one gene for resistance. *Xa-4*, involving both allelic genes, is the major source employed in crosses to combine with other genes for resistance (Table 4).

Prospects

The 1980 bacterial blight epidemic in India should serve as a warning on the potential danger from the disease. The factors relating to the development of an epidemic may not be as simple as described by scientists (9). However, planting very susceptible cultivars in wide acreages has played a significant role in epidemics. Despite continuous efforts at IRRI and many national programs for over a decade, the need for resistance

Table 3. Parental donors having specific gene(s) for resistance to bacterial blight of rice used in the IRRI crossing program.

Gene(s)	Acc. no.	Origin	Gene(s)	Acc. no.	Origin
Xa-3			*xa-5*		
Wase Aikoku	525	Japan	ARC 5756	20200	India
			Aus 32	28895	Bangladesh
Xa-4^a			Aus 251	29043	Bangladesh
CO 10	3691	India	Aus 449	29206	Bangladesh
CO 22	6400	India	Bageri	16193	Bangladesh
Nam-sagui 19	11462	Thailand	BJ1	3711	Nepal
Pelita I/1	14560	Indonesia	Chinsurah Boro II	11484	India
Pelita I/2	14561	Indonesia	DD 48	8620	Bangladesh
Sigadis	6525	Indonesia	Dharial	3396	Surinam
Taothabi	13746	India	DL 5	8593	Pakistan
TKM 6	237	India	Dular	636	India
Gui Do	19745	Korea	DV 29	8816	Bangladesh
Pakheng	30154	Laos	DV 139	8870	Bangladesh
			DZ 192	8518	Bangladesh
Xa-4^b			Hashikalmi	3397	Surinam
Bajang	17183	Indonesia	Kaliboro 600	29367	Bangladesh
Beak Ganggap	17253	Indonesia	Kele	25881	Bangladesh
Bulu Putih	17350	Indonesia			
Bulu Sampang	17352	Indonesia	*xa-5, Xa-7*		
Camor	17366	Indonesia			
Cropak Gede	17687	Indonesia	DV85	8839	Bangladesh
Kentjana	9224	Indonesia	DV86	8840	Bangladesh
Ketan Bas	17830	Indonesia			
Ketan Gondel	17834	Indonesia			
Ketan Gondopuro	17859	Indonesia	*Xa-6*		
Ketan Pandan	17932	Indonesia			
Ketan Temon	17967	Indonesia	Zenith	131	U.S.A.
Lenggang Genuk Bulu	18119	Indonesia	M. Sungsong	38794	Philippines
Padi Tomat	18408	Indonesia			
Palotan Melati	18426	Indonesia	*xa-8*		
Pare Djerah	18458	Indonesia			
Pulo Banrakaja	20073	Indonesia	PI 231129	11113	U.S.A.
Pulut Banda Kaya	27391	Indonesia			
Pulut Bongo	27397	Indonesia	*xa-9*		
Pulut Kemandi	24621	Indonesia			
Rante	18609	Indonesia	Sateng	30193	Laos
Rathu Heenati	15609	Sri Lanka			
Rengesi	18628	Indonesia			
Remadja	679	Indonesia			
Sintawati	18863	Indonesia			

against a destructive disease like bacterial blight has not always been appreciated. Disease control measures should follow an integrated approach, where resistance serves as a base for alternative control measures to fit into the developed disease control and management system. This appears to be the most logical approach when we consider the climate and the diverse cropping systems of rice culture in Asia. High rates of nitrogen fertilizer application are critical because they favor bacterial blight epidemics (9), but with resistance incorporated into the rice cultivars

Table 4. Some selected crosses made at IRRI involving two or more genes for resistance.

Cross no.	Cross	Genes involved
IR4475B	TKM 6/BJ1	Xa-4/xa-5
IR4985B	IR22//M.S./BJ1	Xa-4/Xa-6/xa-5
IR9623	DZ192/TKM 6	xa-5/Xa-4
IR10950	IR30/Zenith	Xa-4/Xa-6
IR14093	DV85/IR28	Xa-5, Xa-7/Xa-4
IR25875	DV85/IR36//IR36	Xa-5, Xa-7/Xa-4//Xa-4

Table 5. Different known genes for bacterial blight resistance used in various combinations in IRRI's crossing program. 1980

Potential gene combination	Cross	
	Number	Frequency (%)[a]
Xa-3, Xa-4	2	0.008
Xa-3, Xa-6	3	0.012
Xa-4, xa-5	698	2.873
Xa-4, Xa-6	261	1.073
Xa-4, Cas 209	43	0.177
xa-5, Xa-6	20	0.082
xa-5, Xa-7	2	0.008
Xa-4, xa-5, Cas 209	1	0.004
Xa-4, xa-5, Xa-7	35	0.144
Xa-4, xa-5, Xa-6	12	0.049
Xa-4, xa-5, Xa-6, Xa-7	1	0.004
Total	1,078	4.435

[a] Of 24,307 crosses potentially having genes for bacterial blight resistance.

farmers could manage the crop better in striving for high yield and low disease damage.

The hybridization program at IRRI, utilizing diverse resistance donors, pyramids the independently segregating genes for resistance. Results of this effort have to be confirmed by genetic analysis. Recent investigations indicate there are distinct pathotypes (races) present in different rice growing countries or even within countries (1, 2, 3, 6). Breeding lines with several genes for resistance should be evaluated through collaboration between scientists from IRRI and national programs.

Literature Cited

1. Eamchit, S., T. W. Mew. 1980. Comparison of virulence of *Xanthomonas oryzae* in Thailand and in Philippines. Plant disease.
2. Ezuka, A., and S. Sakaguchi. 1978. Host-parasite relationship in bacterial leaf blight of rice caused by *Xanthomonas oryzae*. Pl. Prot. Res. 11:93-118.
3. Horino, O., T. W. Mew, G. S. Khush, and A. Ezuka. 1980. Resistance of Japanese and IRRI differential rice varieties to pathotypes of *Xanthomonas oryzae* in the Philippines and Japan. IRRI. Res. Paper Ser. No. 53. Los Baños, Philippines. 11 pp.
4. International Rice Research Institute. 1972. Rice breeding. Los Baños, Philippines. 727 pp.
5. International Rice Research Institute. 1976. Annual Report for 1975. Los Baños, Philippines.
6. International Rice Research Institute. 1980. Annual Report for 1979. Los Baños, Philippines.
7. Lozano, J. 1977. Identification of bacterial blight in rice caused by *Xanthomonas oryzae* in America. Pl. Dis. Reptr. 61:644-648.
8. Mew, T. W., and C. M. Vera Cruz. 1979. Variability of *Xanthomonas oryzae:* Specificity in infection of rice differentials. Phytopathology 69:152-155.
9. Reddy, A. P. K. 1980. A report on bacterial leaf blight epidemics in Punjab State. All India Coordinated Rice Improvement Project (mimeo).
10. Sidhu, G. S., G. S. Khush, and T. W. Mew. 1978. Genetic analysis of bacterial blight resistance in seventy-four varieties of rice, *Oryza sativa* L. Theor. Appl. Genet. 53:105-111.
11. Washio, O., K. Kariya, and K. Toriyama. 1966. Studies on breeding rice varieties for resistance to bacterial leaf blight (in Japanese with English summary). Bull. Chugoku Agric. Exp. Sta. Ser. A 13:55-85.

Selection for Component of Horizontal Resistance to Bacterial Spot of Pepper

R. E. Stall

University of Florida
Gainesville, FL, U.S.A.

Abstract

Fewer lesions formed in leaves of pepper plants of P. I. 271322 than of Early Calwonder after infiltration with inocula of *Xanthomonas campestris* pv. *vesicatoria* containing 10^5 cells ml^{-1}, or less. However, confluent necrosis occurred in leaves of both pepper types with inocula of 10^7 to 10^6 cells ml^{-1}. A plant of P. I. 271322 was crossed with a plant of Early Calwonder and the F_1, F_2, and bcF_1 progenies were obtained. Half-leaves of plants of each progeny were infiltrated with about 2.5×10^3 cells ml^{-1} of *X. camp.* pv. *vesicatoria*. The number of lesions per cm^2 (NL) was determined for each leaf. Segregation for NL occurred in the F_2 population. Low NL was recessive to high NL and appeared to be controlled by 2 genes. Thus, selection for the low NL component of horizontal resistance is possible.

Introduction

Two types of resistance to pathogens have been identified in plants and have been named vertical and horizontal types (10). Vertical resistance is very desirable for use in breeding programs because it is usually simply inherited and is a very high level. However, vertical resistance has the disadvantage of being nullifed by development of races of the pathogen. This is exemplified with a vertical resistance gene for bacterial spot of pepper (1). Change of race in populations of *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Young *et al.* occurred at the rate of 4×10^{-4} mutants per cell division (3). This high rate of mutation for change of race prevented the commercial use of the vertical resistance gene for control of bacterial spot.

Horizontal resistance is usually not nullified by development of races (10). This type of resistance has not been used in breeding programs for control of bacterial spot even though Sowell and Dempsey (9) identified many pepper lines with degrees of resistance to bacterial spot. The primary reasons are the complicated inheritance of genes for horizontal resistance and the difficulty in detecting genetically resistant individuals in the progeny of crosses of susceptible and resistant plants.

Horizontal resistance to fungal plant pathogens has been associated with several components (10). Components for bacterial diseases might be stomatal size and frequency, number of lesions per leaf, length of latent

period, size of lesions, and number of cells in lesions. The use of horizontal resistance in a breeding program for bacterial spot control might be simplified if an important component of horizontal resistance to bacterial spot could be identified. Emphasis could then be directed toward that component.

The purpose of this paper is to report the existence of a component of horizontal resistance that determines the number of lesions of bacterial spot per unit of leaf area. This component exists in the pepper line, P.I. 271322. A method for selection of the component and evidence for heritability of the component are also included.

Materials and Methods

Seeds of P. I. 271322 were obtained from Dr. G. Sowell, Plant Introduction Station, Experiment, Georgia. This line was reported to be highly resistant to *X. camp.* pv. *vesicatoria* (9). Three plants of the line were selfed and plants from the resulting seeds were tested for resistance. A resistant plant of one of the progenies was selected for crossing with a random plant of the susceptible cultivar, Early Calwonder (ECW). Pollen from the resistant plant was transferred to emasculated flowers of ECW. The plant of each parent was selfed and plants from the resulting seeds were used as populations of resistant and susceptible parents. A random plant in the F_1 generation was selected for selfing and backcrossing to a plant of ECW. Pollinations were made in a greenhouse and the seeds from each cross, or self were kept separately.

Seeds of each line were planted in flats and the seedlings in the cotyledonary stage were transplanted to 10-cm clay pots. Fifty of each parent, F_1, and bcF_1 generations and 150 plants of the F_2 generation were planted. The third leaf of each plant was inoculated when the sixth leaf was in the bud stage. The fourth and fifth leaves were inoculated, respectively, 3 and 7 days later. The temperature in the greenhouse during the incubation period fluctuated between 22 and 32°C.

An isolate (80-5) of race 1 of the pepper strain (2) of *X. camp.* pv. *vesicatoria* was used as inoculum. A colony of the bacterium was transferred to 5 ml of nutrient broth and allowed to grow for 24 h at 30°C. The cells were pelleted, resuspended in sterile tap water, and adjusted to 0.3 A in a Spectronic 20 colorimeter at 600 nm wavelength. The concentration of bacteria in the standardized suspension was determined to be 5×10^8 cells ml^{-1}. Inocula of other concentrations were obtained by dilutions from the standardized suspension. The inocula were always suspended in sterile tap water. Suspensions of about 2.5×10^3 cells ml^{-1} were used to inoculate the progenies. The actual numbers of viable cells in those inocula were determined by transferring 0.05 ml to a nutrient agar plate. The mean number of colonies in three plates was considered as the concentration of cells in the inoculum.

Inoculations were made by the infiltration method of Klement *et al.* (7). One half of a leaf, separated by the midvein, was infiltrated. The inoculated area was determined by the dot method (8). Lesions were first noted 7 days after inoculation and were counted near 14 days. Since the number of

viable cells in inocula varied slightly from test to test, the number of lesions per cm^2 (NL) with each leaf was adjusted to correspond to inoculum of 2.5×10^3 cells ml^{-1}. The adjustment was based on the proportion of the number of cells in the inoculum over 2500. This was thought to be valid because of a very high correlation between NL and numbers of bacteria in inocula (R.E. Stall, unpublished). The mean NL of three determinations for a plant was used in the analysis of the inheritance.

Results

Confluent necrosis occurred in leaves of ECW with inocula of 10^7-10^5 cells ml^{-1} and distinct lesions developed with inocula of 10^4-10^2 cells ml^{-1}. About 10 lesions per cm^2 occurred in leaves inoculated with 10^3 cells ml^{-1}. Confluent necrosis occurred in leaves of P. I. 271322 with inocula of 10^7-10^6 cells ml^{-1} and the necrosis occurred at the same time as in leaves of ECW with each concentration. Fewer lesions developed with inocula of 10^5-10^2 cells ml^{-1} in leaves of P. I. 271322 than in leaves of ECW. The average number of lesions in leaves of P.I. 271322 was less than 1 per cm^2 when 10^3 inoculum was used.

The frequency distributions of plants of P.I. 271322 and ECW according to the NL in leaves after inoculation with 2.5×10^3 cells ml^{-1} are presented in Fig. 1. The range, mean, and variance of the NL for each parental population are in Table 1. The range of lesions per cm^2 in leaves of the two populations did not overlap. The means of NL for the progeny of each parent were significantly different ($P = 0.01$). The variance for the NL for the population of resistant plants was equal to the mean which is presumptive evidence for a Poisson distribution of the frequencies (5). The variance for the NL for the population of susceptible plants was less than the mean which is presumptive evidence for a binomial distribution of the frequencies. The frequency distributions for the NL of plants of the F_1 and bcF_1 populations (Fig. 2-3) were similar to those of the susceptible parent. The variances for the NL of these populations were less than the means,

Table 1. The range, mean, and variance of bacterial spot lesions per cm^2 of leaf among populations of pepper plants inoculated with 2.5×10^3 cells ml^{-1} of *Xanthomonas campestris* pv. *vesicatoria*.

Population	Lesions per cm^2 of leaf		
	Range	Mean	Variance
Early Calwonder (ECW)	5.7 — 13.2	9.0	1.8
PI 271322-3 (P_1)	0.5 — 4.8	1.3	1.3
F_1 (ECW x P_1 271322)	7.1 — 13.7	10.7	2.3
F_2 [(ECW x P_1 271322) x self]	1.3 — 15.3	8.4	10.3
bcF_1 [(ECW x P_1) x ECW]	8.1 — 14.8	10.4	1.96

514
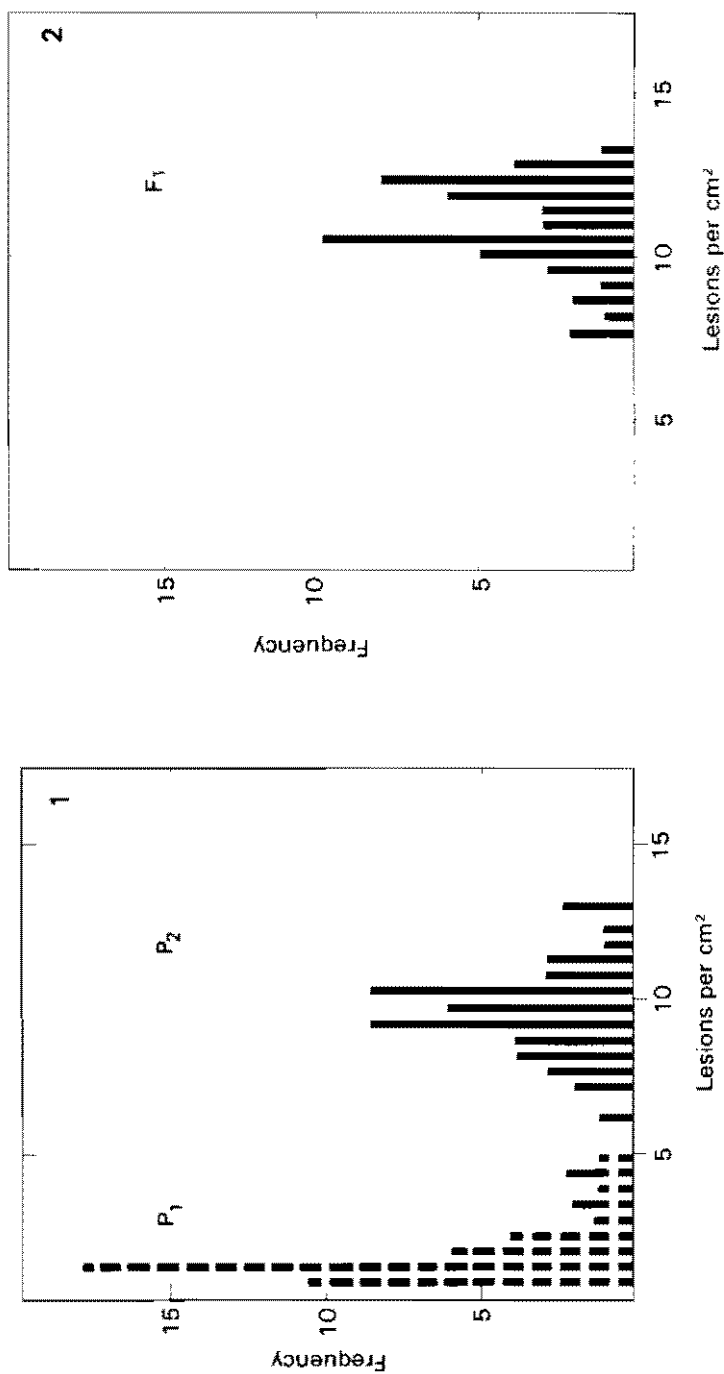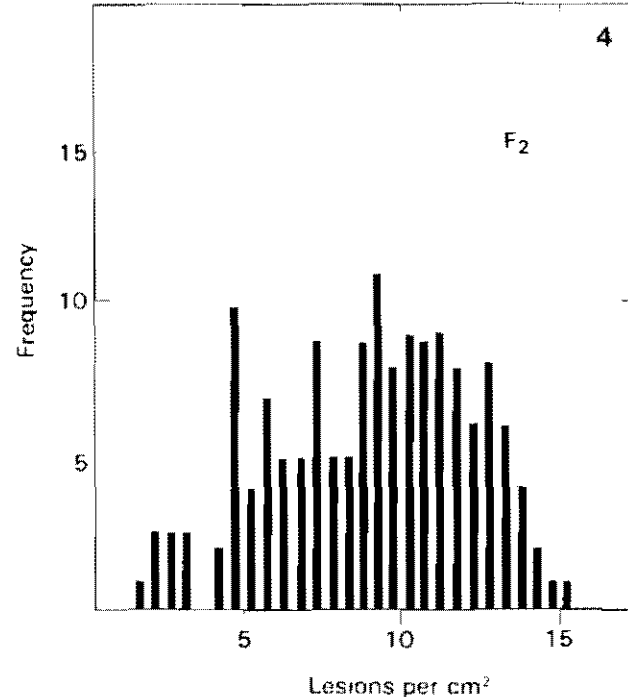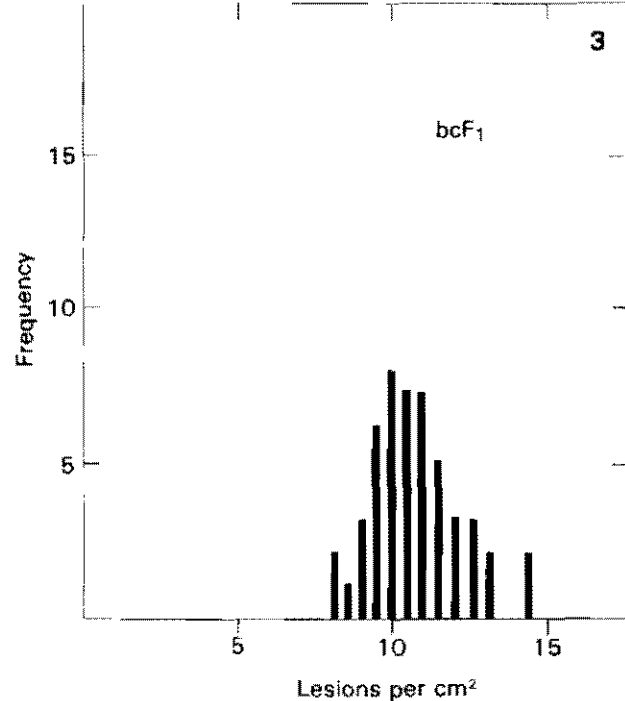
Figs. 1-4. Frequency distributions of plants according to the number of lesions per cm² that developed after infiltration of leaves with 2.5×10^3 cells ml⁻¹ of *Xanthomonas campestris* pv. *vesicatoria*. 1. Distribution of plants of PI 271322 (P_1) and Early Calwonder (P_2). 2. Distribution of plants of F_1 generation ($P_2 \times P_1$). 3. Distribution of plants of the bcF_1 generation ($P_2 \times F_1$). 4. Distribution of plants of the F_2 generation ($F_1 \times$ self).

which was the same result as for the susceptible parent. The frequency distribution for the NL of plants of the F_2 population (Fig. 4) was much wider than that for other populations. The variance for the NL for the population of F_2 plants was greater than the mean. Presumably, the frequency distribution for the F_2 population was arranged as a negative binomial.

Ten plants among the population of 150 F_2 plants were clumped toward the low range of NL. The ratio of those plants to the rest of the F_2 population fits a 15:1 ratio (P>0.95 as determined with the chi square test).

Discussion

A working hypothesis regarding the inheritance of low NL in P.I. 271322 was formulated. The factors for low NL are apparently inherited in a recessive manner, since the NL among populations of F_1 plants was similar to that among populations of the susceptible parent. The non-random frequency distribution of F_2 plants arranged according to NL supports the conclusion that only a few genes are involved in determining the low NL. The number of plants with low NL among the F_2 population was that expected for 2 recessive genes, but this needs corroboration.

The component of low NL was detected with low inoculum (10^3 cells ml^{-1}), but not with high inoculum (10^6 cells ml^{-1}). A susceptible-site hypothesis can be used to explain the different reactions with different inoculum levels (4). The chances are greater that a bacterium will contact a susceptible site on a cell surface if the inoculum is high in numbers of cells. Presumably, fewer susceptible sites exist on cells in leaves of P.I. 271322, therefore a high inoculum level is needed for a bacterium to come in contact with a susceptible site by chance. A susceptible site may only determine if a bacterium can obtain nutrition for growth.

The importance of the component of low NL in field resistance has not been determined. A test to determine importance of low NL should await the development of near isogenic lines with and without the component so that other influencing components are removed (6). Such isogenic lines are under development.

A second component of horizontal resistance apparently is present in P.I. 271322. Different types of lesions were noted among the F_2 population inoculated with *X. camp.* pv. *vesicatoria.* Lesions in some leaves were sunken and watersoaked, whereas lesions in others were raised and appeared as pustules. The former were like the susceptible parent and the latter were like the resistant parent. The two lesion types appeared to be discrete and independent of the component that determines the number of lesions per cm^2.

A combination of horizontal components may be necessary for field resistance to bacterial spot of pepper. However, if inheritance of other components is as simple as inheritance of the NL component seems to be, use of horizontal resistance in breeding programs may be common in the future. Transfer of the genes for the NL component to horticulturally desirable pepper plants should be possible with standard backcrossing.

Literature Cited

1. Cook, A. A., and R. E. Stall. 1963. Inheritance of resistance in pepper to bacterial spot. Phytopathology 53:1060-1062.
2. Cook, A. A., and R. E. Stall. 1969. Differentiation of pathotypes among isolates of *Xanthomonas vesicatoria.* Plant Dis. Reptr. 53:617-619.
3. Dahlbeck, D., and R. E. Stall. 1979. Mutations for change of race in cultures of *Xanthomonas vesicatoria.* Phytopathology 69:634-636.
4. Ercolani, G. L. 1970. Bacterial canker of tomato. IV. The interaction between virulent and avirulent strains of *Corynebacterium michiganense* (E. F. Smith) Jens. *in vivo.* Phytopathologia Mediterranea. 9:151-159.
5. Hastings, N. A. J., and J. B. Peacock. 1975. Statistical distributions. Halsted Press, New York. 130 pp.
6. James, W. C. 1974. Assessment of plant disease losses. Annu. Rev. Plant Pathol. 12:27-48.
7. Klement, Z., G. L. Farkas, and L. Lovrekovitch. 1964. Hypersensitive reaction induced by phytopathogenic bacteria in tobacco leaf. Phytopathology 54:474-477.
8. Marshall, J.K. 1968. Methods for leaf area measurement of large and small leaf samples. Phytosynthetica 2:41-47.
9. Sowell, G., and A. H. Dempsey. 1977. Additional sources of resistance to bacterial spot of pepper. Plant Dis. Reptr. 61:685-686.
10. Vander Plank, J. E. 1968. Disease resistance in plants. Academic Press. New York. 206 pp.

Production of Plants Free of Pathogenic Bacteria

Ib G. Dinesen

National Plant Pathology Institute
Lyngby, Denmark

Abstract

A program for the production of healthy plants is in use in Denmark. Concerning the tests for bacterial diseases two methods are used, the immunofluorescence and ELISA. The sensitivities of the methods are equal, but the ELISA-method is easier to read. Positive and negative controls are necessary for both of the methods, especially for the ELISA where the values are different from time to time. Plant material is prepared for testing by shaking it in sterile water for 16 hours. After that, the suspension is centrifuged to concentrate the bacteria. The results are easy to read, even when the plants are weakly attacked.

Introduction

In Denmark the production of pot plants under glass is increasing. The temperature in Denmark is rather low, and it is necessary to heat the greenhouses. With the increasing oil prices it is very important to have a safe production. For this reason a program called DGP-production has been started. DGP means: "Plants with defined genetic properties and free of defined pathogens." Testing for bacterial diseases is carried out as part of the program. The procedure is:

1. Healthy plants are selected visually from those that are growing best.
2. Cuttings are taken from these plants.
3. The lower 3 cm of the cuttings are used for testing for pathogenic bacteria.

Every time new cuttings are taken the procedure under 3 is carried out until plants are handed over to the Plant Propagation Station.

Examples of cultures are *Dieffenbachia* sp. tested for *Erwinia chrysanthemi* (Burk et al.) Bergey, Kalanchoe *blossfeldiana (Erwinia chrysanthemi)*, and *Pelargonium* x *hortorum* (*Xanthomonas pelargonii* (Brown) Starr and Burkholder).

Testing of Kalanchoe blossfeldiana for *Erwinia Chrysanthemi* **and a Comparison of Methods.**

In 1976 many growers in Denmark experienced severe attack of *E. chrysanthemi* on *K. blossfeldiana*. The general symptoms were strongly

retarded plants with permanently wilted greyish-mat leaves. The first sign of an attack was leaf wilt. Cross sections of stems which were badly attacked revealed a very brown necrotic pith.

Investigations (Dinesen, 1979) showed that this bacterium can be disseminated by cuttings from diseased motherplants. The greatest risk for dissemination is with cuttings where only a few xylem cells have a brownish discoloration and no other symptoms. For that reason it is necessary to find a testing method which is safe and very sensitive.

Materials and Methods

Preparation of Plant Material

As mentioned earlier, the lower 3 cm of the cuttings are tested. These 3 cm are cut in small pieces and transferred to a bottle with 100 ml of sterile water. The bottle is shaken (130 R.P.M.) for 16 hours.

Naturally infected plants are used as positive contols. "Badly attacked" means plants with clear symptoms. "Weakly attacked" means the only signs of attack are brown colored xylem cells. The test is carried out with undiluted liquid from the bottles, and dilutions of 1:10 and 1:100. Prior to use, the liquid is centrifuged twice. The first time, 180 x g for 10 minutes. The second time the supernatant is centrifuged by 6900 x g for 20 minutes. The sediment is resuspended in a phosphate buffer and used for the different serological testing methods. Healthy looking plants without discoloration of the xylem cells were used as negative samples. A pure culture of danish isolate (no. 109) from *K. blossfeldiana* was also included. The dilution was 10^9 cells/ml measured nephelometrically.

Production of Antiserum

Erwinia chrysanthemi (no. 109) was grown for 3 days on nutrient agar (Difco). The cells were washed from the tubes with 0.01 M phosphate (pH 7.2) plus 0.85% sodium chloride (PBS). Cells were washed three times by centrifugation at 6900 x g for 20 minutes and resuspended in PBS. After washing the cells were dialyzed against 2% glutaraldehyd in PBS for 16 hours and then dialysed against PBS at 4°C for 16 hours. A rabbit was given intramuscular injections in the hind legs at weekly intervals for 5 weeks. The injections consisted of 1.5 ml of bacterial suspension (10^9 cells/ml) emulsified 1:1 with Freunds incomplete adjuvant. The injections were given in each of the hind legs. Two weeks after the fifth injection a booster injection was given. Twelve days after the initial injection the bleeding started.

The Immunofluorescence Method

The indirect immunofluorescence method was used.
The procedure was:

1. Add drops of suspension on the glass slide.

2. Air dry.

3. Flood with 95% alcohol - let stand for 10 minutes - flame off and cool.

4. Add middle layer antiserum (anti *Erwinia chrysanthemi* serum diluted 1:200).

5. Incubate for 30 minutes at room temperature and in dark.

6. Rinse carefully with phosphate buffer.

7. Wash 3 times for 5 minutes in a phosphate buffer.

8. Add fluorescein isothiocyanate (FITC)-labeled swine anti-rabbit serum (DAKO-laboratories Copenhagen) diluted 1:100.

9. Incubate for 30 minutes as 5. Repeat 6 and 7.

10. Add mountant and cover slip.

The phosphate buffer was composed of: 80 g NaCl, 2 g KH_2PO_4, 29 g $Na_2HPO_4 \times 12 H_2O$ 2 g KCl, 2 g $NaNO_3$ in 1000 ml distilled water. The slides were examined by Nikon epi fluorescence equipment. A filter of main wavelength of 495 nm and oil-immersion lens was used.

The ELISA-method

The methods described by Clark and Adams (1977) were used. The fractionated γ globulin showed an E_{280} of 1.4. When it was prepared for the testing it was diluted 1:160. The dilution of the conjugated γ-globulin was 1:400. Reading of the microtiterplate was done photometrically on a Titertek multiskan at a wavelength of 405 nm.

Results

In Table 1 the lowest and highest values in five trials, using ELISA, are given. The level of the absorbance values (E_{405}) measured by the Titertek are different from one trial to another. Table 2 shows values from a single trial with badly attacked, weakly attacked, and healthy plants.

The weakly attacked plant is negative when tested without certifugation ($E_{405} = 0.060$) by the ELISA method, but positive ($E_{405} = 1.054$) when centrifugation was used. With immunofluorescence, the bacteria fluoresced much more clearly following centrifugation.

Discussion

By shaking the plant material in sterile water instead of grinding it, the intensity of the fluorescence is improved. The reason is that a lot of tissue debris is avoided. Keeping in mind that the testing of the plants should result in healthy plants, it is very important that all infected plants be identified. If there are plants containing only a few bacteria, these bacteria will multiply during the 16 hours shaking at room temperature so that the chances of finding all infected plants are increased.

As mentioned, the values obtained from the Titertek are different from time to time. Therefore when using the ELISA method it is essential to have a positive and a negative control.

Table 1. Comparisons of the ELISA and immunofluorescence methods, for detection of bacteria in Kalanchoë blossfeldiana.

	ELISA absorbance (E_{405})			Immunofluorescence numbers of bacteria per microscope field		
	undiluted	1:10	1:100	undiluted	1:10	1:100
Weakly attacked	0.060-0.171	0.036-0.131	0.029-0.105	10-50	5-10	0-2
Centrifuged	0.247-1.054	0.230-0.937	0.124-0.131	50	5-20	0-2
Badly attacked	0.186-1.894	0.147-···	0.114-1.161	50	5-20	0-5
Centrifuged	0.273-···a	0.215-···	0.150-0.853	50	10-20	0-2
Healthy plants	0.078-0.139			neg.		
With symptoms but no discoloration of xylem	0.003-0.096	0.030-0.107	0.033-0.103	neg.	neg.	neg.
Centrifuged	0.100-0.106	0.040-0.097	0.032-0.106	neg.	neg.	neg.
Erwinia chrysanthemi no. 109 10^9 cells/ml	0.200-···			50		

a ··· means the absorbence value exceeds 2.000

Table 2. The ELISA and immunofluorescence methods in a single trial with different plant material.

	ELISA absorbance (E$_{405}$)			Immunofluorescence numbers of bacteria per microscope field		
	undiluted	1:10	1:100	undiluted	1:10	1:100
Weakly attacked	0.060	0.036	0.029	10-50	>50	0-2
Centrifuged	1.054	0.937	0.131	>50	10-20	0-2
Badly attacked	0.433	0.225	0.149	25-50	10-20	2-5
Centrifuged	1.465	1.997	0.853	>50	10-20	0-1
Healthy plants	0.019	0.016	0.028	neg.	neg.	neg.
Centrifuged	0.048	0.034	0.028	neg.	neg.	neg.

The investigations also show that with weakly attacked plants it is necessary to use centrifugation in order to concentrate the bacteria. When this is done, the value becomes sufficiently high that there is no doubt about the result (see Table 2) and the reaction is so intense that the reading can be done visually.

When using undiluted material or a dilution of 1:10 from weakly attacked plants the immunofluorescence method is as good as the ELISA method. At a dilution of 1 100 from weakly attacked plants 0-2 bacteria are usually found per microscope field, thus it is necessary to be very careful with the examination because many fields are without bacteria. Keeping that in mind and that it is very tiring and time consuming to look in the microscope in the darkroom, the ELISA method is preferable because it is possible to do the reading automatically. However, ELISA is not faster than immunofluorescence, neither is it more sensitive (see Table 1). With the ELISA and immunofluorescence, one has tools to test material in a safe and sensitive way. To be sure that the plants are completely free of pathogens it is necessary to test plant material several times before it goes to the growers. In Denmark the DGP-plants are tested every time the material is renewed. This means at least four times, before it is handed over to the Plant Propagation Station.

Literature Cited

1. Clark, M. F. and A. N. Adams: Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant virus. J. Gen. Virol. 34:475-485.
2. Dinesen, Ib. G. 1979. A disease of *Kalanchoe blossfeldiana* caused by *Erwinia chrysanthemi*. Phytopath. Z. 95:59-64.

Production of Danish Seed Potatoes Free of *Corynebacterium sepedonicum*

Ib G. Dinesen

National Plant Pathology Institute
Lyngby, Denmark

The production of seed potatoes in Denmark is going to be based on meristem plants or stem cuttings, just to eliminate the virus diseases and be sure that no ringrot is found in the Danish potato production.

The testing procedure for *Corynebacterium sepedonicum* is:

Preparation of the Material

a. Heel ends or stem parts are cut in small pieces.
b. This material is transferred to 100 ml of sterile water and shaken for 18 hours.
c. The fluid is centrifugated twice.

　I. 180 x g in 10 minutes,

　II. The supernatant by 7000 x g for 20 minutes.

d. The sediment is shaken with only a few ml of phosphate buffer.
e. Immunofluorescence testing is done on the sediment.

The Immunofluorescence Procedure

a. Add drops of suspension on the object glass.
b. Air dry.
c. Flood with 95% alcohol; let stand for 10 minutes; flame off and cool.
d. Add middle layer antiserum (anti *C. sepedonicum* serum diluted 1:200).
e. Incubate for 30 minutes at room temperature and in the dark.
f. Rinse carefully with phosphate buffer.
g. Wash 3 times for 5 minutes in a phosphate buffer.
h. Add fluorescein isothiocyanate (FITC)labeled swine anti-rabbit serum (DAKO-laboratories, Copenhagen) diluted 1:100.
　. Incubate for 30 minutes same as e. Repeat f and g.
　. Add mountant and cover slip.

Testing for ring rot (Corynebacterium sepedonicum) in potato

The heel end is tested for ring rot

about 10 p.c. of the tubers in each sample are tested for ring rot

Segments of the stem used for
production of seed potatoes

This stem part is
tested for ring rot
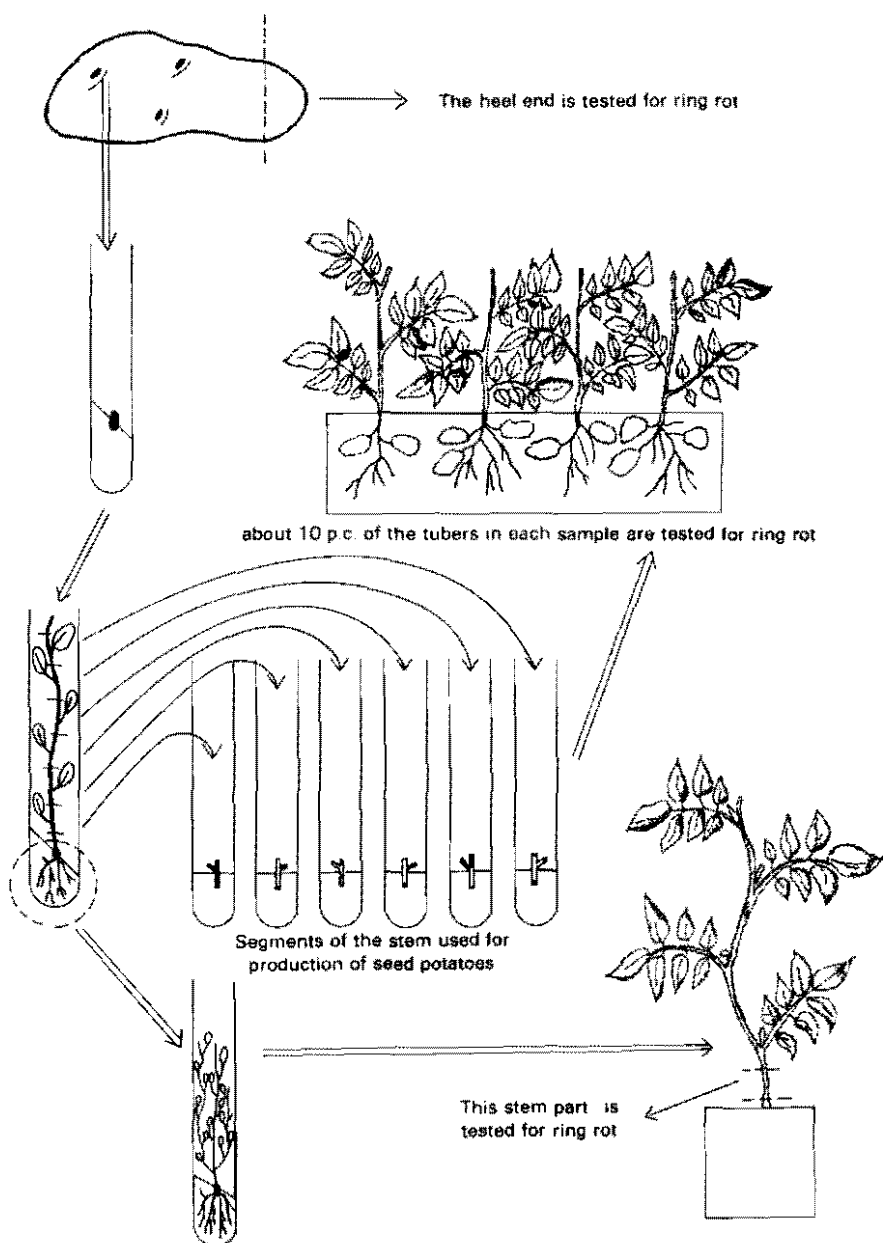
Fig. 1. Testing for ring rot (Corynebacterium sepedonicum) in potato.

Screening for Resistance in *Prunus* to Bacterial Canker

C. M. E. Garrett

East Malling Research Station
Maidstone, Kent, England

Abstract

Resistance to bacterial canker in cherry and plum (*P. avium* and *P. domestica*) is not consistent at the seedling stage and local variation in soil conditions can markedly affect susceptibility. The physiological state of the tree, influenced by prevailing environmental conditions, affects both susceptibility to infection and its subsequent development. An interaction between plum rootstocks and the scions grafted on them affects rootstock response to infection.

Inoculum composition and the timing of its application are also important factors. There are great differences in the susceptibility of some cherry rootstocks and cultivars to different races of the pathogen *Pseudomonas syringae* pv. *morsprunorum*.

Introduction

The absence of effective and cheap bactericides to control plant diseases caused by bacteria puts pressure on plant breeders to produce resistant cultivars. The problem of how to assess resistance to a pathogen most effectively has confronted many workers and has no easy solution. Optimum conditions for testing cannot always be ensured and, since most selection programs aim to reduce considerably the number of seedlings to be carried through for further tests, inocula of higher concentration and purity than are normally encountered in the field are used. Screening in this way can readily eliminate the most susceptible material but a valid assessment of resistance can be made only after several years of field observation.

A search for new stone fruit rootstocks and cultivars was prompted by the demand from growers for dwarf trees, which are essential for intensive systems of fruit growing; there is also a need for new high quality cultivars as well as for a means of reducing losses from bacterial canker. Breeding programs were therefore initiated at East Malling to produce rootstocks with the desired cultural characteristics and bacterial canker resistance.

Some of the problems encountered in screening for resistance to bacterial canker of stone fruits are described.

A prime requisite if screening for resistance is to be successful is a knowledge of the disease in question. Bacterial canker caused by *Pseudomonas syringae* pv. *morsprunorum* is one of the factors limiting the successful production of cherry (*Prunus avium*) and plum (*P. domestica*) in

southeast England. The pathogen lives as an epiphyte on leaf surfaces throughout the growing season. In autumn the leaf scars of cherry become infected when the pathogen is washed into them during rain and, in addition, branch and trunk wounds may become infected. On plum, leaf scars are not susceptible; on this host, wounds on the trunks of young trees are the major avenues of infection. On both hosts, resultant cankers may girdle the branch or trunk and cause die-back.

Autumn sprays of Bordeaux Mixture will virtually eliminate the epiphytic population and thus give a good control of the disease on cherry (1). No comparable chemical control has been found for trunk cankers on plum or cherry.

Methods

A standard technique was used in which a -shaped scalpel wound was made in the stem into which a few drops of inoculum at a concentration of ca 10^8 cells ml^{-1} were inserted; humidity was maintained by binding the wound with polythene tape (2). Inoculations were carried out during the optimum period for infection, December to January (10).

Results

Some of the factors that affected the results of the series of tests are reported on below.

Plant Age

Initially, 1200 cherry seedlings were lined out for screening. Any which became cankered were discarded (Table 1). Many seedlings which survived in the first 2 yr of inoculations were subsequently recorded as susceptible. However, there was a correlation between the results obtained on 3-yr-old rootstocks and the same stocks when reinoculated at 6 yr old (6). No such effect of plant age was detected in screening plum rootstocks. Trajkorski and Lernius (12) in Sweden also found an effect of plant age when they tested cherry for field resistance to bacterial canker without inoculation and assessed performance at 2 yr and 4 yr. They found

Table 1. Resistance of *P. avium* cherry clones of different ages to bacterial canker.

No. clones tested	age (yr)	No. discarded as susceptible	No. resistant	% resistant
1200	1	794	406	33.8
406	2	274	132	32.5
132	3	116	16	12.4
5 + 10 hybrids	3	0	15	100.0
5 + 10 hybrids	6	0	15	100.0

that although 24% were resistant at the earlier assessment, less than one-fifth of these were still recorded as resistant at 4 yr (Table 2).

Assessment should therefore be made on cherry on trees that are at least 3 yr old.

Table 2. Field resistance of cherry *(P. avium)* to bacterial canker.

No. seedlings	Resistant at 2 yrs		Resistant at 4 yrs	
	No.	%	No.	%
7857	1904	24.23		
1904			371	19.48
'Napoleon' cross		18.5		0.6

Data from Trajkorski and Lernius, 1977

Soil Factors

Replicate trees of 132 clones for inoculation at 3 yr were lined out, with two sets of F12/1 and F 1/3 as standards. Cankers on the two standards toward the south end of the double row were only 47% and 77% the length of the same standards near the north end, so the categories denoting high, medium or low resistance of the tests clones had to be adjusted along the rows (6). The differences were not attributable to tree vigor since, although grafted trees of each clone were smaller than nongrafted, there was no consistent difference in canker length. All the clones were derivatives of a single cross. This positional effect has been reported elsewhere (3, 4) and is believed to be due to differences in soil factors and consequent differences in concentration of minor nutrients in host tissues.

Physiological State

The physiological state of the tree is closely bound with the prevailing environmental conditions. It has been established that the trees are immune to infection during most of the growing season. Although cankers will result from inoculation from August to March it is those from December and January inoculations, in the middle of the dormant season, that are ultimately longest (10). Even in the dormant season an interval of 4 weeks between inoculations can make a significant difference to final canker length (6), probably because of changes in host physiological and environmental conditions. An interval of only 3 days between inoculation has resulted in a marked decrease in susceptibility of new cherry cultivars following inoculation with both races of *P. s.* pv. *morsprunorum* and *P. s.* pv. *syringae* (Matthews, pers. comm.).

Cherry rootstock susceptibility was not affected by a susceptible scion grafted on it. In contrast, when a range of plum rootstocks were grafted with the susceptible cultivar 'Victoria' they were significantly more

susceptible than when grafted with the more resistant 'Early Laxton'. There was no reverse effect of rootstock on the susceptibility of the two scion cultivars to inoculation (11).

Inoculum

Clearly the response to a pathogen is dependent upon the inoculum with which it is challenged. The choice of test isolates is always a difficult one to make. A single highly virulent isolate may be selected or a pooled inoculum of isolates of differing virulence, but representative of those which the host might encounter naturally, may be preferred.

It is possible that some of the differences between the results from screening the cherry rootstocks at 2 and again at 3 yr old (Table 1) might have been due to a change in the inoculum used, but the age of the trees was of greater importance.

A variant form of the pathogen, discovered in southeast England in 1970 and designated race 2 (4), differs from the typical race 1 of *P. s.* pv. *morsprunorum* in a number of characteristics, including virulence (Table 3). Its presence was discovered when trees of the canker resistant cultivar 'Roundel' became badly infected and a survey showed that the organism was already quite widely distributed (4). It was therefore necessary to screen for resistance to the new race the selected rootstocks which had been carried through to the 6 yr test and also new cherry cultivars from the breeding program at the John Innes Institute, Norwich. The rootstocks were equally resistant to race 2, with the exception of one *P. avium* cross which was highly susceptible (5). However, many of the new cultivars were susceptible to race 2 and only one-fifth of those selected for the National Fruit Trials were resistant to the new race (7). The screening of 46

Table 3. Distinguishing characters for *Pseudomonas syringae* pv. *morsprunorum* races 1 and 2 and *P. syringae* pv. *syringae*.

Character	*P. syringae* pv. *morsprunorum*		*P. syringae* pv. *syringae*
	race 1	race 2	
Colony on nutrient sucrose agar at 48 h	striated	'fried egg'	striated
Growth in nutrient sucrose broth	white	White, yellow/white	yellow
Hydrolysis arbutin	—	—	+
Tyrosinase activity	+	+	—
Use of tartrate	+	(+)	—
Use of lactate	—	—	+
Sensitivity to A7	+	+	—
Sensitivity to B1	—	+	—

established varieties showed that 32 were susceptible, two-thirds of these being very susceptible, and only 13 (28.3%) were resistant (8).

Although *P. s.* pv. *morsprunorum* is the dominant organism on cherry in southeast England, the pathogen *P. s.* pv. *syringae* has been isolated from the cultivar 'Van'. Inoculations with these isolates have shown that they are much less virulent through leaf scars than *P. s.* pv. *morsprunorum* but are equally virulent through wound inoculation (Garrett, unpublished).

The new cultivars from John Innes have now been screened with *P. s.* pv. *syringae* and some of them are susceptible to this pathogen though resistant to both races of *P. s.* pv. *morsprunorum*. Thus their potential for release to N. America has been reduced.

I have recently made a survey of the fluorescent pseudomonads on plum in England so that representative types can be included in the inoculum when selections of new plum cultivars arise from the current breeding program of Long Ashton Research Station, Bristol. But, however carefully inoculum is selected, the future emergence of a new form or race of the pathogen with altered pathogenicity or host range can threaten new or established resistant cultivars.

Discussion

In breeding resistant cultivars of tree fruits there is inevitably a long lag phase from the initial cross to the release of a new cultivar. In screening for resistance to disease the conditions are weighted in favour of infection and only about 1% of seedlings will be expected to survive the rigorous selection procedures. When the host plants are perennial trees one needs to recognize that response to inoculation at a juvenile stage is not necessarily correlated with that on older trees. In field testing at a single location the variation in soils and environmental conditions from one year to the next or during one season can influence the result of screening tests. In one year conditions were so unfavorable for infection that a screen of plum rootstock was totally valueless. The differences in soil, environment, and strains of pathogen between one locality and another may account for the fact that a cultivar may be recorded as resistant in one and as susceptible in another. A compilation of the rating of pear cultivars for their resistance to fireblight illustrates this clearly (13) although the very susceptible or near immune cultivars perform more consistently than those of moderate resistance.

Greater standardization of conditions could be obtained by use of potted trees in a controlled environment greenhouse. This method is often used for initial testing for fireblight resistance of pear where young growing shoots are inoculated and the results recorded a few days later. Development of bacterial canker takes 4 to 6 mo and difficulties in the practical application, reliability, and correlation with field performance would need to be overcome before such a screening method could be established.

Unless new bactericides are forthcoming, and the prospects for this are not encouraging, the use of resistant cultivars must increase. The development of reliable and more rapid screening procedures is therefore desirable.

Cooperation between plant breeders and crop protection scientists of all disciplines is needed to avoid such catastrophes as the release of mosaic resistant cassava which was highly susceptible to, and decimated by, cassava bacterial blight. The causal organism *Xanthomonas manihotis* was an indigenous but, until then, very minor bacterial pathogen (9). The screening of new cultivars for pest and disease resistance can only be carried out effectively in an integrated program.

Literature Cited

1. Crosse, J. E., and M. Bennett. 1959. The effect of cotton seed oil on the control of bacterial canker of cherry with an early autumn spray schedule of Bordeaux Mixture. Rep. E. Malling Res. Stn. for 1958. Pp. 93-95.
2. Crosse, J. E., and C. M. E. Garrett. 1966. Bacterial canker of stone-fruits. VII Infection experiments with *Pseudomonas mors-prunorum* and *P. syringae*. Ann. appl. Biol. 58:31-41.
3. Crosse, J. E., and N. Shanmuganathan. 1961. Pathogenicity tests with isolates of *Pseudomonas mors prunorum* from leaf surfaces of sprayed and unsprayed cherry trees. Rep. E. Malling Res. Stn. for 1960. Pp. 87-89.
4. Freigoun, S. O. and J. E. Crosse. 1975. Host relations and distribution of a physiological and pathological variant of *Pseudomones morsprunorum*. Ann. appl. Biol. 81:317-330.
5. Garrett, C. M. E. 1975. Selection of resistant rootstocks. Rep. E. Malling Res. Stn. for 1974, 106.
6. Garrett, C. M. E. 1979. Screening *Prunus* rootstocks for resistance to bacterial canker, caused by *Pseudomonas morsprunorum*. J. Hort. Sci. 54:189-193.
7. Matthews, P., and P. Dow. 1976. Variants of *Pseudomonas morsprunorum* race 2. Rep. John Innes Hort. Inst. for 1975. 18 pp.
8. Matthews, P., and P. Dow. 1977. Cherry breeding. Resistance to bacterial canker *Pseudomonas morsprunorum* race 2. Rep. John Innes Hort. Inst. for 1976. pp. 20-21.
9. Persley, G. J. 1976. Report on cassava disease survey in Cameroon, 5-20 June 1976. IITA, Ibadan. 30 pp.
10. Shanmuganathan, N. 1962. Studies in bacterial canker of plum and cherry (*Pseudomonas mors prunorum* Wormald). Ph.D. Thesis, University of London.
11. Shanmuganathan, N., and J. E. Crosse. 1963. Experiments to test the resistance of plum rootstocks to bacterial canker: Rep. E. Malling Res. Stn. for 1962. Pp. 101-104.
12. Trajkorski, V., and B. Lernius. 1977. Breeding for field resistance to bacterial canker in sweet cherry. Lantbrukshogskolans meddelanden 66:276.
13. Van der Zwet, T., and H. L. Keil. 1979. Fireblight and bacterial disease of Rosaceous plants. USDA Agriculture Handbook, No. 510. 200 pp. Science and Education Administration.

Control of Crown Gall (*Agrobacterium tumefaciens* Smith and Townsend) in Nurseries

R. Grimm
Swiss Federal Research Station for
Fruit Culture, Viticulture, and Horticulture
Wädenswil, Switzerland

S. Süle
Research Station for Plant Protection
Herman Otto U.
Budapest, Hungary

Abstract

Crown gall (*Agrobacterium tumefaciens* Smith and Townsend) in nurseries is a problem not to be underestimated. Consequent to *in vitro* experiments and observations in the greenhouse, the possibilities of fighting crown gall were investigated in nurseries with the help of a dipping and dusting method. Seventy-eight thousand rootstocks were examined. They were: apple M4, MM106, M9; Quince, Mirabolen; Wildpeach; and Wildcherry. The efficiency of fighting pathogenic strains of *A. tumefaciens* with the antagonistic strain *A. radiobacter* K84 (Kerr) (10^9 cells/ml) was tested. This was compared with the effect of treatments with streptomycin-sulphate (0.025%) as a standard and with the bactericide CGA 78039 (CIBA-GEIGY, Basle, Switzerland; 1% or 5000 ppm A. I.). The effects of methoxyethyl-Hg (0.012 Hg A. I. in solution) and a slurry of methoxyethyl-Hg (1.75% A. I. total slurry) have been compared. The phytotoxicity of these chemicals was high. It seems that the antagonism between *A. radiobacter* and *A. tumefaciens* is much more efficient in stone fruits (peach and cherries) than in apple rootstocks. After treatment with the antagonist, rootstocks of M9 even showed significantly higher levels of tumors. After double treatment of the same rootstocks (M9, December and April/May 1980/81) with the bactericide CGA 78039, remarkable effects were found.

Introduction

Crown gall (*Agrobacterium tumefaciens* Smith and Townsend) is a phytopathological problem in Switzerland and Hungary. This concerns mainly the rootstock production in nurseries for stonefruits, apples, pears, quince, and the production of berries, not grapewine (3).

No exact data are available for estimating the losses caused by crown gall. It is astonishing that in the USA the loss in plant production by *A. tumefaciens* is one of the most serious plant bacteriological problems (4). In Hungary, the loss of *A. tumefaciens* in nurseries has been estimated to be 10% (Süle, 1981 personal communication).

So far, chemical control of the disease in nurseries has been mostly ineffective (4). Added to this are toxicological problems and problems with buildup of resistance of pathogenic bacteria against antibiotics.

Biological control discovered and developed in Australia (11), however, appeared to be very promising. In the middle of the seventies, a series of experiments fighting the pathogen was started in Hungary, and two years later, in Switzerland. Investigations were made by *in vitro* experiments to test the antagonism between 25 strains of *A. tumafaciens* [isolated in Switzerland, 3 received from A. Kerr, and 3 bought from NCPPP (No. 396-1001, 2270)] and *A. radiobacter* K84, also received from A. Kerr. The tests have been escalated in the greenhouse with tobacco and tomato (3, 8).

It was hoped a practical way could be found for controlling crown gall in nurseries, without treatments of antibiotics or chemicals incorporating mercury.

Materials and Methods

In January, 1980 dipping treatments were made in three Swiss nurseries; 3,271 rootstocks were treated to fight crown gall. All the rootstocks were suspected to be infected with the pathogen. They were one year old and had a diameter of 4 to 5 mm.

The dipping treatment was made with a bacterial suspension of the antagonistic bacteria strain *A. radiobacter* K84 Kerr [(10^8 cells/ml); the other dipping treatments were made with streptomycin-sulphate (0.025%), Pfitzer] as a standard and with the bactericide CGA 78039 (1% or 5000 ppm A. I., CIBA-GEIGY, Basle, Switzerland). The results of these treatments have been compared with the effect of the occasional (but not allowed) dipping treatments of mercury chemicals in nurseries.

After the treatments, the rootstocks were stored separately in a refrigerated chamber, kept there to be planted in May, 1980. Part of the rootstocks (1278 plants) were treated a second time with the same treatment, on the day of planting out in the nurseries (May, 1980). For statistical reasons, it is only possible to present the treatment results of two nurseries (total 2199 rootstocks M9, 717 plants of which were treated twice as described, Table 1).

The plants were evaluated in January, 1981. During the first treatment at the time of harvest of the rootstocks in the nurseries in January, 1980 no roots of the rootstocks were cut. The plants which have been treated a second time had their roots cut one day before the second treatment to a lenght of 1 cm or less.

Observations were made not only to determine if the examined rootstocks showed tumors or not; the applied evaluating system also gave the location of the tumors on the plant and how big they were (8).

In Hungary a total of 69,330 plants have been tested by a dipping and pulverizing method (16). The plants were: apple rootstocks M4 (20,830 plants) and MM106 (10,000 plants); quince (4,000 plants), myrobolan (4,000 plants), wild peach (10,000 plants) and wild cherry (10,000 plants).

The dipping method was made in a bacterial suspension of *A. radiobacter* (K84, Kerr; 10^8 cells/ml) during 15 minutes. The treatment was made in

Table 1. Treatments of 2199 one year old rootstocks M9 and their effect in reducing crown gall in two Swiss nurseries.[a]

Treatment		Nursey 1[a]		Nursey 2[a]	
		evaluated plants	% of plants with galls	evaluated plants	% of plants with galls
Untreated		276	25.75	231	21.9
A. radiobacter K84	1 tr.[b]	140	9.3	110	40.0
A. radiobacter K84	2 tr.[c]	—	—	120	41.4
Streptomycin sulphate	1 tr.	138	20.4	105	8.6
Streptomycin sulphate	2 tr.	136	15.8	123	12.1
CGA 78039	1 tr.	136	16.9	106	13.2
CGA 78039	2 tr.	135	9.6	122	8.2
Methoxyethyl-Hg	1 tr.	138	12.6	102	7.1
Methoxyethyl-Hg	2 tr.	—	—	81	0
	Total	1099		Total 1100	

[a] Nursery No. 1 : Hessen, Wädenswil; No. 2 : Dickenmann, Ellighausen.

[b] 1 tr. : one treatment (January 1980).

[c] 2 tr. : treatments (January and May 1980).

March/April before planting. The plants were evaluated 18 months later, classified as plants with and without galls.

The pulverizing method was done by a sprayer apparatus. The roots were sprayed for total wetness with a bacterial suspension of 10^6 cells/ml and the treated plants were evaluated 18 months later. The tested plant materials and their treatments are listed in Table 2, including the results.

Results

The efficiency of the various treatments to control *A. tumefaciens* in Swiss and Hungarian nurseries is shown in Tables 1 and 2 and Figures 1 and 2.

Almost 26% of the untreated rootstocks M9 in the Swiss nursery 1 (Hessen) were attacked; 21.9% in nursery 2 (Dickenmann). In both cases, a second treatment with the bactericide CGA 78039 (at the time of harvest of the rootstocks M9 in January, 1980 and at the time of planting in May) showed a very good effect; in nursery 2 at a nearly significant level (LSD 0.05; 15, 2), in nursery 1 at a significant level (LSD 0.05; 11.7) (Fig. 1 and 2). The efficiency of treatments of the rootstocks with the bacterial antagonist strain *A. radiobacter* K84 (Kerr) had a significant effect in nursery 1.

Similar and also not homogenous results were found on apple rootstock treatments in Hungarian nurseries (Table 2). In two cases (M4/Idared and M4/Starking) the number of galls from rootstocks M4, treated with the antagonist bacterial strain K84 (Kerr) (dipping treatment), was twice and three times as high as untreated rootstocks.

Treating wildpeach/peach Jerseyland and wildcherry/cherry Bigarreau Burlett with the dipping method showed good efficacy.
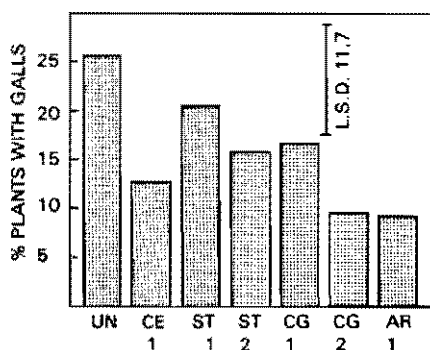
Fig. 1. Results of one treatment of the rootstocks when harvested in January, 1981.
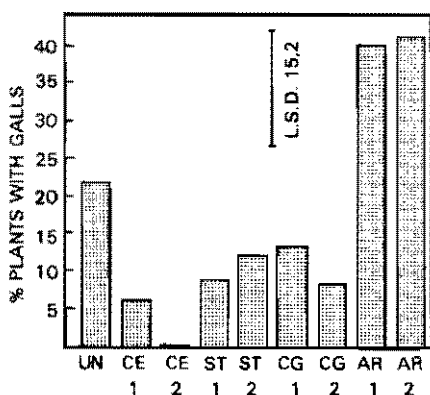
Fig. 2. Results, two treatments of the rootstocks, one when harvested in January, 1980, and one in May, 1980, when they were planted.

Table 2. Treatments to control *A. tumefaciens* and their effect in Hungarian nurseries.

Year of experiment	Method	Plants incubated	No. of plants treated	untreated	Plants with galls treated	o/o	untreated	o/o
1979/80	Dipping	M4/Idared	5,000	5,000	710	14.2	321	6.42
1979/80	Dipping	M4/Starking	2,000	2,000	410	20.5	122	6.1
1976/77	Pulverizing	M4/Starkrimson	500	500	82	16.4	59	11.8
1978/79	Dipping	M4/Jonathan	2,000	2,000	46	2.3	58	2.9
1975/76	Pulverizing	MM106/Idared	5,000	5,000	360	7.2	392	7.84
1977/78	Pulverizing	M4/Redspur	1,165	1,165	35	3	35	3
1977/78	Pulverizing	quince/pear Yellow Williams	2,000	2,000	84	4.2	92	4.6
1977/78	Pulverizing	myrabolan/apricot Rakowsky	2,000	2,000	0	0	38	1.9
1978/79	Dipping	wildpeach/peach Jerseyland	10,000	10,000	0	0	942	9.42
1979/80	Dipping	wildcherry/cherry Bigarreau Burlet	5,000	5,000	42	0.84	412	8.24

Discussion

Until now, young apple rootstocks in some Swiss nurseries were treated to fight *A. tumefaciens* with a solution of methoxyethyl-Hg (0.012 Hg A.I.) or by a slurry of methoxyethyl-Hg (1.75% A. I. total slurry). The objective of these four years of work was to give the nurserymen an alternative control for crown gall, fighting with biological control (1, 2, 6, 7, 9, 10, 11, 12, 13, 14, 15), and to show that a possibility exists for chemical treatment of bacterial plant diseases, not based on Mercury or antibiotics (8, 5).

The results of the experiments fighting *A. tumefaciens* in Swiss and Hungarian nurseries are similar. It is well-known that the control of the pathogen in apple and pear nurseries is much more difficult by the discussed biological method than in stone fruit nurseries.

The reason could be that the bacterial antagonist strain *A. radiobacter* K84 (Kerr) was not isolated from apple or pear nurseries, but from stone fruits. For example, after treatments of rootstocks M9 and M4 with the antagonist K84, the significant inverse effect was found in the cases of nursery 2 (Switzerland, Table 1, Figure 2, and in Hungary, Table 2: M4/Idared and M4 Starking).

It is interesting to see that here the number of diseased rootstocks has doubled or more. It is not clear if the reason for this fact is to be understood as a synergism.

Literature Cited

1. Bazzi, C., U. Mazzucchi. 1976. Possibilita di lotta biologica control il tumore batterico delle drupacee. Inf. fitopathologico 26:21-22.

2. Bazzi, C., U. Mazzucchi. 1978. Biological control of crown gall in cherry, myrabolan and peach rootstocks in Italy. Proc. 4th Conf. Plant Path. Bact., Angers, France.

3. Bosshard, E. 1977. Die Mauke der Reben. Schweiz. Zeitschrift für Obst - und Weinbau 113, 244-247.

4. Egli, T., E. Sturm. 1980. Bacterial plant diseases and their control. Chemie der Pflanzenchutz - und Schadlings-bekampfungsmittel, Band 6, 345-388.

5. Egli, T., W. Zeller. 1981. CGA 78039, a novel bactericide for the control of fireblight. Acta Horticulturae 117:107-111.

6. Faivre-Amiot, A., J. Roux, and M. L. Faivre. 1979. Essai de lutte biologique contre la galle du collet (crown gall) á l'aide d'une souche d'agrobacterium non pathogéne: *Agrobacterium radiobacter* var. *radiobacter* (souche 84 de Kerr). Phytiatrie-Phytopharmacie 28:203-214.

7. Garrett, C. M. E. 1978. Biological control of crown gall, *Agrobacterium tumefaciens*. Proc. Ann. Appl. Biol. 89:96-97.

8. Grimm, R., J. Vogelsanger. 1981. Bekampfung des Wurzelkropfes (*A. tumefaciens*) in schweizer Baumschulen. Schweiz. Zeitschrift für Obst- und Weinbau, in press.

9. Moore, L. W. 1977. Prevention of crown gall on prunus roots by bacterial antagonists. Phytopathology 67 (17):139-144.

10. Moore, L. W. 1979. Agrobacterium radiobacter strain 84 and biological control of crown gall. Ann. Rev. Phytopathol. 17:163-179.

11. New, P. B., A. Kerr. 1972. Biological control of crown gall: field measurements and glasshouse experiments. J. Appl. Bact. 35:279-287.

12. Scheer van der, H. A. Th., J. P. A. van Dieren, and H. J. Wondergem. 1978. Onderzoek naar wortelknobbel bij vruchtbomen. Fruitteelt 68 (8). P. 246.
13. Scheer van der, H. A. Th. Personal communication.
14. Schroth, M. N., and W. J. Moller. 1976. Crown gall controlled in the field with a non-pathogenic bacterium. Plant Dis. Rep. 60, 275-278.
15. Sule, S. 1977. Biological control of crown gall with a peat cultured antagonist. Phytopath. Z. 91:273-275.
16. Süle, S. 1981. Biological control of crown gall in Hungarian nurseries (in press).

Biological Control of Crown Gall on Rose in Spain

M. M. López and M. Miró
Instituto Nacional de Inv. Agrarias (INIA)
Moncada, Valencia, Spain

R. Orive and F. Temprano
INIA, S. José de la Rinconada
Sevilla, Spain

M. Poli
Selection Meilland
Antibes, France

Abstract

Successful application of biological control for *Agrobacterium tumefaciens* was carried out in a commercial rose nursery in Spain during 1979. In spite of the low number of galled plants in untreated plots, 0.1% during the year, plants treated with strain K84 in peat preparations had fewer galls, 0.01%. Previous isolations made from galled plants in this geographical area showed the presence of biotypes 1 and 2. Both Agrocin 84 sensitive and resistant isolates were observed, however, the sensitive isolates were predominant.

A field trial in naturally contaminated soil was initiated, using a mixture of Fredica, Indica, and Manetti rootstock cuttings totalling 40,000. Test treatment included K84, K84 plus captan and captan alone in addition to untreated checks. All K84 inoculum was prepared in peat at a concentration of 5×10^8 viable cells per gram of peat. Inoculations were performed by either amending the   field soil with inoculum where the cuttings were placed, or by dipping 7,500 cuttings into a solution of 2 kg of inoculum in 3 liters of water prior to field planting.

A significant reduction of tumors was observed in K84 treated cuttings and captan treatment appeared to favor tumor development. When *in vitro* compatibility was studied, growth of K84 on solid and in liquid media was inhibited by the addition of captan and TMTD but not benomyl, iprodione and methyl thiophanate. *Agrobacterim tumefaciens*   isolates recovered from tumors were characterized as to biotype and sensitivity to Agrocin 84. Results of these laboratory tests will be discussed.

Introduction

Agrobacterium radiobacter (Beijerinch & Van Delden) Conn, var. *tumefaciens* (Smith & Townsend) Keane *et al.* causal agent of crown gall, produces important economic losses in fruit and rose nurseries in Spain (López and Salcedo, 1979).

Biological control of crown gall on rose has been successfully used in Australia, New Zealand, U.S.A. (Moore, 1979) and Italy (Garibaldi and Raimondo, 1977). Occasionally the method has not been fully effective and

in an experiment carried out in Texas by Lyle (Moore, 1979), 10% more tumors were found in rose plants treated with strain K84 than in control plants inoculated with **A. r.** var *tumefaciens*. The causes of those failures have not been studied.

In Spain, peat inoculum of strain 84, has been prepared from 1979 at the Instituto Nacional de Investigaciones Agrarias (INIA) by mixing broth culture of K84 and peat moss.

Biological control of crown gall on fruit trees has not yet been used commercially, due to the high number of *Agrobacterium* isolates resistant to Agrocin 84 that were obtained in a preliminary survey (López and Salcedo, 1979).

On rose, biological control was first commercially applied in 1979 in an important nursery. In a previous work (Orive *et al.*, 1980) we had studied 66 virulent isolates from 29 rose plants sampled at that nursery. Thirty-three of those isolates belonged to biotype I and the rest of them to biotype 2. Forty-nine isolates were sensitive to Agrocin 84, 12 of them were resistant, and 5 were slightly sensitive. No relationship between biotypes and sensitivity to Agrocin 84 was observed. Our results were in agreement with preliminary data obtained by Faivre-Amiot (Faivre-Amiot, personal communication). Treatment of rose cuttings with K84 was successful in spite of the low incidence of crown gall in 1980. In untreated plots 0.1% of the plants were infected whereas only 0.01% of galled plants were found in treated plots.

The need for standard fungicide treatment at the rose nurseries prompted us to study the *in vitro* and *in vivo* compatibility between biological control of crown gall with strain K84 and the usual fungicide applications. Preliminary data seemed to indicate that K84 was sensitive to some fungicides *in vitro* (Faivre-Amiot, personal communication).

Materials and Methods

Preparation of K84 Inoculum

A 60-liter stainless steel fermenter provided with an aeration system was used to prepare K84 inoculum. The medium used contains: 1.0 g. K_2HPO_4, 0.15 g $MgSO_4$. 7 H_2O, 0.1g $CaCl_2$, 0.7 g KNO_3, 0.3 g $(NHn)_2$, PO_4H, 0.005 g $MnSO_4$. 7 H_2O, 0.005g $FeCl_3$, 10g sucrose, 1 g yeast extract per liter water.

Sterile medium was inoculated by adding 1% of the final volume of 24 h shaken culture of the strain K84 grown in the same medium. Fermentation was carried out at 27-30°C for 60-72 hours. After checking purity and concentration of the broth culture it was throughly mixed with Padul peat moss at 1:1 proportion. The mixture was packed in polyethylene bags and brought to maturity for 10 days at 4°C.

Several storage conditions were assayed and periodical controls of the mixture were carried out by counting viable cells in Petri dishes at an adequate dilution. The data obtained were the average of 3 replications.

Field Trial

An 8836 m² uniform plot infested with *Agrobacterium radiobacter* var. *tumefaciens* was selected for the experiment; of which, 6472 m² were

used for treatments and the rest of the surface was planted with roses and left as a surrounding border. The plot was provided with adequate drainage to minimize possibilities of contamination among different treatments. Three rootstocks (Fredica, Indica, and Manetti) and two varieties (Visa and Sonia) were used in the experiment.

Three inoculation treatments with K84 were tried (cutting inoculation, soil inoculation, and uninoculated control) and every combination was done with and without application of Captan. A randomized design with 36 treatments and 4 replications distributed in two blocks was used for statistical analysis of results.

Rose cuttings were prepared in the standard form for commercial culture. They were de-eyed 24 to 72 h before planting and stored at 4°C. Treatment with Captan was done by dipping cuttings in a 2 g/l aqueous solution of the fungicide 16 h before planting and storing them at 4°C. Treatments with K84 were performed as follows:

— Cutting inoculation: Two kg of peat preparation mixed with 3 l water were used to inoculate 7500 cuttings. The mixture was prepared in a shadowed area and cuttings were dipped immediately before planting.

— Soil inoculation: Two kg of peat preparation mixed with 50 l water were used to inoculate 1 ha. Suspension was carefully distributed in furrows immediately before planting.

— Uninoculated control: Cuttings were dipped in a suspension of 2 kg peat moss in 3 l water.

Planting was carried out in December, 1979. During spring, 1980 half of the plants were budded with var. Sonia and the other half were budded with var. Visa. The plants in the border were budded with var. Red Success. The plants were lifted in january 1981.

Analysis of variance was used for statistical interpretation of results. Data were transformed in $\sqrt{n+1}$ for counting data and in arc sen $\sqrt{p}$ for percentage data.

Bacteriological methods

Isolation of A.r. var tumefaciens. The media used for isolation from tumors were the selective media of Schroth, Thomson and Hildebrand (1965), those of New and Kerr (1971), and PYGA medium.

Biotype Characterization. The following tests were used for biotype characterization. 3 Ketolactose, growth in 2% NaCl, Litmus milk, acid production from erythritol and melezitose, alkali production from malonate and growth in selective media, according to the techniques described by Kerr and Panagopoulos (1977).

Sensitivity to Agrocin 84. The Stonier's method (Stonier, 1960) with the modifications introduced by López (1978) was used.

Pathogenicity Tests. Pathogenicity of *Agrobacterium* isolates was tested in tomato plants according to the technique described by López (1978).

Sensitivity of K84 to Fungicides *in vitro.* Tubes containing 4.5 ml liquid medium were added with 1 ml of an aqueous solution of fungicide and then

inoculated with one drop of a bacterial suspension prepared by resuspending a 48 h old culture of K84 in 5 ml of sterile distilled water. The following liquid media were used: Nutrient Broth Difco, PYG, and LPG medium with two replications per tube.

The following fungicides and final concentrations were used: Captan, 3000 ppm; iprodione and TMTD 1500 ppm; and methylthiophanate and benomyl 1000 ppm. Cultures were incubated at 25°C in a shaking bath and viable cells were counted in New and Kerr medium at 24, 48, 72, 96 and 168 h. Data obtained were the average of 2 replications per treatment.

Effect of Fungicides on Avirulence and Agrocin 84 Production of Strain K84. Bacteriocin production by K84 was tested by the method described by Stonier (1960). One ml of fungicide solution was put into each Petri dish and then the culture medium, kept at 50°C, was added and throughly mixed with the fungicide. Final concentrations were those previously indicated. Immediately after it solidified, the medium was treated with chloroform by the tecnique described by Stonier (1960), and then inoculated with K84.

Three pathogenic strains of *Agrobacterium* isolated from rose tumors were used to test bacteriocin production, two of them belonged to biotype 1 and were Agrocin 84 sensitive and the third one belonged to biotype 2 and was Agrocin resistant.

Avirulence of K84 after fungicide treatment was tested by inoculation of tomato plants with 5 colonies reisolated from broth cultures that had been added with fungicides as previously described. Avirulence could not be tested in culture treated with captan or TMTD because those fungicides inhibited growth of K84 in shaken cultures.

Results

Quality of K84 Inoculum

The average concentration of viable cells in the broth culture was 5.28 x 10^9 cells/ml and final pH of the medium was 6.43. Twenty-five batches of peat inoculum stored at 4°C for 10 days gave an average concentration of 4.2 x 10^9 viable cells/g, that we considered adequate for K84 inoculation.

Survival of K84 in Padul Peat Inoculum

Variation in the number of viable cells in peat inoculum along a 22-week storage period at 4°C and at 30°C is shown in Fig. 1. At 4°C, the number of viable cells after 22 weeks was higher than 10^8 cells/g of peat inoculum, whereas at 30°C concentration was only 10^6 bacteria/g.

Effects of Inoculation with K84 and/or Treatment with Captan on Incidence of Crown Gall

A high number of cuttings died, probably due to late planting and inadequate rooting conditions. A total of 16,037 plants survived. No significant difference was found between percentage of galled plants of the Visa and Sonia varieties in the different treatments. Thus, data from both varieties were grouped.
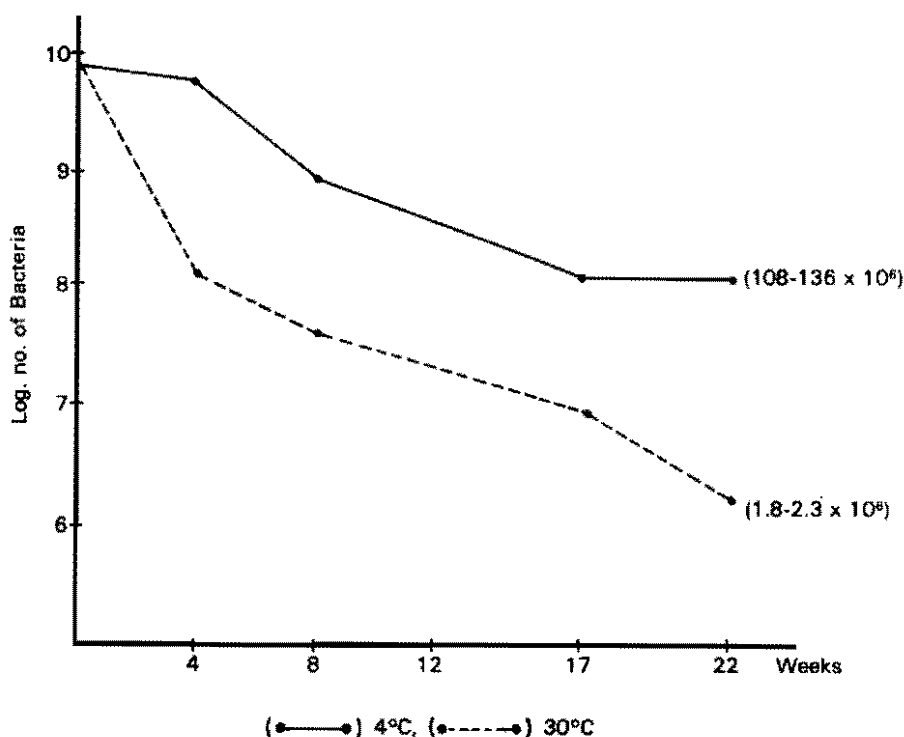
Fig. 1. Survival of strain 84 in Padul peat sterilized by dry heat (90°C, 24 h).

The effect of the different treatments on the number of galled plants is shown in Table 1. Significant differences (P=0.01) were found between percentage of galled plants on Fredica rootstock and plants on the other two rootstocks, the higher incidence being on Fredica. No significant difference could be detected between percentage of galled plants on Indica and Manetti rootstocks.

A higher incidence of crown gall was found on uninoculated plants than in plants treated with K84, the difference being significant at P=0.01. Soil inoculation with K84 resulted in a higher percentage of galled plants than cutting inoculation, the difference being significant at P= 0.05. No significant difference in crown gall incidence was observed between inoculated and uninoculated plants when the treatment with captan was omitted but in plants treated with captan, inoculation with K84 produced a significant (P =0.01) reduction in percentage of galled plants.

No significant effect of captan treatment on the crown gall incidence was observed on plants inoculated with K84. Higher incidence of crown gall was observed on uninoculated control plants treated with captan than on untreated plants, the difference being significant at P= 0.01.

542

Table 1. Effect of inoculation with strain 84 and/or treatment with or without captan, on crown gall incidence on three rose rootstocks.[a]

Treatment	Rootstock[b]	No. healthy plants	No. galled plants
Cutting inoculation with	F	978	3
strain 84	I	984	0
	M	811	0
Soil inoculation with	F	1015	6
strain 84	I	358	0
	M	721	0
	F	890	12
Control	I	975	1
	M	822	0
Treatment of cuttings	F	841	0
with captan and inocu-	I	1040	0
lation with strain 84	M	799	0
Soil inoculation with	F	998	34
strain 84 and treatment	I	1219	2
of cuttings with captan	M	782	0
Treatment of cuttings	F	776	127
with captan	I	877	5
	M	961	0
Total		15847	190

[a] Cuttings were planted on December 1979 in soil naturally infected with *A.r. var tumefaciens* and disease incidence was assessed on January 1980.

[b] F: Fredica;   I: Indica;   M: Manetti.

Statistical analysis of mean weight of tumors per plant on Fredica rootstock confirmed the results obtained when the percentage of galled plants was considered, the only difference being the level of significance in some of the factors studied. Results are shown in Table 2.

Number, Size, and Distribution of Tumors in Individual Rose Plants

A total of 221 tumors were counted in the 190 galled plants obtained in the experiment. Most of the plants had a single tumor (86.7%), but some of them had 2 tumors (10.9%), 3 tumors (1.9%), or even 4 tumors (0.5%).

Size distribution of tumors was as follows: less than 2 cm diameter 25.6%, 2 to 5 cm 54%, 5 to 10 cm 19.9% and more than 10 cm 0.5%.

The most frequent situations of tumors on affected plants were at the crown and the base of the cuttings (Fig. 2).

Biotype and Sensitivity to Agrocin 84 of A. r. var *tumefaciens* Reisolated from Tumors

Forty out of the 65 isolates studied belonged to biotype 1 and were sensitive to Agrocin 84 and 13 of them were biotype 1 but resistant to

Table 2. Dry weight (g) of gall tissue in Fredica rootstock after treatment with or without captan and/or inoculation with strain 84.

Treatment	Wt (g) of gall tissue	No. of galled plants	Mean of dry wt(g) of gall tissue in living plants
Cutting inoculation with strain 84	1'13	3	0'0012
Soil inoculation with strain 84	59'52	6	0'0583
Control	416'80	12	0'4621
Treatment of cuttings with captan and inoculation with strain 84	0'00	0	0
Soil inoculation with strain 84 and treatment of cuttings with captan	290'80	34	0'2810
Treatment of cuttings with captan	2562'90	127	2'83
Total	3330'55	172	
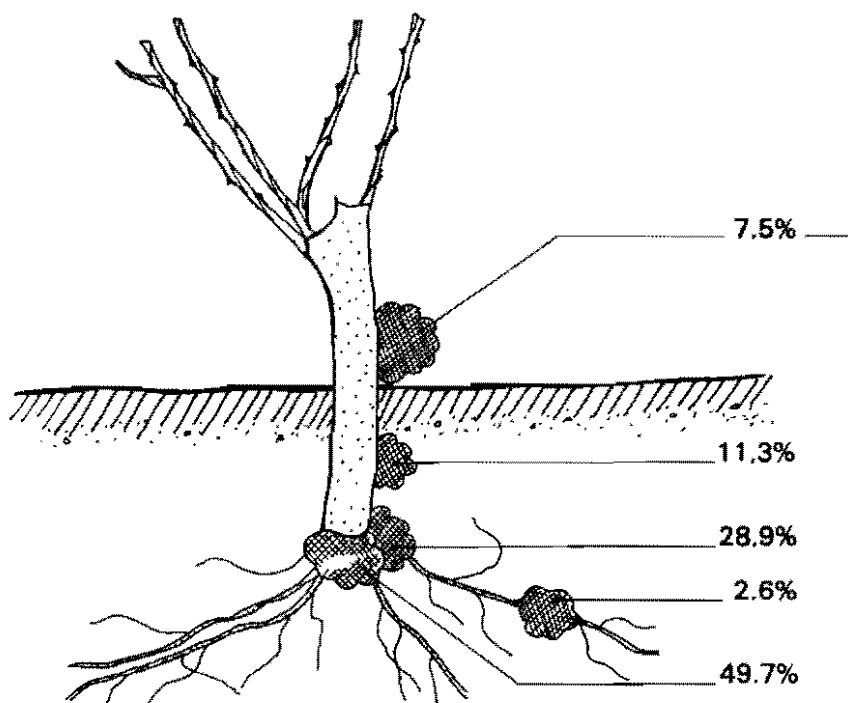
Fig. 2. Distribution of tumors on the rose plants.

Agrocin 84. Twelve isolates of the biotype 2 were obtained; 8 of them were sensitive to Agrocin 84 and 4 of them were resistant (Table 3).

Bacteriocin production was tested on 10 isolates resistant to Agrocin 84. Six of them were bacteriocin producers, of which 5 were biotype 1 and 1 was biotype 2. Three of the 6 isolates came from plants treated with captan and inoculated with K84, and the other three from uninoculated control plants (2 from plants treated with captan and 1 from an untreated plant). Comparative studies on those bacteriocins and Agrocin 84 are now in course.

No relationship was observed between the treatments of the plants and the characteristics of *Agrobacterium* reisolated from tumors. Strains of different biotype and different sensitivity to Agrocin 84, were isolated from a single tumor. Characteristics of Agrobacterium reisolated were not related to the external aspect of the tumor (smooth or rough surface).

Effect of Fungicides on Growth of K84

The effect of several fungicides on the number of viable cells of K84 is shown in Fig. 3. Captan (3000 ppm) and TMTD (1500 ppm) completely inhibited growth of K84. Only slight inhibition was observed with iprodione (1500 ppm), methylthiophanate(1000 ppm) and benomyl (1000 ppm).

Effect of Fungicides on Avirulence and Bacteriocin Production of K84

Addition of iprodione, methylthiophanate or benomyl to the Stonier medium did not change the ability of K84 to produce Agrocin 84. Captan

Table 3. **Characteristics of** *A.r. var tumefaciens* **isolated from tumors after treatment of rose cuttings with or without captan and/or inoculation with strain 84.**

Treatment	No. of studied isolates	Biotype 1		Biotype 2	
		Agrocin 84 sensitive	Agrocin 84 resistant	Agrocin 84 sensitive	Agrocin 84 resistant
Cutting inoculation with strain 84	—	—	—	—	—
Soil inoculation with strain 84	7	6	1	—	—
Control	9	2	2	5	—
Treatment of cuttings with captan and inoculation with strain 84	—	—	—	—	—
Soil inoculation with strain 84 and treatment of cuttings with captan	19	10	4	2	3
Control with captan	30	22	6	1	1
Total	65	40	13	8	4
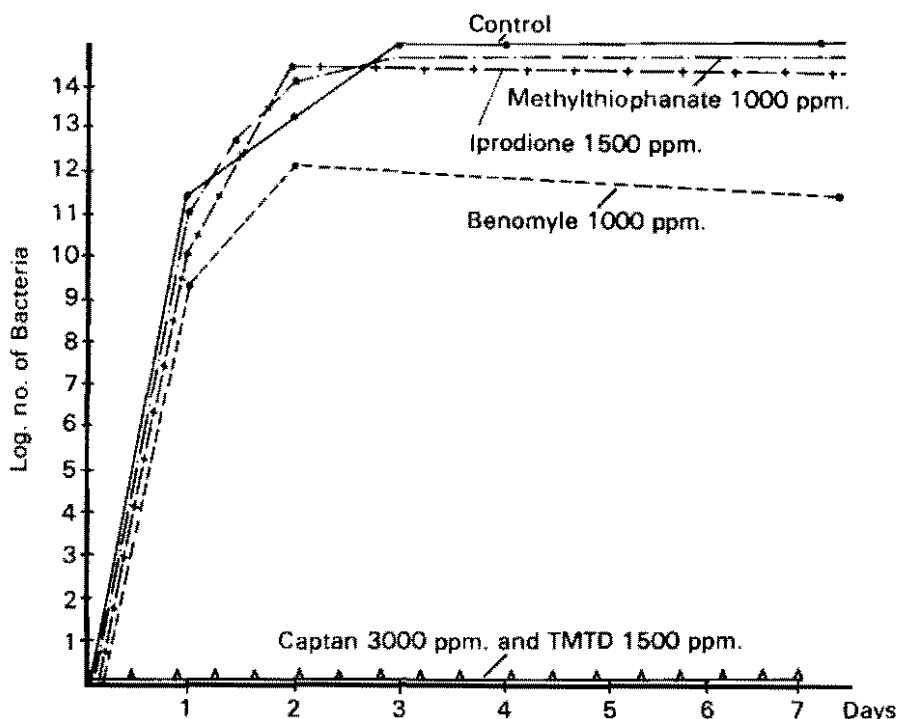
Fig. 3. Effect of different fungicides on the growth of strain 84.

and TMTD greatly inhibited growth of K84 in Stonier medium, but no change in bacteriocin production was observed. Avirulence and bacteriocin production of K84 were not affected when grown in a broth culture added with those fungicides.

Discussion

Results obtained on Fredica, that is an extremely sensitive rootstock, show that biological control of crown gall can be successfully used in Spain, despite the relatively high frequency of *Agrobacterium* isolates resistant to Agrocin 84 (López and Salcedo, 1979; Orive *et al.*, 1980).

In our experiment, rose varieties did not affect rootstock susceptibility to crown gall.

Inoculation of cuttings with K84 gave better results than soil inoculation in the field experiment.

When a fungicide treatment was not applied, no significant difference in crown gall incidence could be detected between plants inoculated and not inoculated with K84. This could be due to the extremely low incidence of crown gall in plants not treated with captan. Field experiments to confirm the effect of fungicides in biological control by K84 are now in course.

The inhibiting effect of captan on K84 *in vitro* could not be confirmed by field results.

This can be partially due to dilution of fungicide when cuttings are dipped in the suspension of K84 as well as to the different environmental conditions. Nevertheless a breakdown of biological control on tomato plants was observed when captan was added to a mixture of K84 and one Agrocin 84 sensitive strain and inoculated on tomato stem (López, unpublished data).

The higher incidence of crown gall observed in uninoculated plants treated with captan compared with non treated plants, could be explained by inhibition of *Agrobacterium* competitiors by this fungicide. Thus, care should be taken in applying this fungicide in soils heavily infested with *Agrobacterium.*

Since avirulence and bacteriocin production of K84 were not altered *in vitro* by any of the fungicides tested, biological control with K84 could be compatible with fungicide application. Nevertheless, field tests with K84 inoculated and uninoculated plants should be done before K84 is used extensively.

The presence of *Agrobacterium* isolates sensitive to K84 in most tumors, suggests an incomplete colonization of cuttings by K84.

Isolation of some *Agrobacterium* producing bacteriocin in plants inoculated with K84 as well as in uninoculated plants indicates that bacteriocin-producing isolates were present naturally in the field. The possibility of some gene transfer from K84 to natural isolates as described by Panagopoulos, Psallidas, and Alivizatos (1979) cannot be excluded.

Literature Cited

1. Kerr, A., C. G. Panagopoulos. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. Phytopath. Z. 90:172-179.
2. Lopez, M. 1978. Characteristics of French isolates of *Agrobacterium.* Proc. 4th Int. Conf. on Plant Path. Bact. Angers, 1978.
3. López, M. M., C. Salcedo. 1979. Primeros resultados sobre control biológico de *Agrobacterium tumefaciens.* IV Jornadas Grupo Fitopatología. S. E. M. Córdoba. Diciembre 1979. (Abstract).
4. Moore, L. W. 1979. Practical use and success of *Agrobacterium radiobacter* strain 84 for crown gall control. Edited by B. Schippers and W. Gams. Academic Press, London, New York, S. Francisco.
5. New, P. B., and A. Kerr. 1971. A selective medium for *Agrobacterium radiobacter* biotype 2. J. Appl. Bact. 34:233-236.
6. Orive, R., M. M. López, F. Temprano, R. Millan, and C. Salcedo. 1980. Balance inicial del primer año de lucha biológica contra *Agrobacterium tumefaciens* en rosal. V Jornadas Grupo Fitopatología S.E.M. Zaragoza, Noviembre, 1980. (Abstracs).
7. Panagopoulos, C. G., P. G. Psallidas, and A. S. Alivizatos. 1979. Evidence of breakdown in the effectiveness of biological control of crown gall. In soil borne plant pathogens. Edited by B. Shippers and W. Gains. Academic Press. London, New York, S. Francisco.
8. Stonier, T. 1960. *Agrobacterium tumefaciens* Conn., II. production of an antibiotic substance. J. Bacteriology 99:889-898.

Acknowledgments

Control of Bacterial Diseases of Plants with Plant Crudes

N. Mukherjee and P. Biswas

Department of Plant Pathology
BCKVV, Kalyani,
West Bengal, India

Abstract

Plant chemicals or crude extracts occasionally have been tested for control of bacterial diseases of crops. But not much is available in the way of good and recommendable chemicals for such purposes. Antibiotics recommended are costly besides having other disqualifications. In this context we tested crude extracts of 25 selected plants, screened at different concentrations against two plant pathogens, *Xanthomonas campestris* and *Erwinia carotovora carotovora*, following standardized methods, and identified some plant oils and crudes that have potentialities. Selected inhibitors have been tested against black rot of cabbage *(X. campestris)* and soft rot of potato *(E. carotovora)* as post-inoculation spray. Plant oils from *Taaraktogenos kurzii* and *Elacis quineensis* seeds and water and ethanol extracts of *Casuarina equisetifolia* leaves and *Coriandrum sativum* seeds successfully protected plants from their respective pathogens to a great extent. Tubers were equally protected by oils of *Cybopogen nardus* and *Madhuca indica* and extracts of seeds of *C. sativum* and leaves of *C. equisetifolia* and *Piper betle*. The oils and crudes have been equally successful in field trials on control of black rot of cabbage and citrus canker *(X. campestris citri)* so far. Repeated applications at lower doses, both individually and in mixture with others, have given better or similar results.

Necessity of using a higher dose (2-5%) of such oils appears to be the first problem to be overcome. Considering all other aspects, however, the prospect of using such plant oils and crudes appears to be bright.

Introduction

Plant chemicals and crude extracts of plants have been reported to have antibacterial properties against a number of plant pathogenic bacteria *in vitro*. The literature in this area has revealed that inhibitors detected *in vitro* have not resulted in identification of usable products. Actually, few have so far been tried for controlling bacterial diseases of plants.

Plant crudes from over 300 plant species appear to have been screened and reported to have antibacterial principles (1, 2, 3, 4, 5, 10, 11, 12, 13, 16, 17, 21, 22, 23, 24, 26).

Most of these plant chemicals and extracts remain to be tried for disease control. Elaborate studies with extracts from *Allium sativum* (18) have revealed that a 15% solution of instant garlic powder can prevent the decay

of carrot due to *E. carotovora* var. *carotovora*. In another study *Azadirachta* cake solution prepared at 12 lb per 4 gal and sprinkle-sprayed on citrus nurseries suffering from canker (*X. citri*) checked the disease (27). Extraction from the cake, however, required soaking in water for a week and 10 to 12 sprays/year were recommended. The work was later confirmed (6) and an *Azadirachta* cake solution (1 kg/20 l) was used successfully by another group of workers (20). The results seem to be consistent and suggest a good and safe control measure for this widespread disease on the Indian continent.

The reported control of vascular bacteriosis of crucifers due to *X. campestris* with crude plant extracts termed "phytoncides" prepared from *Cochlearia armoracia, Allium sativum,* and *Brassica napus* (8) is another such achievement showing definite yield increase. Accounts of different "phytoncides" and their uses in agriculture and particularly in plant protection have been compiled (7, 25). Use of a purified plant chemical named citrinin for treating cabbage seeds to make them free of *X. campestris* (19) is noteworthy.

Further attempts to control bacterial diseases of plants with plant crudes have been scanty. Present work is a continuation of some previous work with plant chemicals (4, 13, 15) in a changed direction, considering the fact that attempts for using purified plant chemicals for plant disease control have been difficult and highly uneconomic for most of the agricultural crops. The present program is aimed at the use of plant crudes for economically practical controls.

Materials and Methods

In vitro assays of the sensitivity of the bacteria to plant crudes have been conducted mainly with *X. campestris* and *E. carotovora carotovora* following standardized fishspine method of assay of antibacterial activity (14). Among the large number of plants screened the following are important for such purposes: *Eichhornia crassipes* (Pontederiaceae), *Allium cepa* (Liliaceae), *Raphanus sativus* and *Brassica napus* (Cruciferae), *Piper betle* (Piperaceae), *Casuarina equisetifolia* (Casuarinaceae), *Coriandrum sativum* (Umbelliferae), *Lantana camara* (Verbenaceae), *Areca catechu cocos nucifera,* and *Elacis quineensis* (Palmae), *Bamboosa aurandinaceae* (Graminae), *Azadirachta indica* (Maliaceae), *Taaraktogenos kurzii* (Flacortiaceae), *Cymbopogon nardus* and *Trigonella foenum-graecum* (Leguminosae), *Sapindus saponaria* (Sapindaceae), *Ricinus communis* (Euphorbiaceae) and *Madhuca indica* (Sapataceae). Extraction of oils was accomplished through pressing methods in a country pressing-mill operated electrically. Other extracts have been prepared by crushing fresh plant materials in a blender mixing with water in 1:1 wt/wt ratio. The preparation was strained and a volume made up to be used fresh in an assay program. Cold alcohol extracts of crushed plant tissues were prepared, dipping crushed plant material in absolute ethyl alcohol for at least 72 h. Alcohol extracts were brought into water by drying out of alcohol and were used fresh for assay. Solutions of plant oils and extracts were prepared in water by addition of a wetting agent at 0.1 to 1% by weight.

In vivo assays were conducted at the first stage with 2 months old cabbage plants grown in 8" diameter earthenware pots. Plants were inoculated by pin-pricking and spraying with plant crude preparations with a hand sprayer 24 h after inoculation. The plants were incubated for 24 h in a humid cabinet and thereafter in an incubator (30°C ±1.0) to allow disease development. Necessary uninoculated and inoculated unprotected controls were maintained.

Disease intensities were measured with standard score charts, developed for the purpose and converted to percentage control of disease using the following formula:

$$\% \text{ disease control} = \frac{\text{total number of plants - no. of plants killed}}{\text{total number of plants inoculated}} \times 100$$

In the case of soft rot, the whole tubers were inoculated following a method developed from Harris (9). Plant crudes were spray applied in polythene bags, sealed air-tight and incubated at 30 ± 1°C for disease development. Disease intensity was measured with the help of a score card, developed for the purpose. Percent disease control was calculated in the same way, counting number of tubers rotten.

Dust formulations of the crudes were prepared in clay and talc, taking 5 ml crudes in absolute alcohol, impregnating in 15 g clay, and finally mixing with 80 g of talc. The inoculated tubers were dusted and incubated as usual.

The trial for control of citrus cankers was conducted in a 5-year-old sweet orange orchard at the Mondouri Farm on the University following a randomized block trial with 6 treatments and 4 replications. An antibiotic mixture containing streptomycin sulfate, terramycin hydrochloride, and carrier material in the 16:1:83 ratio (wt./wt) was used as standard.

Results

Sensitivity *In Vitro* of Plant Bacteria to Plant Crudes

Among the large number of plant oils and extracts screened against two plant pathogenic bacteria, *X. campestris* and *E. carotovora* (Table 1), oils from the seeds of *Taaraktogenos kurzii* and the leaves of *Cymbopogon nardus* are quite inhibitory to both bacteria. Oils from the seeds of *Azadirachta indica*, *Pongamia glabra*, and *Elacis quineensis* are quite inhibitory to *X. campestris*, while those from seeds of *Madhuca indica*, *Ricinus communis*, and *Brasica napus* are more or less similarly inhibitory to *E. carotovora*. However, the inhibition by these oils generally required a higher concentration i. e., 3-20%.

The crude extracts of plants prepared either in water or ethyl alcohol were comparatively less inhibitory. Among them, nuts of *Areca*, seeds from *Casuarina* and *Trigonella*, and leaves of *Casuarina* have been inhibitory to *X. campestris*. *E. carotovora* was inhibited by seeds of *Trigonella*, *Areca* nut

Table 1. *In vitro* sensitivity of two plant pathogenic bacteria to plant oils and extracts. (Average of 6 replicates.)

Plant crudes		Plant part	Inhibitory concentration (%)	
			X. campestris	*E. carotovora*
Oils :	*Azadirachta*	seed	10	—[a]
	Cymbopogon	leaf	5	10
	Taaraktogenos	seed	3	3
	Cocos	seed	25	—
	Pongamia	seed	10	—
	Madhuca	seed	30	5
	Brassica	seed	35	20
	Elaeis	seed	15	—
	Ricinus	seed	—	10
Extracts :				
	Areca	nut	20	20
	Areca	shell	25	15
	Sapindus	fruit	—	20
	Piper	leaf	30	15
	Casuarina	leaf	20	20
	Casuarina	seed	20	—
	Coriandrum	seed	25	20
	Lantana	leaf	35	25
	Trigonella	seed	20	15
	Allium	bulb	35	20
	Raphanus	root	30	5
	Eichhornia	leaf	—	15

[a] no inhibition.

shell, bulb of *Allium*, modified roots of *Raphanus*, fruits of *Sapindus*, and leaves of *Piper, Casuarina*, and *Eichhornia*.

Concentration of Crudes and Control of Bacterial Diseases

Plant crudes were first tested against that two plant diseases caused by two bacteria at three different concentrations, 3, 5 and 10%, with a low percentage of detergent (0.1%) being mixed with them. The results appeared promising. Black rot causing bacterium was more or less checked by the plant oils from *Taraaktogenos* and *Elacis* and by the extracts from *Coriandrum, Casuarina*, and Sapindus even at 3% concentrations (Table 2). Bacterial soft rot was generally checked by the plant oils tried but not by the extracts (Table 3).

Table 2. Control of black rot of cabbage with different dosages of plant crudes. (Average of 8 replicates)

Plant crudes	Percent disease control at dosages					
	3°/o		5°/o		10°/o	
	Initial	Final	Initial	Final	Initial	Final
Oils:						
Azadirachta	P[a]	P	P	P	P	P
Cymbopogon	P	P	P	P	P	P
Taaraktogenos	60	20	70	40	100	50
Pongamia	P	P	P	P	P	P
Elaeis	50	0	70	10	90	40
Extracts:						
Areca nut	40	5	60	5	70	20
Sapindus	50	20	70	20	80	40
Casuarina leaf	60	30	80	40	100	50
Corianddrum	50	35	60	20	90	40
Lantana	50	70	70	20	80	20
Trigonella	40	10	50	10	60	20

[a] P = Phytotoxic.

Table 3. Control of bacterial soft rot of potato with different dosages of plant crudes. (Average of 8 replicates.)

Plant crudes	Percent disease control at dosages					
	3°/o		5°/o		10°/o	
	Initial	Final	Initial	Final	Initial	Final
Oils:						
Cymbopogon	70	20	100	50	100	50
Taaraktogenos	60	10	70	10	100	50
Madhuca	70	10	100	30	100	30
Brassica	40	0	60	30	70	30
Extracts:						
Piper	50	10	60	10	70	45
Casuarina leaf	70	0	90	30	100	50
Coriandrum	40	0	50	0	80	50
Trigonella	45	0	60	0	70	10
Allium	60	10	70	10	90	10
Eichharnia	40	0	50	0	60	5

Successful Plant Crudes

Successful plant crudes were tested further, generally at 5% concentrations, in a comparatively large scale taking at least 25 plants/tubers per replication. The crudes were prepared by mixing them with a higher percentage (1%) of the detergent to improve upon performance, particularly of the oils. The results, in Table 4, were better than the *in vitro* performances of the crudes. Oils of *Taaraktogenos* and extracts from seeds of *Coriandrum* and *Trigonella* and leaves of *Casuarina* successfully

Table 4. *In vivo* sensitivity of *X. campestris* and *E. carotovora* to some plant crudes at 5°/o conc. (Average of 4 replicates).

| Plant crudes | Percent protection of host[a] time against | | | |
| | *X. campestris* | | *E. carotovora* | |
	Initial[b]	Final[c]	Initial	Final
Oils:				
Azadirachta	P[d]	P	—[e]	—
Cymbopogon	P	P	100	50
Taaraktogenos	100	50	100	50
Pongamia	P	P	—	—
Madhuca	—	—	100	30
Brassica	—	—	100	0
Elacis	90	40	—	—
Extracts:				
Areca nut	70	0	—	—
Sapindus	80	30	—	—
Piper	—	—	90	50
Casuarina leaf	100	50	100	60
Coriandrum	90	30	100	50
Lantana	80	0	—	—
Trigonella	60	10	70	10
Allium	—	—	100	—

[a] Cabbage for *X. campestris* and potato for *E. carotovora*.

[b] Initial -- 2 days after treatment.

[c] Final -- 15 days after treatment.

[d] P = phytotoxic.

[e] — = not tried.

checked both the diseases. Black rot was also checked by *Elacis* oil and *Sapindus* extracts in addition. On the other hand, soft rot was also controlled by *Cymbopogon* and *Madhuca* oils. At the dosage used the oils from *Azadirachta, Pongamia,* and *Cymbopogon* were phytotoxic to cabbage plants.

Number of Applications of Crudes and Disease Control

The lowest possible dose (3%) was tried in the next stage of trials by repeated applications. Results (Table 5) indicated that application at lower concentration of the crudes required no less than 2 or 3 applications at 5-day intervals for successful disease control.

Table 5. Control of black rot of cabbage and soft rot of potato with plant crudes in different number of applications (Average of 6 replicates).

Plant crudes	Percent disease control by number of applications					
	Black rot			Soft rot		
	1	2	3	1	2	3
Oils:						
Cymbopogon	—[a]	—	—	30	50	80
Taaraktogenos	10	80	95	—	—	—
Madhuca	—	—	—	30	70	90
Elacis	0	60	100	—	—	—
Streptocycline	30	80	100	20	70	80
Control (inoculated)	0	0	0	0	0	0

[a]— = not tried.

Further Trials on Control of 4 Bacterial Diseases with Plant Crudes

These 4 trials also were conducted in a larger scale in fields or godown-floors. Trials on black rot and citrus canker with 7 and 5 treatments, respectively, were conducted at the University farm on UP-sweet orange varieties, replicated four times. The trials on soft rot diseases were conducted with kufri chaandramukhi (potato) and local (zinger) varieties in yucca stores under natural conditions of storage in gunny bags. Results as presented in Table 6 showed that plant oils successfully checked soft rot of both zinger and potato though not as well as the antibiotic mixture. Mixtures of oils with "antibiotic M" at half dosages were also successful in cases of *Cymbopogon, Taaraktogenos,* and *Elacis.* Mixture of *Cymbopogon* and *Madhuca* also could check zinger soft rot. Spraying of oils of *Taaraktogenos, Elacis* and a mixture of the two successfully checked both black rot and citrus canker.

Table 6. Further studies on bacterial disease control with plant crudes. (Averag of 4 replicates).

Plant crudes etc.	Percent control of diseases			
	Black rot	Soft rot of potato	Citrus canker	Zinger soft rot
Cymbopogon spray	—[a]	40	—	60
Taaraktogenos spray	90	—	31	—
Madhuca spray	—	40	—	85
Elacis spray	60	—	54	—
Cymbopogon dust	—	30	—	—
Madhuca dust	—	20	—	—
Streptocycline spray	70	100	57	100
Streptocycline dust	—	100	—	—
Cymbopogon + Madhuca	—	30	—	80
Taaraktogenos + Elacis	70	—	29	—
Cynab + Streptocycline	—	100	—	100
Taaraktogenos + Streptocycline	70	—	40	—
Madhuca + Streptocycline	—	100	—	100
Elacis + Streptocycline	65	—	58	—
Untreated control	0	0	0	0

[a]— = not tried.

Discussion

Two plant pathogenic bacteria seemed sensitive to many oils and extracts from plants belonging to the families Graminae, Flacourtiaceae, Leguminosae, Sapindacae, Maliaceae, Palmae, Piperaceae, Casuarinaceae, Umbelliferae, Sapotaceae, etc. at higher concentrations. Besides identification of these plant bacteriostats, the diversity of sources of such inhibitors opened up wide possibilities. Antibacterial activities of the essential oils from *Cymbopogon nardus* (17) and extracts from *Casuarina, Allium, Ficus,* and *Areca* against *X. oryzae* (13) were reported earlier. They have been confirmed and in addition tried successfully for control of bacterial diseases. In fact, good sources of plant crudes, that can be tried for disease control have been located.

The preliminary exploration of possibilities of using plant oils have met with encouraging success. Plant species *Taaraktogenos, Elacis, Sapindus casuarina,* and *Coriandrum* for black rot of crucifers or canker of citrus and *Cymbopogon, Taaraktogenos, madhuca, Piper, Casuarina* and *Coriandrum* for soft rot of zinger or potato have been well identified. They need

extensive trials now because being biogenic and biodegradable, they have an edge over commercial bactericides or antibiotics. The crudes that have been tried, are rurally produced and may be cheap. The requirement of higher concentrations of such crudes seemed to be a problem. Relatively poorer performance of such crudes compared to standard antibiotics seemed to be another problem. However, the merits overcome the shortcoming in the particular contexts of large farm areas in countries like ours, with small farm units, poor economic conditions of the farmers, low value of the crops concerned, and lack of sufficient standard antibiotics. Plant crudes thus stand a chance for trial in control of bacterial diseases of plants.

Literature Cited

1. Ark, P., and J. P. Thompson. 1968. Antibiotic properties of the seeds of wheat and Barley. Plant Dis. Rep. 42 (3) 959-962.
2. Bhatt, S. K., and V. K. Saxena. 1979. Efficacy of successive extracts of seeds of *Derris robusta* against some human and plant pathogenic bacteria, Indian Drugs; 17 (2):42.
3. Bhatt, S. K., and V. K. Saxena. 1980. Screening of antibacterial activity of various extractives of seeds of *Albizzia amara* and *Ipomea purpuree.* Indian Drugs, 17 (8)239-241.
4. Choudhuri, A., N. Mukherjee, and N. Aditya Choudhury. 1974. Sensitivity of some plant pathogenic fungi toward plant metabolites; Antifungal activity of some chalcones, dihydrochelcones and flavonones. Experientia, 30:1022.
5. Chopra, I. C., B. N. Khajuria, and L. C. Chopra. 1957. Antibacterial properties of volatile principles from *Alpinia galanga* and *Acorus calemus,* Antibiotics and Chemotherapy, 7:378.
6. Dakshinamurty, V., and D. K. Rao, 1959. Preliminary studies on the control of citrus canker on acid lime. Andhraagric J. 6 (3):145-148.
7. Dorobotko, V. G. (Ed.). 1960. Phytoncids in medicine, agriculture, and food industry. Kiev. Pub. of Unrainian Acad. Sci; pp. 200.
8. Dorozkhin, N. A., A. I. Kustova, and V. I. Nitievskaya. 1973. The effect of Trichodermin antibiotic and phytoncides on infection of cabbage by diseases. Refer. Zhur., 12:1302.
9. Harris, R. I. 1978. A method for testing potential bactericide for the protection of soft rotting potato tubers. Potato Res. 21(3):231-233.
10. Hayes, L. E. 1946. Survey of higher plants for presence of antibacterial substances. Bot. Gaz, 108 408.
11. Malekzada, F., and T. P. Pirone. 1962. An antibacterial substance from cauliflower seeds. Phytopathology: 52(8):721-758 (Abstr.)
12. Melkumyan, I. S. 1966. Antimicrobial activity of some plants in the Armenian flora in relation to phytopathogenic bacteria. Bot. Ll. Armen, 19(7):64-68.
13. Mondal, R. K. 1976. Sensitivity of plant pathogenic bacteria towards different chemicals. Ph.D. thesis, Bidhan Chandra Krishi Viswavidyalaya, West Bengal.
14. Mondal, R. K., and N. Mukherjee. 1978. Sensitivity of three plant pathogenic bacteria to antibiotics and drugs. Z. fur. Pfl. und Pfltz. 85(10):607-616.
15. Mukherjee, N., P. K. Gupta, and N. Adity Choudhuri. Studies of antifungal activity of some pterocarpans and conmestans. Sc. and Cult. 40(5):176-201.

16. Ozek, I. 1948. Effect of garlic on bacteria. Istarel Seriyati. 27:156-161.

17. Pathak, R. K., S. C. Chourasis, and K. V. Singh. 1979. Antibacterial activity of essential oils of two medicinal plants. Indian Drugs Pharm. Indi. 14(5):7-8.

18. Prodesimo, A. N., and L. L. Ilag. 1976. Toxicity of garlic juice to plant pathogenic organisms. Kolikasam. 5(7): 251-258.

19. Rao, K. R., and M. J. Thirumalachar. 1960. Control of black rot of cabbage with citrinin. Hindusthan Antibiotics. Bull. 2:126-127.

20. Reddy, G. S., and Papa Rao. 1960. Control of canker in citrus nurseries. Andhra agric. J. 7(5)175-188.

21. Rudolph, K., and O. Cinar. 1971. Bacteriostatic compounds from bean leaves; Acta Phytopathologic Acad. Sci., Hungary. 6(1-4):105-113.

22. Sampurna, T., and S. S. Nigam. 1979. Antibacterial activity of some Indian essential oils. Indian Perfume. 23 (3 & 4): 205-207.

23. Shrivastava, R., A. Omray, and K. C. Verma. 1980. Microbial growth inhibition by essential oil of *Cyathocline lyrata* Cass. Indian Perfume. 24:219-221.

24. Stall, R. E., and A. A. Cook. 1968. Inhibition of *Xanthomonas vesicatoria* in extracts from hypersensitive and susceptible pepper leaves. Phytopathology 58 (12):1584-1587.

25. Tokin, B. P. 1957. Phytoncides, their roles in nature. Leningrad Univ. Cited in Gorlenko, M. V. 1965. Bacterial diseases of Plants; Oldborne press; London.

26. Uppalapati, L., and R. J. Thirumala. 1979. Chemical and antimicrobial studies of the extracts of *Dillenia indica* L. Chem. Petro-Chem. J. 10(8):21-23.

27. Vaheeuddin, S., C.B. Rai, G.S. Reddy, and A. P. Rao. 1957. Control of canker in citrus nurseries. Proc.Seminar on Dis.of Hort. Plants, Simla. June. pp. 94-109.

Efficiency and Phytotoxicity of Some Bactericides Against *Pseudomonas* spp. and *Erwinia* spp.

I. Palazon, J. Meynard, M. Herrero, M. P. Martinez

Centro de Investigación y Desarrollo Agrario del Ebro
Zaragoza, Spain

Introduction

The major problems caused by aerial bacteria on pome fruits in Spain lie in the presence of *Pseudomonas syringae* and geographical proximity of *Erwinia amylovora.*

Efficiency of streptomycin, mainly against *E. amylovora,* is well known but its use, as the use of other antibiotics, has legal restrictions in Europe. Since little information is available on the application of other bactericides in agricultural practices (Van der Zwet and Keil, 1979), active ingredients that have less efficiency such as copper derivates and some fungicides, are used.

In our weather conditions, March and April are the months of highest risk of infection by both bacteria. Later in the year, dry weather and high temperatures should prevent their development. Trees need treatment at blossom time and this work investigates possible adverse effects on the flowers and young leaves. The toxicity of several fungicides to pollen has been widely examined (3). Information is lacking, however, on the toxicity of bactericides to the flower, except for the work of McDaniels *et al.* (8) and Ries (11), using apple pollen.

This paper deals with the efficiency of a range of bactericides against *Pseudomonas* spp. and *Erwinia* spp. Toxicity of these products to leaves and flowers of pear trees also is studied.

Materials and Methods

Efficiency of the Products

Most microorganisms used were isolated from *Pyrus communis* (pear tree), except for *Erwinia chrysanthemi* which proceeds from *Zea mays* (maize). They came from France (J. A. Paulin, INRA), except *Pseudomonas syringae* H 012 P, which came from Huesca, Spain. According to Paulin (1980), *E. amylovora* has a sensibility to bactericides comparable with *E. chrysanthemi.* Thus a rather accurate idea of efficiency of these products for eventual use against 'Fire Blight' may be obtained from this work.

These bacteria were tested with bactericides at constant concentrations according to the type of product. Thus, in the efficiency test, all the copper-

based products (copper sulphate, Bordeaux mixture simple or with oil, copper oxide, oxichloride and oxiquinoleate, and Tri-Miltox[R]) were used at concentrations of 5 g, 2 g, 0.5 g, 0.05 g and 0.005 g of copper metal per liter of medium; the antibiotics (streptomycin, kasugamycin and polyoxin) at concentrations of 64, 32, 16, 8, 4, 2, 1 and 0.5 ppm; disinfection products (sodium hypochloride, Quatex[R] and Catigene[R]), 2, 0.8, 0.2, 0.02 and 0.002 percent a.i., and the other products at 2, 1, 0.5, 0.01 and 0.005 g a.i. per liter of medium.

These products were aseptically incorporated into an LPGA medium, then placed in Petri dishes. They were inoculated with a Stirs replicator system with 0.01 ml of 10^8 and 10^6 cells/ml of bacterial suspension according to MacFarlands levels. Two repetitions were made for each concentration of product and bacterium. After incubation during 48 to 72 h, according to the type of product, bacterial growth was observed in inoculated spots.

Phytotoxicity on Leaves

A phytotoxicity test was conducted at concentrations different from the ones listed later, which had been used in an attempt fo find out the consequences of excess concentrations of the products on the leaves. Thus, for example, the concentrations tested for the copper products were 1 g, 2 g, and 5 g of copper metal per liter (except for Tri-Miltox[R], 0.48, 1.44, and 2.4 g/l, as it contains another active agent). In general, the amounts used were the recommended concentration plus two higher values equivalent to that concentration times 2 to 5.

The plant material tested consisted of pear tree branches of different cultivars, Passe Crassane, Williams, and Blanquilla. These branches were sprayed on April 9 and May 7.

The observations were made 24 h, 48 h and 7 days after each treatment and were based on discolorations and burns on leaves.

Phytotoxicity to Flowers

To evaluate phytotoxicity to the pistil, flowers from cultivars Passe Crassane, Williams and Beurré Hardy, were picked one day before anthesis. The flowers were emasculated and placed on moist florists' foam (Savanna, Steekterweg 92, Alphen a.d. Rijn Holland). Twenty-four hours later, five flowers of each cultivar were sprayed to run off with one of the range of bactericides listed in Table 6. Two hours after spraying (3) stigmas were pollinated with a pollen mixture from untreated cultivars. The pollen tube was allowed to grow for 4 days at room temperature (20 - 23°C) and subsequently pistils and receptacles were preserved in 5% sodium sulphite, softened by autoclaving, stained in 0.1% aniline blue in 0.1 N $PO_4 K_3$ and examined by fluorescent microscopy (6).

To estimate phytotoxicity to pollen in the anther, flowers with undehisced anthers were depetalled, placed in moist florists' foam and sprayed as before: 30 flowers per cultivar and per treatment were used. Twenty hours later the anthers were collected in Petri dishes and left to complete dehiscence for 24 hours. The Petri dishes were then sealed and deep freeze stored (-20°C). Pollen was sown on a medium containing 15% sucrose, 1% agar, and 10 ppm boric acid.

Results

Efficiency of the Products

The efficiency of the products was measured by the lowest amount of a. i. (C.M.I.) which, when incorporated to a medium, prevented bacterial growth. For security purposes this test was repeated at least twice and every concentration of product and bacterium was placed on two dishes each time.

Antibiotics. Kasugamycin and polyoxin gave disappointing results, for they were not efficient before the concentration of 64 ng of a.i./ml was applied, which excludes their use for these bacteria. On the other hand, streptomycin seemed to act against tested germs; it killed at concentrations close to 4 to 8 ng/ml. That explains its widespread use as farming bactericide against *Erwinia amylovora* for example.

Copper Products. As shown in Table 2, copper products present a great homogeneity in their efficiency against bacteria. Most of them were active at a concentration ranging between 0.5 and 2 g of copper metal per liter, which represents an average efficiency. On the other hand, Copper oxyquinolate seemed completely inefficient even at the highest concentration used (5 g of copper metal per liter) as it did not prevent the growth of the colonies. Two other products seemed to be more efficient: copper sulphate, which was active on all germs between 0.05 and 0.25 g of copper metal/l and especially Tri-Miltox[R] which killed bacteria between 0.005 and 0.05 (except for *E. chrysanthemi* from 0.05 to 0.25). It must be noticed, however, that Tri-Miltox[R] not only contains copper but also mancozeb.

Disinfection Products and Others. Among these products, three appear to give interesting results. These are experimental products not commercialized yet. CGA 78039, of Ciba-Geigy, responded positively at all tested concentrations. Coll 0411-1/80, of the firm José Collado S.A., was efficient at 1 g/hl. As for MCST 7938, of Merck, Sharp, and Dohme, it was usually efficient at 5 g/hl, that is, its action is still interesting. Unfortunately, it is difficult to dissolve in water, even at small concentrations. To solve this problem dilutions were made in a small quantity of alcohol, which improved solubility.

Phytotoxicity on Leaves

The phytotoxicity on leaves of some products is closely related to climatic data. Thus, the following results must be interpreted in connection with temperature and rain data of April and May in Zaragoza, Spain (see Table 3).

As for the copper products, the sprayed leaves were very sensitive to weather influences. Thus, after the first spray, temperature was high and the rainfall very low, and there was no phytotoxicity on copper sprayed leaves. But the three days following the second spray were rainy and cold, so burns appeared. For copper compounds, the worst burns occurred with copper sulphate. However, Bordeaux mixture, either prepared in commercial firms or in the field, seemed to cause little or no toxicity, like in the case of oxyquinolate. On the cultivars Passe Crassane and Williams, Tri-Miltox[R] can be used but Blanquilla had a very negative response to being sprayed with this product.

Table 1. Efficiency *in vitro* of three antibiotics against some bacterial species.   x : lower dose in ng/ml of a.i. killing 10^8 bacteria/ml.

	1147 *P. syringae*	4012 P *P. syringae*	1592 *P. viridiflava*	1500 *E. chrysanthemi*	B 983 *E. herbicola*
Streptomycin	$4 < x < 8$	$4 < x < 8$	$2 < x < 4$	$4 < x < 8$	$2 < x < 4$
Kasugamycin	$32 < x < 64$	$32 < x < 64$	$32 < x < 64$	$16 < x < 32$	$16 < x < 32$
Polyoxin	$64 < x$	$64 < x$	$64 < x$	$64 < x$	$64 < x$

Table 2. Efficiency in vitro of copper compounds against some bacterial species. x: lower dose of Cu metal (g/l) Killing 10^8 bacteria/ml.

	1147 *Ps. syringae*	H 012 P *Ps. syringae*	1592 *Ps. viridiflava*	1500 *E. chrysanthemi*	B 983 *E. herbicola*
copper sulfate	$0,05 < x < 0,25$	$0,05 < x < 0,25$	$0,05 < x < 0,25$	$0,05 < x < 0,25$	$0,05 < x < 0,25$
bordeaux mixture	$0,5 < x < 2$	$0,5 < x < 2$	$0,5 < x < 2$	$0,25 < x < 0,5$	$0,25 < x < 0,5$
bordeaux mixture "in situ"	$0,5 < x < 2$	$0,5 < x < 2$	$0,5 < x < 2$	$0,5 < x < 2$	$0,25 < x < 0,5$
bordeaux mixture + oil	$0,5 < x < 2$	$0,5 < x < 2$	$0,5 < x < 2$	$0,5 < x < 2$	$0,25 < x < 0,5$
copper oxychloride	$0,5 < x < 2$	$0,5 < x < 2$	$0,5 < x < 2$	$0,25 < x < 0,5$	$0,25 < x < 0,5$
copper oxide	$0,5 < x < 2$	$0,5 < x < 2$	$0,5 < x < 2$	$0,25 < x < 0,5$	$0,5 < x < 2$
copper oxiquinoleate	$5 < x$	$5 < x$	$5 < x$	$5 < x$	$5 < x$
Trimiltox[R]	$0,005 < x < 0,05$	$0,005 < x < 0,05$	$0,005 < x < 0,05$	$0,05 < x < 0,25$	$0,005 < x < 0,05$

Table 3. Efficiency in vitro of some bactericides against some bacterial species. x: lower dose in g a.i./l Killing 10^8 bacteria/ml.

	1147 *Ps. syringae*	4012 P *Ps. syringae*	1592 *Ps. viridiflava*	1500 *E. chrysanthemi*	B 983 *E. herbicola*
Terlai[R]	$10 < x < 20$	$10 < x < 20$	$10 < x < 20$	$10 < x < 20$	$10 < x < 20$
CGA 78039	$x < 1$	$x < 1$	$x < 1$	$x < 1$	$x < 1$
Sodium hypochloride*	$1 < x < 2$	$1 < x < 2$	$1 < x < 2$	$1 < x < 2$	$1 < x < 2$
Quatex[R]*	$0,02 < x < 0,1$	$0,02 < x < 0,1$	$0,2 < x < 0,8$	$0,2 < x < 0,8$	$0,2 < x < 0,8$
Catigene[R]*	$0,2 < x < 0,8$	$0,2 < x < 0,8$	$0,2 < x < 0,8$	$0,2 < x < 0,8$	$0,2 < x < 0,8$
MSCT 7938	$5 < x < 10$	$1 < x < 5$	$5 < x < 10$	$1 < x < 5$	$1 < x < 5$
Coll 0411-1/80	1	1	1	1	1
Mancozeb	$10 < x < 20$	$10 < x < 20$	$20 < x < 50$	$10 < x < 20$	$20 < x < 50$

* in o/o.

Table 4. Threshold of phytotoxicity of some bacteria on leaves of Pear.

	Cultivar		
	Passe Crassane	Williams	Blanquilla
Streptomycin	—	—	—
Kasugamycin	—	—	—
Polyoxin	—	—	—
Terlai (R)	—	—	—
CGA 78039	—	—	—
Sodium hypochloride	$>0.5\%$	$>0.5\%$	$>0.5\%$
Quatex (R)	*	*	*
Catigene (R) 50	*	*	*
Catigene (R) 70	*	*	*
Catigene (R) 70-S	*	*	*
MSCT 7938	—	—	—
Coll 0411-1/80	—	—	—
Tri-Miltox (R)	—	—	*
Bordeaux mixture	—	—	>200 g.Cu/Hl
Bordeaux mixture "in situ"	—	—	—
Bordeaux mixture + oil	—	>200 g.Cu/Hl	*
Copper sulphate	*	*	*
Copper oxychloride	—	>200 g.Cu/Hl	>200 g.Cu/Hl
Copper oxide	—	>100 g.Cu/Hl	>200 g.Cu/Hl
Copper oxyquinoleate	—	—	>100 g.Cu/Hl

* Strong phytotoxicity at all doses.

The antibiotics did not result in damages on pear leaves. Leaves sprayed with Catigene[R] and Quatex[R] presented a strong phytotoxicity at all concentrations, as did the sodium hypocloride. The remaining products did not cause any damage.

Phytotoxicity on Flowers

The 'in vitro' germination of pollen from sprayed undehisced anthers is recorded in Table 6. Passe Crassane has been the most affected cultivar; all copper treatments but Tri-Miltox[R] reduced germination of its undehisced pollen. On the other hand, copper oxyquinolate and Catigene[R] reduced germination in all cultivars and no significant effect was observed after treatment with Tri-Miltox[R], streptomycin, kasugamycin, CGA 78039, and Quatex[R].

Table 5. Climatic data[a] in Zaragoza, Spain, in April and May 1981.

| | APRIL | | | | MAY | | |
| | Temperature | | Rain | | Temperature | | Rain |
Day	Max.	Min.	mm	Day	Max.	Min.	mm
1	23.6	6.0		1	20.4	8.8	
2	16.4	5.2	1.1	2	19.0	9.6	
3	15.2	8.4	3.6	3	13.8	9.0	
4	21.4	5.6		4	17.0	5.2	
5	24.2	6.0		5	21.4	9.8	
6	25.2	9.0	3.0	6	27.4	6.8	
7	22.0	8.6		7	22.0	10.6	1.5
8	22.8	8.2		8	22.6	8.0	8.0
9	24.6	13.0		9	15.8	12.0	9.7
10	19.0	10.2	1.9	10	17.2	5.6	10.0
11	24.6	8.0		11	17.6	7.0	
12	25.0	8.8		12	21.2	6.6	
13	21.6	12.2	4.5	13	20.6	8.8	
14	20.6	11.6		14	20.8	9.0	
15	22.0	6.0		15	23.0	7.4	
16	21.0	10.6	6.8	16	23.2	13.2	
17	20.0	11.4		17	22.8	7.6	
18	19.0	7.0		18	29.4	7.8	
19	20.6	2.8		19	30.0	11.2	
20	14.8	5.0	0.4	20	24.0	13.2	0.9
21	12.8	3.4	20.7	21	24.6	11.4	
22	10.6	7.0	49.3	22	25.0	8.6	
23	17.4	8.6		23	29.6	9.0	
24	15.6	7.4		24	28.6	13.6	
25	20.4	3.0	1.6	25	18.8	14.8	4.0
26	13.0	4.0		26	19.6	9.4	1.4
27	15.6	2.0	1.1	27	21.4	9.6	
28	19.6	6.2		28	27.0	9.4	
29	21.0	4.0		29	24.0	11.8	
30	19.2	5.0		30	30.8	8.6	
				31	30.0	15.2	0.2

Bactericide	Cultivar		
	Passe Crassane	Williams	Beurré Hardy
Bordeaux mixture (0.5 g Cu/l)	24**	73	76
Bordeaux mixture 'in situ' (id.)	31*	77	64
Copper oxychloride (id.)	33*	65	56
Copper oxyde (id.)	24**	72	61
Bordeaux mixture + oil (id.)	16***	76	75
Copper oxyquinolate (id.)[a]	34*	36**	55
Tri-Miltox (R) (0.05 g Cu/l)	61	74	84
Streptomycin (200 ppm)	70	73	77
Kasugamycin (40 ppm)	50	57	73
Polyoxin (20 ppm)	60	49*	90
Coll 0411-1/80 (10 g/hl)	56	50*	75
CGA 78039 (10 g/hl)	61	68	68
Quatex (R) (0.2%)	59	57	66
Catigene (R) (0.2%)	40	54	42**
Unsprayed control	61	75	73

*, **, *** significantly different from pooled for cultivar 5%, 1% and 0.1% levels respectively.

[a] Average of all cultivars significantly lower (at 5% level) than control average.

Changes in anther appearances could be noticed 20 hours after spraying, when dehiscense had already begun. Some anthers looked brown, especially after treatment with polyoxin and Quatex[R], others appeared wet and translucent, mainly after treatment with Catigene[R], and some showed a gray coloration when treated with Catigene[R], Coll 0411-1/80 and Bordeaux mixture.

Burning of petals occurred on treated flowers. This happened in all cultivars but especially in Beurre Hardy. Although this was a general response after using these bactericides, symptoms were more severe after treatment with cupric oxide and especially with Quatex[R] and Catigene[R], when necrotic lesions in pistils could also be detected.

A reduction of pollen germination on sprayed stigmas could be observed after treatment with some bactericides. Stigmas with less than the minimum number of pollen tubes usually found on untreated stigmas were considered to have been adversely affected by the bactericide (4). In this experiment the number of pollen tubes in hand pollinated stigmas varied between 50-150 and results are given as percentage of stigmas with more than 50 pollen tubes (Table 7). In situ Bordeaux mixture, copper oxide,

Table 7. Percentage of hand pollinated stigmas with more than 50 pollen tubes.

Bactericide	Cultivar		
	Passe Crassane	Williams	Beurré Hardy
Bordeaux mixture (0.5 gCu/l)	40**	80	62
Bordeaux mixture 'in situ' (id.)[b]	33**	46**	25**
Copper oxychloride (id.)	83	37**	88
Copper oxyde (id.)[a]	57*	33**	46*
Bordeaux mixture – oil (id.)[b]	46*	32**	37**
Copper oxyquinolate (id.)	85	24**	83*
Tri-Miltox (0.05 gCu/l)[b]	83	0***	21**
Streptomycin (0.2 ppm)	39**	58*	92
Kasugamycin (40 ppm)	55*	72	83
Polyoxin (20 ppm)[a]	33**	43**	67
Coll 0411-1/80 (10 g/hl)	67	50**	76
CGA 78039 (10 g/hl)	96	72	100
Quatex (0.2%)	52*	68	90
Catigene (0.2%)[b]	24**	44**	21**
Water control	92	96	91

*, **, *** significantly different from pooled control for cultivar, at 5%, 1% and 0.1% levels respectively.

[a, b] Average of all cultivars significantly lower, at 5% and 1% levels, than control average.

Bordeaux mixture plus oil, and Catigene ᴿ reduced pollen germination in all cultivars; however, no significant effect could be detected after spraying with CGA 78039.

In all control pistils examined an average of 3 to 6 pollen tubes reached the base of the style. However, after treatment with some bactericides, a decrease in the number of pistils containing pollen tubes (Table 8) and in the mean number of tubes per pistil (Table 9) could be observed. Passe Crassane seemed the most sensitive cultivar and Quatex ᴿ and Catigene ᴿ most deletereous treatments, impairing the arrival of pollen tubes to the ovary in all pistils. This was probably due to necrotic lesions produced in the pistils, since pollen tubes were stopped upon arrival at a necrotic area.

Discussion

As the efficiency tests have been done *in vitro* the results must be modulated for field use. It is likely that the bacterial resistance is more

Table 8. Percentage of hand pollinated pistils with pollen tubes reaching the base of the style.

Bactericide	Cultivar		
	Passe Crassane	Williams	Beurré Hardy
Bordeaux mixture (0.5 gCu/l)	100	96	100
Bordeaux mixture 'in situ' (id.)[b]	66**	96	32**
Copper oxychloride (id.)	58**	91	100
Copper oxyde (id.)	77*	91	100
Bordeaux mixture + oil (id.)	20**	100	96
Copper oxyquinolate (id.)	91	84	72*
Tri-Miltox (R) (0.05 gCu/l)	65**	79*	96
Streptomycin (200 ppm)	71*	83	100
Kasugamycin (40 ppm)	86	72*	100
Polyoxin (20 ppm)	92	76*	92
Coll 0411-1/80 (10 g/hl)	60**	76*	100
CGA 78039 (10 g/hl)[a]	50**	88	96
Quatex (R) (0.2%)[c]	0	0	0
Catigene (R) (0.2%)[c]	0	0	0
water control	100	100	100

*, ** Significantly different from pooled control for cultivar at 5% and 1% levels respectively.

[a,b] Average of all cultivars significantly lower, at 5% and 1% levels, than control average.

[c] Not included in statistical analysis.

important and efficiency of products less important in the field. So the concentrations to use would be a little higher to reduce this eventuality and to palliate spray irregularity.

Only six products have shown a good efficiency against bacteria tested: streptomycin, copper sulphate, Tri-Miltox[R], Coll 0411-1/80, MSCT 7938, and CGA 78039, the latter in spite of our references of little action against *Pseudomonas syringae* (5).

Unfortunately the phytotoxicity is important for some of these products. Streptomycin, CGA 78039, Coll 0411-1/80, and MSCT 7938 are not phytotoxic on leaves but copper sulphate on all cultivars tested and Tri-Miltox[R] on Blanquilla have shown strong phytotoxicity, the last probably because of presence of mancozeb in its composition. Neutralization with lime of copper sulphate in the Bordeaux mixture reduces greatly its phytotoxicity on leaves but decreases its bactericide activity. We must remember the increasing of 'russeting' if copper products are used in blossom after 'stade B-C'' (1).

Table 9. Mean number of pollen tubes per pistil reaching the base of the style after hand pollination.

Bactericide	Cultivar		
	Passe Crassane	Williams	Beurré Hardy
Bordeaux mixture (0.5gCu/l)	1.7	2.4	3.9
Bordeaux mixture 'in situ' (id.)	1.2	1.9	0.9
copper oxychloride (id.)	0.8	1.2	3.9
copper oxyde (id.)	1.2	3.1	5.2
Bordeaux mixture + oil (id.)	0.2	3.6	5.1
copper oxyquinolate (id.)	1.9	1.9	2.4
Tri-Miltox[R] (0.05gCu/l)	1.1	1.8	6.2
streptomycin (0.2 ppm)	1.2	6.1	4.3
kasugamycin (40 ppm)	2.3	1.6	6.3
polyoxin (20 ppm)	2.6	2.7	6.1
Coll 0411-1/80 (10g/hl)	1.4	3.1	4.2
CGA 78039 (10g/hl)	0.5	3.4	10.4
Quatex[R] (0.2o/o)	0	0	0
Catigene[R] (0.2o/o)	0	0	0
water control	3.5	3.9	5.4

The worst products are sodium hypochloride, Quatex[R], and Catigene[R], which have shown poor efficiency and strong phytotoxicity on leaves and flowers.

Generally, the incidence of all products on pollination is negative. Passe Crassane has proven the most susceptible cultivar to bactericides used in the blossom period. This fact associated with the great susceptibility of this cultivar to fire blight (12) should be taken into consideration.

The number of pistils with pollen tubes in the ovary was reduced to 29, 35, 40, and 50 percent for streptomycin, Tri-Miltox[R] Coll 0411-1/80, and CGA 78039, respectively. MSCT 7938 has not been tested in this way. However, it is necessary to work further on the practical influence of this negative effect.

The present survey shows that development of necrotic lesions in the pistil, caused by Quatex[R] and Catigene[R], is the most important factor preventing fertilization. Church and Williams (3) pointed out that although pollen germination on the stigma gave a good guide on the crop limiting potential of a fungicide, a more comprehensive investigation was needed since, besides germination, pollen tube growth and fertilization could be affected too. This proves to be the case when treating with Quatex[R]. Under this treatment, while pollen germination on the stigma is little affected, pollen tube growth is stopped due to necrotic lesions in the style.

On the other hand, a poor germination at the stigma does not necessarily mean a reduction in the number of pollen grains reaching the ovary, as is the case in Williams and Beurré Hardy after treatment with copper oxyde, Bordeaux mixture + oil, and Tri-Miltox[R]. This is in agreement with the lack of correlation found by some authors (10) between low pollen germination and low fruit set when using some fungicides.

These results should be interpreted in terms of potential damage since more product is deposited on the flowers than in the field (3) and in the orchard more flowers and pollen are available (11). However, an indication may be drawn on which products are more harmful to the fertilization process as well as on the varietal susceptibility to the different products.

Literature Cited

1. Bordoux, P. 1967. Stades d'induction de la rugosité chez la varieté Golden D. aprés pulverization de fongicides a base de cuivre et de souffre. Phyt. Phytopharm, 16. p. 12
2. Bristow, P. R. 1981. Effect of triforine on pollen germination and fruit set in highbush blueberry. Plant Dis. 65:350-353.
3. Church, R. M. and R. R. William. 1977. The toxicity to apple pollen of several fungicides as demonstrated by *in vivo* and *in vitro* techniques. J. Hort. Sci. 52 (3):429-436.
4. Church, R. M. and R. R. William. 1978. Fungicide toxicity to apple pollen in the anther. J. Hort. Sci., 53 (2):91-94.
5. Egli, T. and M. Zeller 1980. CGA 78039: a new bactericide.   Meeting on Fireblight. KIEL (RFA).
6. Linskens, H. F. and K. L. Esser. 1957. Uber eine spezifische Anfarbung der Pollenschlauche une die Zahl kallosepropfen nach selbstung und fremdung. Naturwiss., 44, p. 16.
7. Marcucci, M. C. and D. Iragazzini. 1980. Influenza della Dodina sulla germinabilita del polline e sulla fertilitá del pero inrapporto all'epoca di trattamento fiorale. Riv. Ortoflorifrutt. It., 64:475-487.
8. McDaniels, L. H., E. M. Hildebrandt and H. F. Burrel. 1933. The effect of various bactericides on the set of fruit and the germination of the pollen of the apple. Proc. Am. Soc. Hort. Sci., p. 30.
9. Paulin, J. P. 1981. Personal communication.
10. Ramina, A. 1974. Effeti di alcuni fungicidi ad azione sistemica sulle fecondazione e sulla allegagione del melo. Riv. Ortoflorifrutt. It., 58 (5):342-349.
11. Ries, S. M. 1978. Germination of apple pollen as influenced by fungicides. Fruit Var. Jour., 32 (1):12-16.
12. Van der Zwet, T., and H. L. Keil. 1979. Fire Blight. A bacterial disease of Rosaceous plants. Agric. Handbook 510, U.S.D.A., 200 pp.

Relation of Cardamom Capsule Disease *Bacillus megatherium* to Time of Crop Maturity in Southern Sri Lanka

R. H. S. Rajapakse and V. L. Kulasekera

Ruhuna University
Matara, Sri Lanka

Abstract

Data on the time of harvest and the incidence of capsule rot in a trial at Sri Lanka suggested that the time of crop maturity is an important determinant of capsule disease losses. Late maturing selections, in which most of the crop was harvested after mid October, showed a lower incidence of capsule rot *Bacillus megatherium* than those in which more of the harvesting took place during the period June to mid October.

Introduction

Cardamom (*Elettaria cardamomum* L. Maton. Fam. Zingiberaceae) is a spice that is grown in Sri Lanka which has a potential for expansion and intensification in the minor export crops diversification program (De Silva, 1978). Diseases such as capsule rot have led to a loss of about the whole crop in certain cardamom plantations (1).

The primary symptoms of the disease appear as watersoaked spots on affected capsules. These spots enlarge rapidly imparting a dull greenish brown color to the infected capsules. In the later stages, the diseased capsules decay, emitting a foul smell, and fall off. Under field conditions capsules are infected at all stages of maturity.

The present paper emphasizes the incidence of the disease in relation to crop maturity. This experiment was conducted at the Illumbakanda Cardamom Plantation, Deniyaya, and at Department of Agronomy, Faculty of Agriculture, Ruhuna University, Mapalana, Kamburupitiya, Sri Lanka during 1980-81.

Materials and Methods

Capsule rot losses are high during two periods in the southern region of Sri Lanka. The first is in June and July and the second, in late September and early October. The exact period of capsule rot epidemic depends on the

distribution of the main periods of rainfall and this varies from year to year.

In the present study the period of high capsule rot incidence has been taken as July 1 to October 15. In the field trials, losses were classified into two categories, diseased capsules and pest-damaged capsules. Most of the diseased capsules were affected by capsule rot and the pest damage was of minor significance.

The first trial at Deniyaya consisted of 20 selections of the cultivar Malabar of cardamom, planted in different states in the district. These selections differ in vegetative habit, in capsule size and number, and in the distribution of capsules. All these characters influence the level of capsule rot losses.

Three areas of mature cardamom were studied. In one area, the Malabar cultivar had been planted in 1972 from seeds collected from Matale

Table 1. Pattern of cropping and incidence of pod disease at Deniyaya 1980 — 1981 July.

Selection	Overall o/o diseased	April-June	June-Mid October		Mid October-February	
		o/o pods	o/o pods	o/o diseased	o/o pods	o/o diseased
1	117	6.2	13.0	25.5	80.8	8.9
2	13.9	6.5	22.3	24.0	71.2	10.4
3	15.6	6.4	19.9	32.2	73.7	11.6
4	15.7	4.1	9.2	44.9	86.7	12.3
5	21.3	9.9	19.7	39.6	70.4	16.9
6	21.6	9.2	27.6	35.4	63.2	15.9
7	21.6	11.8	23.6	40.7	64.6	15.3
8	22.9	8.6	18.7	46.5	72.7	17.2
9	23.1	11.2	18.2	40.1	70.6	19.0
10	23.5	9.8	21.4	43.7	68.8	17.9
11	24.4	7.9	22.9	43.2	69.2	19.2
12	25.0	11.5	22.8	43.3	65.7	20.1
13	25.0	11.4	18.6	51.0	70.0	18.4
14	25.0	3.9	20.8	50.5	75.3	17.8
15	25.8	9.0	13.2	54.1	77.8	19.8
16	27.0	10.9	19.3	62.5	69.8	17.2
17	27.7	15.1	20.7	69.5	64.2	16.8
18	27.9	15.8	21.9	49.2	62.3	20.5
19	30.1	10.2	19.8	64.8	70.0	21.4
20	30.6	8.9	21.1	65.7	70.0	20.6

Research Station. The other areas were planted in 1976 and 1975 and included the Malabar and Mysore cultivars and their selections.

Results

In this experiment, disease losses ranged from 24.0% to 69.5% in the July and mid October period and from 8.9% to 21.4% in the mid October to February period (Table 1, Fig. 1). Generally, selections with low losses in the first period also had low losses in the second period. Certain selections such as SLS 1 and SLS 2 had low losses throughout the year; they bore a high proportion of their crop after mid October. All the selections which bore less than 70% of their crop after mid October had an overall disease level greater than 20%. In this trial, there was a clear relationship between cropping pattern and overall disease incidence (Fig. 1).
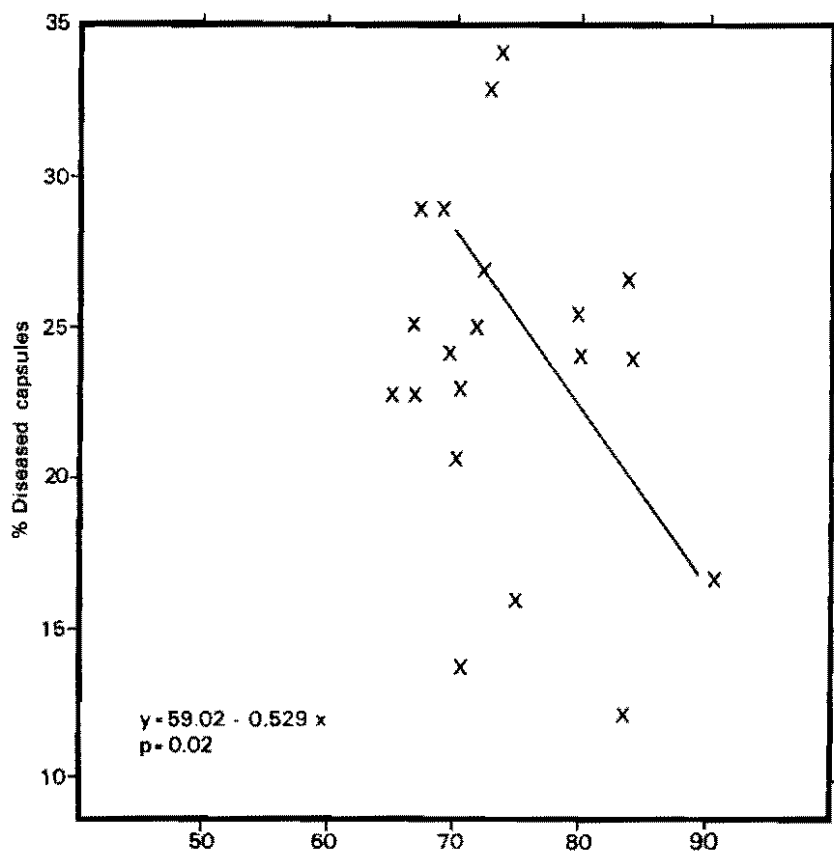

Fig. 1. Relationship between pattern of cropping and incidence of capsule disease.

Discussion

None of the selections in the trial was immune to capsule rot. Some had low overall losses, but it was not clear how much of this was due to direct genetic resistance. Disease losses on SLS 2 were low in relation to its cropping pattern, suggesting that it is less susceptible to capsule rot than the other selections in the trial. All the selections with a low overall level of disease losses produce most of their crop outside the main capsule rot epidemic period.

Breeding for cropping pattern to reduce overall disease losses would avoid some of the difficulties of working with weak source of resistance to capsule rot of cardamom. The difficulties are increased because cultivars for farmers must be distributed as seed and not as selections. Plants which produced most of their crop after mid October would largely escape infection and if few capsules were carried through the dry season, the pests that attack the capsules, would be retarded.

The period of maximum harvest varied in different areas in Sri Lanka. The results reported here apply only to one locality in the southern region and these selections may show somewhat different cropping patterns when moved out of that region. In the southern region ideal selections should crop late in the year with capsules maturing over a short period and have genetic resistance to capsule rot.

Literature Cited

1. Alles, W. S. 1979. The resurgence of Sri Lanka's traditional spice crops (a review). Proc. Sri Lanka Assoc. Adv. Sci. Colombo, Sri Lanka.
2. De Silva, N. 1978. Cardamom development in Knuckles region. Agric. Diversification Div. Peradeniya, Sri Lanka.

Acknowledgments

Effect of Antibiotics and Fungicides on Bacterial Wilt of Tomato

R. Kishun

Indian Institute of Horticultural Research
Bangalore, India

Abstract

Field trials were conducted for three consecutive seasons to control bacterial wilt of tomato. Bleaching powder was most effective of substances tried in decreasing wilting of tomato plants. Percentages of wilting in bleaching powder treated plots for three seasons were 5.3, 13.9, and 11.5; while in untreated control plots the wilting percentages were 59.9, 72.5, and 82.8%. Average yield of tomato fruits in bleaching powder treated plots (22.5g/15 m²) for three seasons was 24.45, 23.65, and 23.33 while in control plots it was 13.77, 14.05 and 13.45. The next most effective chemical for increasing the yield was Agrimycin-100.

Introduction

Bacterial wilt caused by *Pseudomonas solanacearum* Smith is one of the important diseases of tomato in temperate, subtropical, and tropical countries of the world (4). The disease is serious in many states of India and sometimes it causes complete failure of the crop. At present, there are no effective controls except resistant varieties and cultural practices. Little information is available on control of bacterial wilt by chemicals under field conditions (1, 2, 7). In India no attempt has been made to control this serious disease with antibiotics and fungicides. Trials were conducted to find out their effectiveness. Results are presented in this paper.

Materials and Methods

Trials were conducted at the Indian Institute of Horticultural Research Farm, Bangalore, India, during the summers (March to June) of 1979 and 1980 and in Kharif (July to September) of 1980. One month old seedlings of tomato cv. Pusa Ruby were transplanted in bacterial infected soil in 15 m² 5 x 3 m) plots. The design of the experiments was a randomized block with three replications. The treatments were Agrimycin-100, Paushamycin, potassium, penicillin (100 ppm), Bavistin (1000 ppm), Ceresan Wet (2000 ppm), bleaching powder (15 kg/ha), control-A (Agrimycin-100 spray) and control B (without treatment). All the chemicals were applied as seedling dip before transplanting except bleaching powder which was incorporated in the soil before transplanting. The tomato nursery was given one spray of

Agrimycin-100 within 15 days of sowing in the nursery bed. Data on wilting of plants and yield of tomato fruits were recorded and analyzed statistically.

Results

Among all the antibotics and fungicides tested, bleaching powder was significantly superior in reducing the disease in all experiments. Percentage of wilting in bleaching powder was 5.3, 13.9, and 11.5, compared to 59.9, 72.5, and 82.8 in the control during summer, 1979, 1980, and Kharif season of 1980, respectively (Table 1). Next most effective chemical for reducing the disease was Agrimycin-100 in both the seasons of 1980 (35.8 and 35.8%, respectively) but during 1979, second effective chemical was Plantomycin (19.1%). The least effective chemical was potassium penicillin in summer seasons of 1979 and 1980, whereas it was Plantomycin in Kharif, 1980. Percentage of disease control was maximum in bleaching powder treated plots (85.7%), followed by Agrimycin 100 seedling dip (52.5%).

Yield of tomato fruits was higher in bleaching powder treated plots in all experiments. The average numbers of fruits per plot in bleaching powder treated plots were 492.6, 732.6, and 650.6 whereas in control plots the averages were 334, 419.3, and 317 during summer 1979, summer 1980, and Kharif 1980, respectively (Table 2). The number of fruits was lowest in ceresan wet treated plots during summer and Kharif seasons of 1980 but in summer, 1979, potassium penicilin treated plots yielded lowest. Percentage increase in number of fruits was more (75.27%) in bleachingpowder plots followed by Agrimycin-100 spray in nursery bed (control-A, 50.32%).

The average weights of tomato fruits in bleaching powder treated plots were 24.45, 23.65, and 23.33 kg/plot (Table 2). This is significantly superior to control in summer 1979 and Kharif 1980. There was no significant difference between these two treatments in summer 1980. The average weight of tomato fruits was 13.77, 13.98, and 13.45 kg/plot in control plots, during the three seasons of testing. The next best effective chemical in respect to yield (weight), was Agrimycin-100 spray (control-A) as yield was 20.70 and 22.71 kg/plot, in the trials of 1980. Bavistin was ranked next to bleaching powder in only one season (1979). Minimum yield of tomato fruits was recorded in ceresan wet treated plots in both the experiments of 1980 but in 1979 lowest yield was in potassium penicillin treated plots. Percentage increase in weight of tomato fruits was maximum (73.41%) in bleaching powder followed by Agrimycin-100 (Control-A, 54.47%).

Discussion

Bleaching powder was found most effective when incorporated into the soil before transplanting of tomato seedlings. So far there is no report in the literature regarding the use of bleaching powder against bacterial wilt of tomato. However, bleaching powder is reported to be effective against

Table 1. Effect of antibiotics and fungicides on the incidence of bacterial wilt of tomato.

| Sl. No. | Treatment | Per cent wilted plants | | | Average | Per cent reduction in disease over control |
		1979 Summer	1980 Summer	Kharif		
1.	Potassium Penicillin	46.9 (43.22)*	60.7 (51.50)	50.1 (45.34)	52.5	26.7
2.	Agrimycin-100	30.4 (33.47)	35.8 (36.94)	35.8 (36.94)	34.0	52.5
3.	Plantomycin	19.1 (25.78)	50.1 (45.34)	60.7 (51.50)	43.3	39.6
4.	Bavistin	27.0 (31.32)	39.9 (39.42)	51.8 (46.33)	39.5	44.9
5.	Ceresan Wet	37.4 (37.61)	50.0 (45.28)	41.7 (40.33)	43.0	40.0
6.	Bleaching Powder	5.3 (13.07)	13.9 (22.27)	11.5 (20.23)	10.2	85.7
7.	Control-A	41.1 (39.89)	45.9 (42.89)	38.7 (38.77)	41.9	41.5
8.	Control-B	59.9 (50.78)	72.5 (58.76)	82.8 (65.95)	71.7	—
	S.E.M.	(2.60)	(2.82)	(2.86)		
	C.D. at 5o/o	(7.89)	(8.57)	(8.69)		
	C.D. at 1o/o	(10.96)	(11.90)	(12.06)		

* Figures in parenthesis are angular values.

Table 2. Effect of antibiotics and fungicides on yield of tomato in bacterial sick plots.

SI. No.	Treatment	Yield									
		No. of fruits				Per cent increase over control	Weight in kg				Per cent increase over control
		1979	1980		Average		1979	1980		Average	
		Summer	Summer	Kharif			Summer	Summer	Kharif		
1.	Potassium Penicillin	323.3 (2.49)*	475.6 (2.50)	397.6 (2.58)	398.8	11.80	15.06	16.20	20.35	17.20	25.27
2.	Agrimycin-100	406.6 (2.60)	586.0 (2.72)	492.3 (2.68)	494.9	38.74	18.94	15.20	18.41	17.51	27.60
3.	Plantomycin	417.3 (2.57)	523.0 (2.67)	461.3 (2.66)	467.2	30.97	18.18	14.77	18.20	17.05	24.18
4.	Bavistin	521.3 (2.70)	665.3 (2.80)	421.3 (2.62)	535.9	50.23	21.70	20.69	16.55	19.64	43.04
5.	Ceresan Wet	433.0 (2.61)	442.3 (2.58)	328.0 (2.49)	401.1	12.44	18.40	13.43	11.38	14.40	4.88
6.	Bleaching Powder	492.6 (2.69)	732.6 (2.85)	650.6 (2.81)	625.2	75.27	24.45	23.65	23.33	23.81	73.41
7.	Control-A	443.3 (2.63)	642.0 (2.74)	523.3 (2.71)	536.2	50.32	20.24	20.70	22.71	21.21	54.47
8.	Control-B	334.0 (2.49)	419.3 (2.52)	317.0 (2.48)	356.7	—	13.77	13.98	13.45	13.73	—
	S.E.M.	(0.05)	(0.12)	(0.05)			2.30	0.71	2.30		
	C.D. at 5o/o	(0.15)	(0.38)	(1.73)			6.97	13.66	8.37		
	C.D. at 1o/o	(0.22)	(0.53)	(2.40)			9.68	18.95	11.62		

* Figures in parenthesis are transformed values.

bacterial leaf blight of rice (6), stalk-rot of maize (9, 10), soft-rot of cauliflower (3), and soft-rot and blackleg of potato (8). Other chemicals used in the present investigations were not tested earlier against bacterial wilt of tomato. However, Bavistin sprays (0.07%) have been reported effective against Hooghly wilt of Jute caused by *Macrophomina phaseoli, Pseudomonas solanacearum,* and *Fusarium solani* (5) but in our studies it was used as seedling dip.

Literature Cited

1. Anonymous. 1976. Possible bacterial wilt control. Alafua Agric. Bull. 1. 12 pp.
2. Enfinger, J. M., S. M. McCarter, and C. A. Jaworski. 1979. Evaluation of Chemicals and application methods for control of bacterial wilt of tomato transplants. Phytopathology 69. (6):637-640.
3. Kang, M. S., B. S. Thind, and C. S. Malhi. 1976. Soft-rot-A serious disease of Cauliflower. Progressive Farming.
4. Kelman, A. 1953. The bacterial wilt caused by *Pseudomonas solanacearum.* N. C. Agric. Exp. Stn. Tech. Bull. 99, 194 pp.
5. Mishra, C. B. P., and T. Ghosh. 1978. Evaluation of systemic fungicide (Bavistin) in controlling Hooghly wilt of Jute. Pesticides 12 (8):41-42.
6. Padmanabhan, S. Y., and S. S. Jain. 1966. Effect of Chlorination of water on control of bacterial leaf blight of rice, caused by *Xanthomonas oryzae* (Uyada and Ishiyama) Dowson. Curr. Sci. 35 (24)610-611.
7. Rosenherb, M. M. 1962. Report of the Hawaii agricultural Experiment Station for the biennium ending June 30, 1962. Honolulu, Univ. Hawaii. 94 pp.
8. Saini, L., and R. D. Parasar. 1980. Efficacy of stable bleaching powder in controlling soft rot and black leg of potato. Indian Phytopath. 33 (3):409-414.
9. Sharma, R. D. 1980. Control of bacterial stalk rot disease of maize through chemicals. Food Farming and Agric. 12 (11)263-266.
10. Thind, B.S., and M. M. Payak. 1972. Antibiotic and bleaching powder in the control of bacterial stalk rot of maize. Hindustan Antibiot. Bull. 15 (1 and 2) 9-13.

Inheritance of Resistance in Mungbean to Bacterial Leaf Spot

H. C. Yadav, J. N. Chand, G. S. Saharan

Haryana Agricultural University
Hissar, India

Abstract

The F_1, F_2 and F_3 progenies of crosses made between resistant and susceptible parents showed that the resistance in Jalgaon-781, ML-8, and ML-10 varieties was governed by a single recessive gene situated on the same locus.

Introduction

Bacterial leaf spot disease of mungbean (*Vigna radiata* L. Wilezek) caused by *Xanthomonas phaseoli* (E. F. Smith) Dowson mungbean strain is widely distributed in India (9) causing considerable loss. Several mungbean varieties which are relatively resistant to this disease (1, 3, 10) are low yielding and late in maturity. The present work on inheritance of resistance pattern is aimed at determining suitable breeding procedure for developing high yielding, early maturing, and bacterial leaf spot resistant varieties.

Materials and Methods

Culture of mungbean leaf spot organism (*Xanthomonas phaseoli*) used in the present studies was the same as identified and employed in screening of mungbean varieties (1). It was maintained on nutrient dextrose agar at 6 to 8°C in a refrigerator. For preparing the inoculum, the organism was grown on NDA at 25 to 30°C for 48 h. The growth was scraped from the petri plates and the bacterial suspension (approximately 10^7 cells/ml) was prepared by diluting it in sterile water.

The crosses were made following the technique of Boling *et al.* (2). The emasculation was done in the evening and pollination on the next day, before 7 a.m. The mungbean lines, Jalgaon-781, ML-8, and ML-10 were used as resistant parents, and PS-7, PS-16, and Pusa Baisakhi, as susceptible ones. Crosses were also made between resistant and resistant parents as well as between susceptible and susceptibles (Table 1). F_1, F_2, and F_3 progenies were tested in field at Haryana Agricultural University Farm, Hissar, India under epiphytotic conditions created according to

Table 1. Reaction of F_1 and F_2 progenies of various mungbean crosses to bacterial leaf spot organism *Xanthomonas phaseoli.*

Crosses	Type of cross	Reaction of no. of plants F_1	F_2 R	S	Ratio	$r^2 = \sum \dfrac{(O-E)^2}{E}$
PS - 7 X Jalgaon - 781	S X R	S	54	157	1:3	0.0339
PS - 7 X ML - 8	S X R	S	36	106	1:3	0.0094
PS - 7 X ML - 10	S X R	S	30	108	1:3	0.7525
PS - 16X Jalgaon - 781	S X R	S	52	162	1:3	0.0560
PS - 16X ML - 8	S X R	S	64	218	1:3	0.7989
PS - 16X ML - 10	S X R	S	69	237	1:3	0.9802
PB X Jalgaon - 781	S X R	S	25	88	1:3	0.4984
PB X ML - 8	S X R	S	68	219	1:3	0.2612
PB X ML - 10	S X R	S	38	120	1:3	0.0749
PS - 7 X PS - 16	S X S	S	—	192	—	—
PS - 7 X PB	S X S	S	—	188	—	—
PS - 16X PB	S X S	S	—	238	—	—
J - 781X ML - 8	R X R	R	185	—	—	—
J - 781X ML - 10	R X R	R	215	—	—	—
ML-8 X ML - 10	R X R	R	214	—	—	—

R = Resistant; S = Susceptible; PB = Pusa Baisakhi.

Sundaram (12) on 20-day old plants having 2 or 3 leaves. Water was sprayed on the plants in the evening in order to have sufficient humidity the next morning to facilitate inoculation by bacterial cell suspension (10^7 cells/ml). Both the lower and upper surfaces of the leaves were inoculated using a knap-sack sprayer. The inoculated seedlings were sprayed with water twice a day for 4 days to maintain sufficient humidity on the leaf surface. Symptoms appeared within 7 to 15 days of inoculation. Disease reaction was recorded at an interval of one week till pod formation.

Results and Discussion

The F_1 individuals of mungbean parents made between susceptible (PS-7, PS-16 and Pusa Baisakhi) and resistant varieties (Jalgaon-781, ML-8 and ML-10) were susceptible, suggesting that the susceptibility was completely dominant over resistance.

F_2 progenies of Susceptible x Resistant crosses segregated into 3S : 1R (Table 1), indicating that the resistance in Jalgaon-781, ML-8, and ML-10 was controlled by a single recessive gene. The F_3 progenies (Table 2) segregated in accordance with 1 R:2 Seg : 15 ratio which further confirmed the conclusion. In the rest of the crosses viz., S x S and R x R, no segregants in F_2 individuals were observed. This suggests that the single recessive resistance gene in Jalgaon-781, ML-8, and ML-10 was situated on the same locus and did not overlap.

Contrary to the above findings, Singh and Patel (11) found resistance in Jalgaon-781 governed by a single dominant gene. Such conflicting results are expected in the light of earlier findings (5, 7, 8) where the changes in

Table 2. Reaction of F_3 progenies of various crosses to bacterial leaf spot organism *X. phaseoli.*

Cross		Type of cross	Reaction of No. of plants			Ratio
			R	Seg	S	
PS - 7	X Jalgaon - 781	S X R	6	14	8	1:2:1
PS - 7	X ML-8	S X R	8	15	7	1:2:1
PS - 7	X ML-10	S X R	6	13	9	1:2:1
PS - 16	X Jalgaon - 781	S X R	7	12	9	1:2:1
PS - 16	X ML-8	S X R	9	14	7	1:2:1
PS - 16	X ML - 10	S X R	5	14	8	1:2:1
PB	X Jalgaon - 781	S X R	7	14	8	1:2:1
PB	X ML - 8	S X R	7	14	9	1:2:1
PB	X ML - 10	S X R	6	17	7	1:2:1

R = Resistant; S = Susceptible; Seg = ?; PB = Pusa Baisakhi

the inheritance pattern were attributed to the influence of environment when screening was carried out in different agroclimatic conditions. Secondly (6), the influence of the differences in the physiological stages of the plant cannot totally be ruled out. Susceptibility or tolerance of beans to common bacterial blight caused by *X. phaseoli* depends greatly upon the stages of development of the plants, as plants are more susceptible in the reproductive stage than in the vegetative stage (4).

Therefore, in our studies it can be concluded that the inheritance of resistance to bacterial leaf spot in mungbean is governed by a single recessive gene and there are no modifying genes. As the resistance character is inherited independently, so it is possible to breed mungbean cultivars having a short duration, desired maturity period, and resistance against bacterial leaf spot disease.

Literature Cited

1. Beniwal, M. S. and R. D. Parashar. 1979. Studies on bacterial leaf spot of mung (*Vigna radiata* L.). Globios 6:27.
2. Boling, M., D. A. Sander, and R. S. Matlock. 1961. Mungbean hybridization technique. J. Agron. 53:54-55.
3. Chand, J. N., O. P. Yadav, and H. C. Yadav. 1977. Reaction of genetic stocks of mung bean to bacterial blight (*Xanthomonas phaseoli*). Indian Phytopath. 30:465.
4. Coyne, D. P., M. L. Schuster, and K. Hill. 1973. Genetic control of the reaction of common blight bacterium (*X. phaseoli*) in relation to stage of plant development and bacteria multiplication in leaves (*Phaseolus vulgaris* L.). J. Am. Soc. Hortic. Sci. 98 (1):94-99.
5. Hen, M. H., T. T. Chang, and H. M. Beachell. 1968. The inheritance of culm length, panicle length, duration of heading and bacterial leaf blight reaction in rice cross Sigadis x Taichung (Native) 1. Japan J. Breed. 18:7-11.
6. Moses, G. J., Y. P. Rao, and E. A. Siddig. 1975. Studies on physiological changes in relation to resistance susceptibility reaction of rice plants to bacterial blight. Indian Phytopath. 28:508-11.
7. Moses, G. J., Y. P. Rao, and S. A. Siddig. 1976. Effect of environment on inheritance of resistance to bacterial blight in rice. Indian J. Gen. 36:396-402.
8. Murty, V. V. S., and G. S. Khush. 1972. Studies on the inheritance of resistance to bacterial leaf blight in rice varieties; pp. 301-305, in Rice Breeding, International Rice Research Institute, Los Baños, Philippines.
9. Patel, P. N., and J. K. Jindal. 1970. Bacterial diseases in seed legumes in 1968 and 1969. Paper presented at Fourth Annual Workshop Conference on Pulse Crops held at Ludhiana in April.
10. Patel, P. N., J. K. Jindal, and D. Singh. 1972. Studies on inheritance in crops to bacterial disease in India. IV. Resistance in mung bean (*Phaseolus aureus*) to *X. phaseoli*. Indian Phytopath. 25:526-29.
11. Singh, Dharam and P. N. Patel. 1977. Studies on resistance in crops to bacterial disease in India. VIII. Investigations on inheritance of reactions to bacterial leaf spot and yellow mosaic diseases and linkage if any with characters in mung bean. Indian Phytopath. 30 (2):202-206.
12. Sundarum, N. V. 1965. Technique to create epiphytotics of angular leaf spot (*X. malvacearum*) of cotton (ii) Field trials. Madras Agri. J. 52 (3):95-105.

A New *In Vitro* Inoculation Method for Citrus Canker Diagnosis

M. M. López and L. Navarro
Instituto Nacional de Inv. Agrarias
Moncada, Valencia, Spain.

Abstract

To avoid the possible introduction of citrus canker (*Xanthomonas citri*) (Hasse, Dowson) in citrus growing areas, it is important to have a rapid and safe method for diagnosis. At present, pathogenicity tests are the only methods for diagnosis; specific biochemical tests are not available and serological detection lacks specificity.

Inoculation of plants growing *in vitro* were evaluated as a method for detecting the bacteria. Twenty day old Marsh grapefruit (*Citrus paradisi* Macf.) and Parson Brown sweet orange (*C. sinensis* (L.) Osbeck) seedlings growing under sterile conditions in Murashige and Skoog salt solutions were inoculated by placing a drop of bacterial suspension on wounded and unwounded leaves. Inoculated seedlings were incubated at 27°C under 16 h daily exposure to 1,000 lux illumination.

Seven to ten days following inoculation, typical symptoms were observed in all inoculated plants. Disease expression appeared sooner and was more severe in wounded than in unwounded plants. No symptoms were observed in plants inoculated with a *Pseudomonas syringae* strain isolated from citrus or with a *Xanthomonas* spp. that was pathogenic to non-citrus hosts. Advantages and disadvantages of the method are discussed.

Introduction

Citrus canker, caused by *Xanthomonas citri* (Hasse) Dowson, is a very serious disease of citrus. It produces lesions on fruit, leaves, and twigs and causes defoliation, dieback, and fruit drop in most commercial varieties. (Fawcett, 1936; Rosetti, 1977).

The disease is present in several countries of Asia, South America, Africa and Oceania (Garnsey *et al.*, 1979). The areas free of *X. citri* are threatened by its introduction. To avoid this, a quick, specific, and safe method of diagnosis is needed, to be used in quarantine procedure or to identify suspicious symptoms in the field.

At present, pathogenicity tests have to be used for diagnosis. Biochemical tests and accurate serological detection methods are not available. The standard pathogenicity tests, using plants growing in pots, require sophisticated security systems to avoid dissemination of the bacteria, especially in those countries where it is not present.

In this study, inoculation of plants growing *in vitro* was evaluated as a method for diagnosis of citrus canker, because plants growing in test tubes

can be handled easily without danger of disseminating diseases. Routine diagnosis of *Corynebacterium fascians* is carried out by inoculation of plants growing *in vitro*. Tissue cultures by *in vitro* methods have been used to study the effect of bacterial toxins in callus and cells (Bajaj and Saettler, 1970; Kalil *et al.*, 1971) and the production of toxins by suspension cultures (Hsu and Goodman, 1978).

Materials and Methods

Marsh grapefruit (*Citrus paradisi* Macf) and Parson Brown sweet orange (*C. sinensis* (L.) Osbeck) were used as indicator plants, because both species are very susceptible to citrus canker (Fawcett, 1936; Namekata and Balmer, 1973).

Seeds were peeled (both seed coats were removed), disinfected by immersing them for 10 minutes in a 0.5% sodium hypochlorite solution containing 0.1% Tween 20 wetting agent, and rinsed three times with sterile distilled water. One seed was sown per tube of 25 x 150 mm containing 25 ml of the plant cell culture salt solution of Murashige and Skoog (1962) solidified with 1% Bacto-agar. Tubes were placed in a culture room at 27°C under a 16 h daily illumination with 1000 lux Sylvania Gro Lux light, to allow seed germination.

After 20 days the third fully expanded lead of each seedling was inoculated under sterile conditions with a suspension of *X. citri* var. *asiatica* strain no. 1209 CNBP. The suspension was prepared from a 48 h culture grown at 25°C on Difco Nutrient Agar supplemented with 0.5% yeast extract and 1% dextrose, and adjusted to 10^9 cells/ml with sterile distilled water.

Inoculations were made by adding a drop of bacterial suspension on the main vein of wounded and unwounded leaves. The wound consisted of two incisions made on the main vein with a scalpel under sterile conditions. After inoculation, tubes were cupped and sealed with plastic tape (Fig. 1) and cultured again under the same conditions.

Following the same procedure, seedlings were inoculated with sterile distilled water, *Pseudomonas syringae* Van hall, *X. vesicatoria* (Doidge) Dowson, *X. ampelina* Panagopoulos, *Erwinia carotovora* (Jones) Bergey, and *Agrobacterium tumefaciens* (Smith) Townsend.

Results and Discussion

Similar symptoms were observed on Marsh grapefruit and Parson Brown sweet orange seedlings inoculated with *X. citri* and grown *in vitro*. Symptoms appeared sooner and were more severe on wounded than on unwounded leaves (Fig. 2 and 3). On unwounded leaves of sweet orange, first symptoms appeared 1 week after inoculation, whereas on grapefruit they appeared after 2 weeks. In both cases, initial symptoms were light brown spots that slowly enlarged and became brownish yellow, corky lesions (Fig. 2). All seedlings of sweet orange showed symptoms 2 weeks
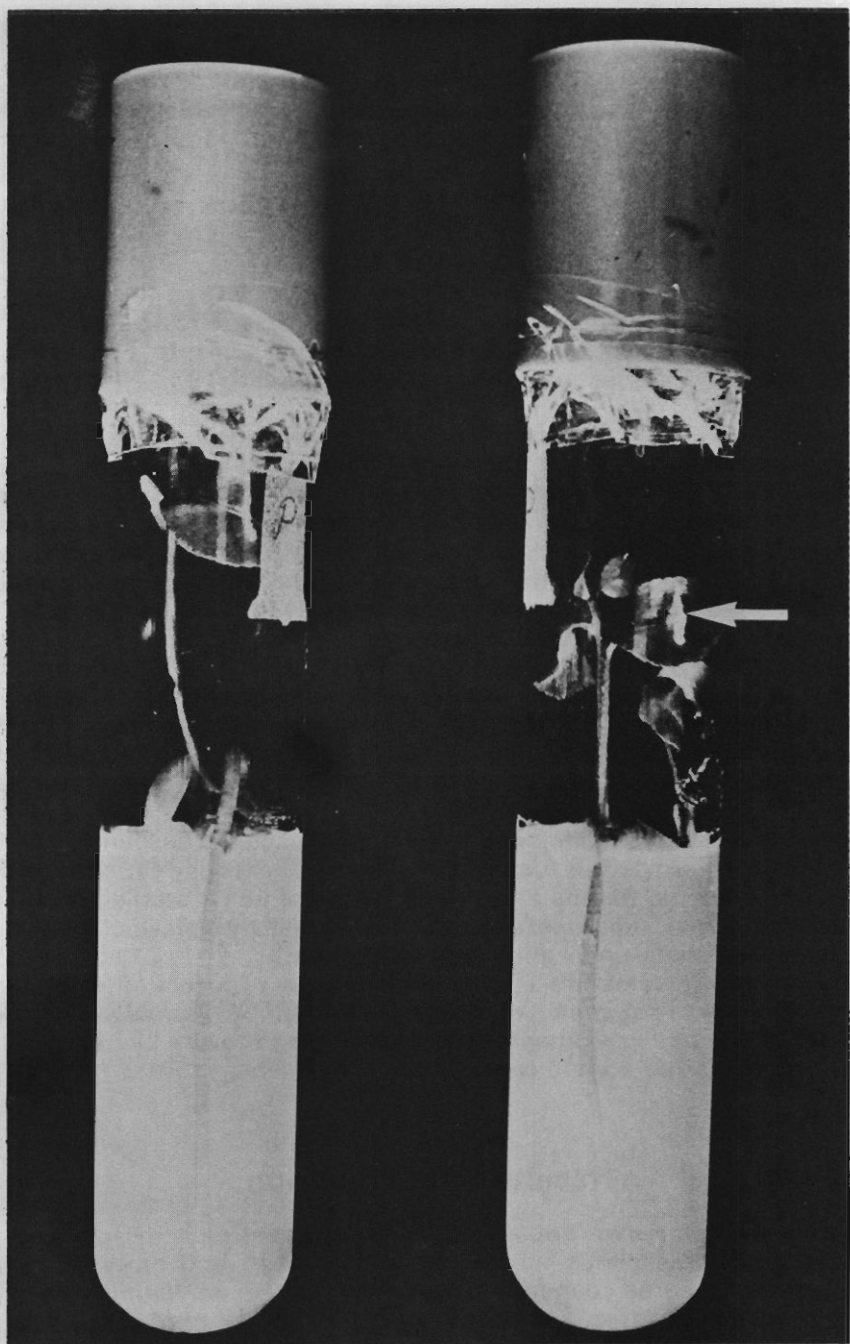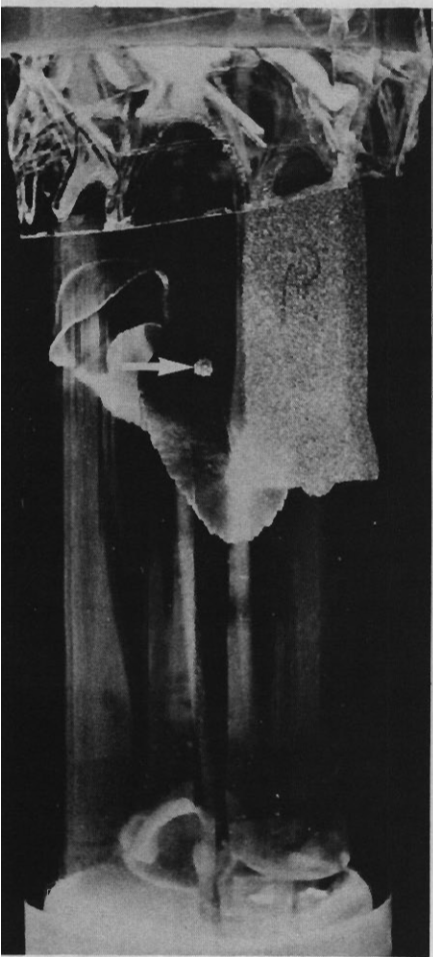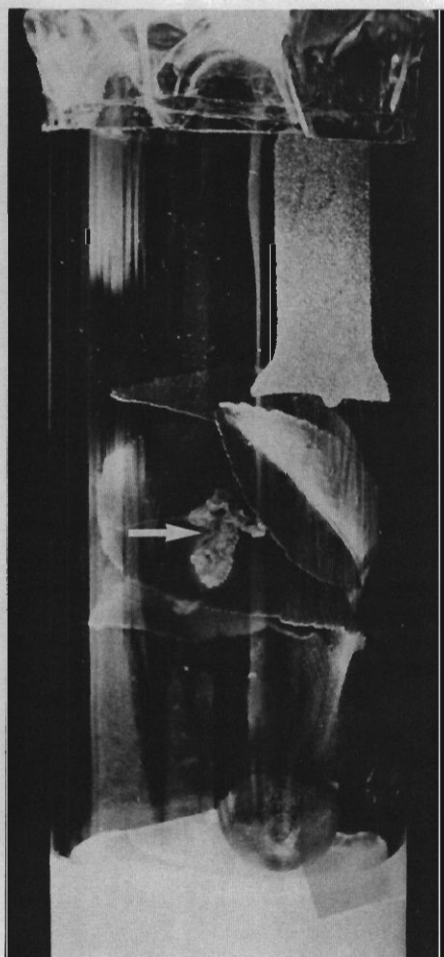
Fig. 1. Marsh grapefruit seedlings 14 days after wound inoculation. Left, inoculated with sterile distilled water; right, inoculated with *X. citri* (arrow indicates lesion).

Fig. 2. (A). Symptoms of *X. citri* on Marsh grapefruit seedling one month after inoculation on unwounded leaf. (B) Symptoms of *X. citri* on Marsh grapefruit seedling one month after inoculation on wounded leaf (arrows indicate lesions).
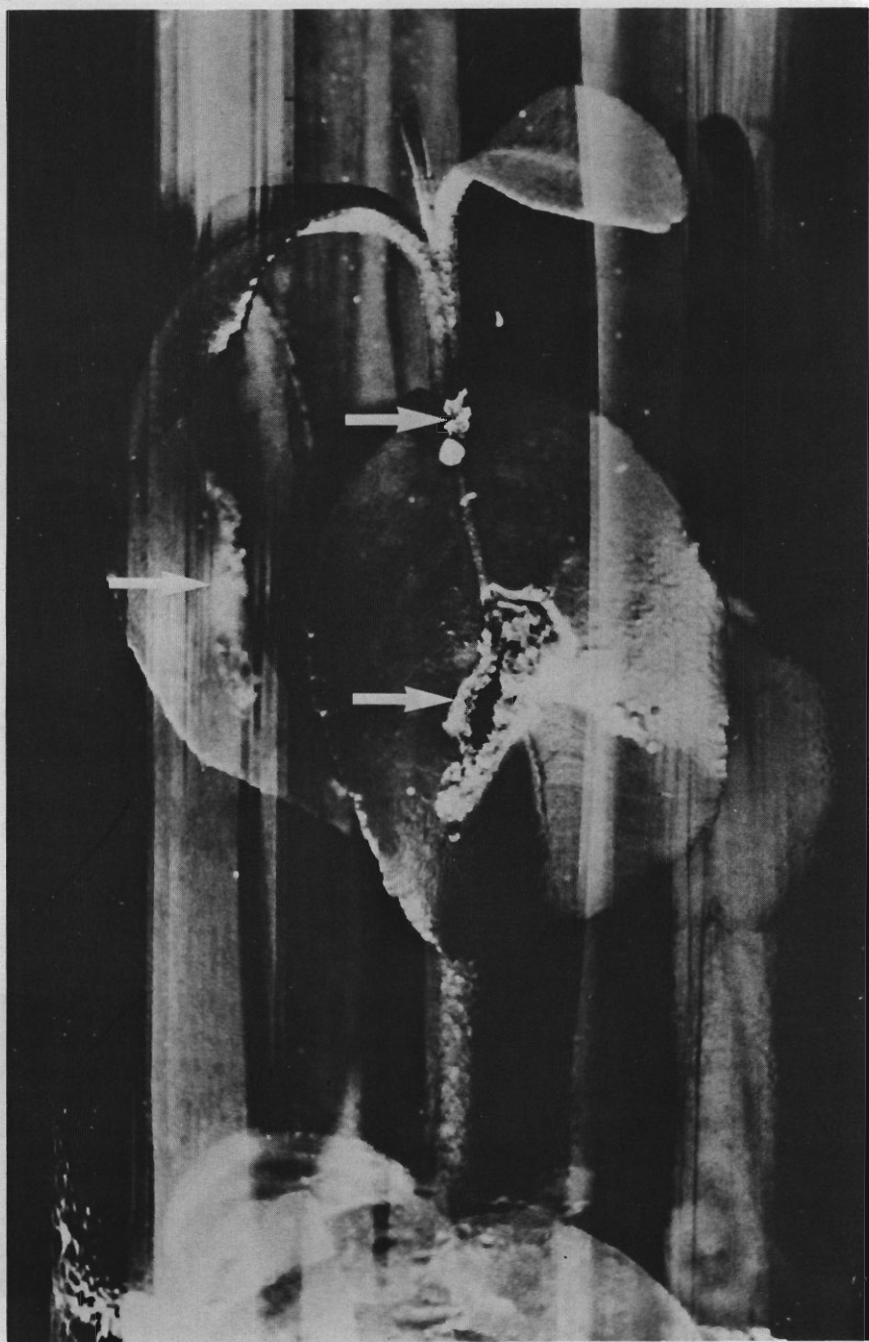
Fig. 3. Systemic lesions on Parson Brown sweet orange 1 month after wound inoculation with *X. citri* (arrows indicate lesions).
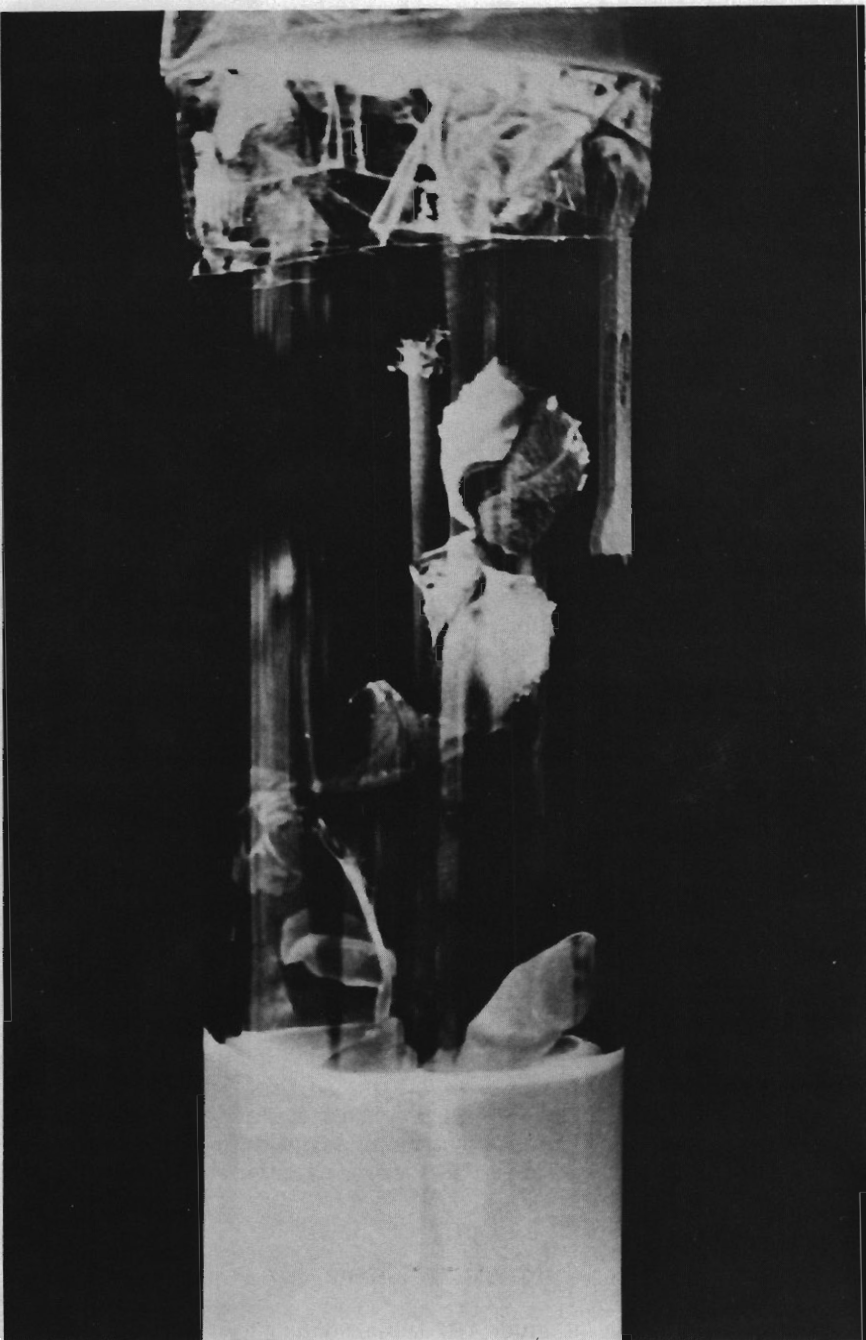
Fig. 4. Symptoms of *X. citri* on Parson Brown seedling two months after inoculation on wounded leaf.

after inoculation, whereas all grapefruit seedlings had symptoms 3 weeks after inoculation.

On wounded leaves of both varieties lesions appeared on all plants 1 week after inoculation, but symptoms were more severe on Parson Brown than on Marsh grapefruit. On the edges of the wounds a few small light brown lesions were formed initially. Lesions quickly proliferated, covering the whole edge, and they became darker.

In sweet orange, new systemic lesions appeared after one month of leaf blades, petioles and stem (Fig. 3) and some leaves died and dropped. Some plants infected for two months showed severe defoliation and dieback (Fig. 4). These severe symptoms were not observed on plants inoculated on unwounded leaves. No symptoms were observed after 2 months in plants inoculated with sterile distilled water or other phytopathogenic bacteria.

These results show that *X. citri* induce symptoms on plants grown and inoculated *in vitro* and that this inoculation method is specific for diagnosis of citrus canker, since no symptoms were observed on seedlings inoculated with other phytopathogenic bacteria.

The incubation period and symptom expression on seedlings inoculated *in vitro* are similar to those described with conventional methods of inoculation (Koizumi, 1966; Namekata and Balmer, 1973). However, the *in vitro* method has several advantages. It is safer in countries where *X. citri* is not present, because test tubes can be tightly closed and easily handled without risk of bacteria dissemination. For this reason, it can be recommended for use in quarantine procedures for citrus, especially in connection with other *in vitro* methods for virus elimination (Navarro *et al,* 1975; Roistacher *et al,* 1977), that may make citrus introduction a safe operation.

Another advantage of the *in vitro* method for *X. citri* diagnosis is that it requires very little space; greenhouses and growth chambers are not necessary. The method can be useful in study of host-parasite relationships because plants are grown under sterile conditions. Nutrition, light and temperature can be easily controlled and humidity inside the test tube is practically 100%.

The difference in symptom intensity of Parson Brown sweet orange and Marsh grapefruit indicate that this method can also be used to study sensitivity of citrus varieties to different pathotypes of *X. citri.* These studies could be carried out even in laboratories located outside growing areas and without facilities to grow citrus.

It could be interesting to try similar *in vitro* inoculation methods for other bacteria causing severe diseases and subject to quarantine regulations, such as *Erwinia amylovora* and *Pseudomonas solanacearum.*

Literature Cited

1. Bajaj, Y. P. S., A. W. Saettler. 1970. Effect of halo toxin containing filtrates of *Pseudomonas phaseolicola* on the growth of Bean Callus tissue. Phytopathology, 60 (7):1065-1067.
2. Fawcett, 1936. Citrus diseases and their control. McGraw Hill Co. New York and London 656 pp.

3. Garnsey, S. M., E. P. Ducharme, J. W. Lightfield, C. P. Seymour, Griffieths. Preventive Action to Protect the U. S. Citrus Industry. The Citrus Industry, Jan. 1979:5-13.

4. Hsu, S., R. N. Goodman. 1978. Production of a Host-Specific, wilt Induced Toxin in Apple Cell suspension Cultures Inoculated with *Erwinia amylovora*. Phytopathology 68:351-35.

5. Kalil, Millicent L. and A. C. Hildebrant. 1971. Effects of *Agrobacterium* sp. on cell cultures of tomato. Phyton 28 (2):177-186.

6. Koizumi, M. 1966. Method for inoculation of *Xanthomonas citri*. Annals of Phytopath. Soc. of Japan, 32:299-300.

7. Murashige, T., F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

8. Namekata, T., and E. Balmer. 1973. Comparative studies on pathogenicity among causal agents of the three citrus canker. Proc. I Congreso Mundial de Citricultura. Murcia, Valencia. España:659-662.

9. Navarro, L., and C. N. Roistacher. 1975. Improvement of shoot-tip Grafting *in vitro* for virus-free Citrus. J. Amer. Soc. Hort. Sci. 100(5):471-479.

10. Roistacher, C. N., E. C. Calavan, and L. Navarro. 1977. Concepts and procedures for importation of citrus budwood. Proc. Int. Soc. Citriculture. 1:133-136.

11. Rossetti, V. 1977. Citrus canker in Latin America: A review. Proc. Int. Soc. Citriculture. 3:918-924.

Abstracts

RESISTANCE OF POTATO TO TUBER ROT INDUCED BY *Erwinia chrysanthemi* **AS INFLUENCED BY TEMPERATURE AND LENGTH OF STORAGE.** Hidalgo, O.A., and E. Echandi. Dept. of Plant Pathology, N.C. State University, Raleigh, N.C., U.S.A.

Tubers of 11 clones of *Solanum tuberosum* subsp. *andigena* (andigena) and 12 cultivars of *S. tuberosum* subsp. *tuberosum* (tuberosum) were inoculated with water suspensions (5.5×10^4, 10^5, 10^6, CFU/ml) of *Erwinia chrysanthemi* immediately after harvest and after 6 and 16 weeks of storage at 4 and 23°C. Tuber rot in andigena and tuberosum increased as inoculum concentration increased. Clones of andigena were classified as resistant, intermediate or susceptible and cultivars of tuberosum were classified as intermediate or susceptible based on tuber rot incidence. Rot increased in all tubers stored at 4°C and only in tuberosum tubers stored at 23°C. Electrolyte leakage (EL), total sugars (TS), reducing sugars (RS), non-reducing sugars (NRS) and dry matter (DM) were determined in non-inoculated tubers. There was a significant positive correlation between the rate of EL, concentration of RS and tuber rot in andigena and tuberosum. Tuber rot and DM were negatively correlated in andigena tubers but were not correlated in tuberosum. Andigena clones with low EL, TS, and RS, but high DM were resistant to tuber rot, and the incidence of tuber rot in these clones was influenced less by temperature and length of storage. The influence of temperature and length of storage on *E. chrysanthemi* susceptibility may be explained by increased membrane permeability; increased leakage of accumulated sugars in potatoes stored at 4°C could favor bacterial proliferation resulting in more disease.

(Paper presented at the Conference; complete text being published elsewhere.)

INFLUENCE OF POTATO CULTIVAR AND SOIL TYPE ON EFFECTIVENESS OF PLANT GROWTH-PROMOTING RHIZOBACTERIA. Howie, W.J., and E. Echandi. Dept. of Plant Pathology, N.C. State University, Raleigh, N.C., U.S.A.

The influence of potato cultivar and soil type on effectiveness of plant growth-promoting rhizobacteria (PGPR) was examined. Rhizobacteria were isolated from potato roots and tubers from fields with a history of high potato yields. Fluorescent rhizobacteria were selected for antibiosis against *Erwinia carotovora* subsp. *carotovora* and growth-promoting activity on potatoes. These bacteria were identified as *Pseudomonas putida* and *P. fluorescens*. In greenhouse tests, PGPR treated potato seedpieces and stem cuttings increased shoot dry weight 23 - 100% and root dry weight 27 - 178%. Survival of PGPR in the rhizosphere was monitored using antibiotic resistant strains. Populations of these strains decreased from 3.6×10^9 colony forming units per g (CFU/g) dry root weight to 4.5×10^5 CFU/g dry root weight 4 wk after treatment. In field trials, PGPR strains

were applied to seedpieces of cultivars Kennebec, Pungo, Red Pontiac, and Superior and planted in Cape Fear loam, Plymouth loamy sand, and Delanco sandy loam. Significant yield increases of 17 - 37% were obtained in two of three field trials. Variability in plant growth-promoting activity was observed between greenhouse and field trials, and no given treatment combination of PGPR, potato cultivar, and soil type was consistently better than another.

(Paper presented at the Conference; complete text being published elsewhere.)

POTENTIAL ROLE OF LECTINS IN RESISTANCE OF TOMATO TO *Pseudomonas solanacearum*. Lin, C. Y. Dept. of Agriculture and Forestry, Taiwan Provincial Government, Nantou, Taiwan, Republic of China.

The titer of lectins elicited in tomato plants by the introduction of *Pseudomonas solanacearum* was related to the susceptibility of the host plant, virulence of the inoculated bacteria, incubation time after inoculation, and the bacterial strains used to determine titer. The tomato plants contained relatively low amounts of lectins, which agglutinated *P. solanacearum* prior to inoculation regardless of their susceptibility to this pathogen. Plants inoculated with incompatible strains of *P. solanacearum*, i. e., resistant VC-8 and susceptible C-28 plants inoculated with avirulent mutant #64-B or resistant plants inoculated with virulent strains #64, showed significant increase in lectin titer 48-72 h after inoculation. Susceptible C-28 plants inoculated with virulent strain #61 of this bacterium showed only slight increases in lectin titer even after prolonged incubation. Lectin titers of preparations from eight tomato lines inoculated with virulent strain #64 were higher in resistant than in susceptible lines and were slightly higher when lectins were combined with cells of avirulent mutant #64-B than with those of virulent strain #64. Agglutination titers of lectin preparation from the same eight tomato lines inoculated with avirulent mutant #64-B were much higher regardless of susceptibility when lectins were combined with virulent strain. Titers of lectin preparations from the resistant VC-8 and susceptible C-28 tomato lines inoculated with avirulent #64-B mutant were similar and much higher when lectins were combined with five *P. solanacearum* mutants avirulent to tomato than when combined with ten strains virulent to tomato. Titers were much higher for lectin preparations from resistant VC-8 than from susceptible C-28 plants inoculated with the virulent strain #64, regardless of the virulence of the strain used for lectin determination.

(Paper presented at the Conference; complete text being published elsewhere.)

BREEDING PEPPERS FOR RESISTANCE TO THE PEPPER STRAIN OF *Xanthomonas campestris* **pv.** *vesicatoria*. Ribeiro, R. de L.D., O. Kimura, F. Akiba, S. Sudo, C. F. Robbs, P. S. T. Brioso, O. C. de Almeida, and J. P. Pimentel. Universidade Federal Rural do Rio de Janeiro, Seropedica, R.J., Brazil.

A distinct pathogenic group of *Xanthomonas campestris* pv. *vesicatoria,* causing bacterial blight on pepper but not on tomatoes, occurs in Brazil. Apparently this group has not been reported from other regions of the world. A breeding program was initiated in 1970 to develop new pepper cultivars with resistance to the pathogen. A selection from Santaka, a small-fruited, "hot" pepper originally from Japan, was used as source of resistance. Advanced breeding lines are now available which are highly resistant to the bacterium and which produce "sweet" fruits of commercial quality. Resistance is a recessive trait and appears to be poligenically inherited. The resistant reaction is expressed through: (i) a markedly reduced number and size of leaf lesions, and (ii) a delayed rate of defoliation. Stems and fruits of resistant lines do not develop lesions under conditions of natural infection in the field.

(Paper presented at the Conference; manuscript not received in time to publish.)

NEW METHOD FOR PRODUCTION AND MULTIPLICATION OF BACTERIA-FREE PLANTS OF CASSAVA FROM PARTIALLY INFECTED STEMS. Takatsu, A. Dept. of Biol. Vegetal 70910, Universidade de Brasília, Brasília, D.F., Brazil.

A new method for production and multiplication of healthy cassava plants (*Manihot esculenta* Crantz) from stems partially infected by *Xanthomonas manihotis* was developed. Stem cuttings of 3 to 4 cm were sprouted in shade, in well moistened soil, in 1000 cc capacity plastic bags, covered with a plastic sheet for 8 to 10 days. Under these conditions the temperature varied from 20 to 23°C at 2 cm below the soil surface. This gave better disease break-out in infected stem cuttings than direct sun light, in which the higher temperature allowed most of infected stem cuttings to produce shoots without disease symptoms. After the cuttings sprouted, they were moved in their bags to the plastic (polyethylene sheet) house and the plants were allowed to grow for 2 months. Plants were watered by hand to avoid bacterial contamination through water splash. All plants that showed disease symptoms were eliminated immediately. At 2 months, plants were pruned at the ground, leaving only one bud. Infected plants that did not show external symptoms until after the 2 months were identified and eliminated after pruning when new shoots showed the disease symptoms. The remaining plants were considered healthy 40 days after pruning and transplanted to the field. Multiplication of healthy plants obtained after one year of observation under field conditions was done with short stem cuttings (3 or 4 cm) sprouted by the same method, but in direct sun light, covered with a black polyethylene sheet. Under these conditions the temperature varied from 22 to 40°C, which allowed fast and uniform sprouting (5 to 6 days) even of immature stem cuttings. The polyethylene sheet was then removed and plants were allowed to grow for 20 to 30 days, then transplanted to the field. From each adult plant it was possible to obtain 100 to 180 new plants instead of 20 to 30 by traditional field methods. The new method is simple and reliable and can be carried out by farmers without expensive materials and installations.

(Abstract provided but paper was not presented at the Conference.)

INTERACTION AMONG BACTERIA INHABITING COTTON LEAF.
Verma, J. P., P. P. Sinha, and R. P. Singh. Div. of Mycology and Plant
Pathology, Indian Agricultural Research Institute, New Delhi 110012,
India.

Species of *Aeromonas* (2 spp), *Flavobacterium* (3 spp) and *Pseudomonas*
(2 spp) comprising 14.2% of the total phylloplane bacterial (P1B) population
of cotton (*Gossypium hirsutum*) leaf, on preinoculation (8-20 hr), protected
the leaf from infection by a virulent isolate of *Xanthomonas campestris* pv.
malvacearum (Xcm) not only in a highly susceptible cv., but also in
differentials [isogenic lines with different bacterial blight resistant (BBR)
genes] used for race characterization. The presence of BBRG did not alter
significantly the preinoculation protective effect (PPE) of P1B. Heat killed
cells of P1B and their Str^r mutants (1000 ppm) afforded similar PPE (50-
60%). At 20:1 (P1B:Xcm) ratio, protection was complete on a few cvs, while
on others it was 80-92%. Further, at this ratio a fair degree of protection
(about 40%) was obtained at time of inoculation. The inhibitory effect of
P1B/its culture filtrate (CF) could be demonstrated by food poisoned
technique but not by filter paper disc or cross streaking. Moreover, when
Xcm and P1B were mixed *in vitro* the former could be recovered by dilution
plating after 8 h of incubation even at 10:1 (Xcm:P1B) ratio, thereby
indicating that the growth of Xcm could be inhibited by a fairly low
concentration of P1B, which itself multiplied very rapidly in the mixture.
However, P1B multiplied rapidly in the cotton leaves only in the presence of
Xcm. It was concluded that the latent phase of Xcm induces P1B
multiplication which later inhibits the growth of Xcm and development of
bacterial blight (BB) symptoms. Spraying of P1B reduced BB disease
severity on all 12 cvs tested. The CF of P1B possessed PPE and 8-16 h CF
afforded maximum protection at 8 h preinoculation. The CF was also
inhibitory to the growth of Xcm *in vitro*. Heat inactivated CF retained its
activity. Preliminary studies have indicated that of the two (?)main
protection factors (elicitors?) at least one is a polypeptide, which is capable
of inducing phytoalexin production in cotton leaves.

(Abstract provided but paper was not presented at the Conference.)

PLANT MYCOPLASMA CONTROL WITH ANTIBIOTICS. J. de la R.
García, Pfizer, S.A., Mexico 19, Mexico.
Since the discovery of mycoplasma-like organisms (MLO's) in plants in
1967 it has been established that many diseases which for more than 80
years have been considered to have been of viral etiology were actually
caused by mucoplasmas or spiroplasmas.
A trial was conducted lasting 4 years, in different regions of Mexico, and
in cultivars of tomato (*Lycopersicum esculentum* Mill.), Chile pepper
(*Capsicum frutescens* L.), watermelon (*Citrullus vulgaris* Schrad.), melon
(*Cucumis melo* L.), and papaya (*Carica papaya* L.) to determine effects of
oxitetracycline on the mycoplasma-like organisms. The symptoms of
yellowing and shrinking leaves, chlorosis, and stunting, disappeared
temporarily after 3 to 4 sprayings with oxitetracycline at 100 ppm. Results
indicate the symptoms and damage caused by these organisms can be

minimized by spraying every five days, starting 20 to 30 days before flowering, with a minimum of four applications.

The typical symptomatology caused by these microorganisms in plants and the action of tetracyclines on them is accepted as evidence at present that the causal agent could be a mycoplasm instead of a virus.

(Paper presented at the Conference; manuscript not received in time to publish.)

BIOLOGICAL CONTROL OF FIRE BLIGHT BY *Erwinia herbicola.* S. V. Beer, J. R. Rundle, and J. L. Norelli, Department of Plant Pathology, Cornell University, Ithaca and Geneva, NY, U.S.A.

Fire blight, caused by *Erwinia amylovora,* is a devastating disease of pear, apple, and other rosaceous plants. Control measures available currently are insufficient to insure adequate and economical disease control. The closely related nonpathogenic bacterium, *E. herbicola,* often is isolated from the same niches where *E. amylovora* is found. The possibility that *E. herbicola* might be introduced into fire blight infection courts and thereby suppress the subsequent development of fire blight is being explored.

Initially, a strain of *E. herbicola* was selected for trial based on its production, *in vitro,* of a bacteriocin that inhibits *E. amylovora, in vitro.* This strain reduced the incidence of fire blight to the same extent as streptomycin (100 mg/1) when dilute suspensions (10^8 cfu/ml) of log-phase cultures were sprayed into apple (*Malus pumila*) blossoms in a research orchard one day before and three days after inoculating the blossoms with *E. amylovora.* In a subsequent experiment, the importance of *in vitro* bacteriocinogenicity to the suppressive ability of *E. herbicola* was tested. All combinations of the bacteriocinogenic strain and a non-bacteriocinogenic mutant of *E. herbicola* and strains of *E. amylovora* that were either sensitive or resistant to the bacteriocin resulted in the same degree of suppression of fire blight. These data suggested that bacteriocinogenicity, as detected *in vitro,* is unimportant to the suppressive ability of *E. herbicola* in the orchard.

Therefore, a screening procedure that uses immature pear fruits to detect antagonism of nonpathogenic bacteria against *E. amylovora* was developed. The ability of strains of *E. herbicola* to suppress the development of fire blight in pear fruit was correlated with the activity of the same strains in apple blossoms.

The physiological state of *E. herbicola,* when introduced into host tissues, affects its ability to suppress the development of fire blight. Application of reconstituted frozen concentrates of *E. herbicola* to pear fruits was less effective in reducing the incidence of fire blight than application of suspensions of log-phase cultures of equivalent viable cell concentration. Reconstituted frozen concentrate of *E. herbicola* applied to apple blossoms in one season resulted in substantially less control of fire

blight than was realized in previous seasons when suspensions of log-phase cultures were used. The mechanism(s) involved in the suppression of fire blight by *E. herbicola* is under investigation.

SCREENING FOR DURABLE RESISTANCE TO CASSAVA BACTERIAL BLIGHT, *Xanthomonas campestri,* pv. *manihotis.* Lozano, J.C., and R. Laberry, CIAT, Cali Colombia.

Durable resistance to *Xanthomonas campestris* pv. *manihotis* in cassava (*Manihot esculenta* Crantz) was identified during four cycles of field evaluation in a cassava bacterial blight endemic area. The durability of resistant genotypes was correlated to the resistant reaction to the pathogen and to the production and sanitary conditions of propagating material (cuttings) during each cycle. However, some genotypes rated resistant, based on foliar symptoms only under controlled conditions were eliminated by this bacterial pathogen after 2, 3 or 4 cycles of continuous cultivation.

Special Contributions

Annotated List of Bacterial Plant Pathogens in Brazil

C. F. Robbs
Universidade Federal Rural do Rio de Janeiro
Rio de Janeiro, Brazil

J. R. Neto
Instituto Biologico de Sao Paulo
Sao Paulo, Brazil

R. de L. D. Ribeiro, O. Kimura
Universidade Federal Rural do Rio de Janeiro
Rio de Janeiro, Brazil

Abstract

A list of phytopathogenic bacteria occurring in Brazil is presented along with their geographical distribution in the Country, host range, and pathogenic races or strains. The list is organized on the basis of the "Approved Lists of Bacterial Names - 1980" and in conformity with recent proposals for nomenclature and classification of bacterial plant pathogens at the infra-subspecific level. Only recognized species and pathovars are included.

Introduction

Most of the records of phytopathogenic bacteria in Brazil are published in obscure journals or in the form of abstracts (in Portuguese) within proceedings from meetings of national scientific societies. Appropriate identification, nomenclature, and information on the pathogenicity of plant bacteria, up to the infra-subspecific level, are important for pathologists, breeders, and for enforcement of legislation dealing with plant quarantine. Also, this information is essential for preparation of maps showing the geographical distribution of plant diseases. Recent developments concerning the characterization and naming of bacteria further increase the need for a review of the previously published lists of bacterial plant pathogens in Brazil (40, 41, 43).

Materials and Methods

The list of bacterial plant pathogens is organized on the basis of the "Approved Lists of Bacterial Names" (56). The nomenclature system adopted is in conformity with the recent proposals by Young et al. (62, 63). All the pathovar names included are among those recognized by Dye et al. (10).

All pathogens have been examined in pure culture by authorities in Phytobacteriology. Cultures of a number of the pathogens were deposited in the National Collection of Plant Pathogenic Bacteria (NCPPB), Harpenden, United Kingdom and/or in the Culture Collection of the Plant Disease Division (PDDCC), Auckland, New Zealand. Reference numbers of those cultures are given.

For identification purposes, the following literature was consulted: Bergey's manual of determinative bacteriology, 8th. edition (1) for general uses: Keane *et al.* (17) and Panagopoulos *et al.* (28) for *Agrobacterium tumefaciens* (Smith and Townsend) Conn.; Dickey (4) and Dye (6) for the "carotovora" group of Erwinias; Dye (7) for the "herbicola" group of Erwinias; Dye and Kemp (9) for *Corynebacterium spp.;* Lelliott *et al.* (22) and Hildebrand and Schroth (16) for the fluorescent pseudomonads; Hayward (15), Lozano and Sequeira (23), and French and Sequeira (12) for biovars and races of *Pseudomonas solanacearum* E. F. Smith; Hale & Wilkie (14) for the "gramineous" non-fluorescent pseudomonads; Dye (5) and Dye & Lelliott (8) for the xanthomonads.

Klement's recommendations about inoculation procedures (21) were followed. Natural plant hosts are listed sequentially in a decreasing order of economic importance of the diseases they suffer from each pathogen. The literature involving reports of bacterial plant pathogen occurrence in Brazil, which has been cited in previous reviews (40, 41, 43), is not cited here. Mycoplasma-and Rickettsia-like organisms that cause plant diseases were not included.

Geographical distribution of the various pathogens relates to the Region and, in certain cases, to the States of Brazil (Fig. 1) from which they have been reported. These are: North Region (N)- States of Amazonas (AM), Pará (PA), and Territories of Roraima (RR), Amapá (AP), and Rondonia (RO); Northeast Region (NE) - States of Maranhao (MA), Piauí (PI), Ceará (CE), Rio Grande do Norte (RN), Paraiba (PB), Pernambuco (PE), Alagoas (AL), Sergipe (SE), and Bahia (BA); Southeast Region (SE),— States of Espírito Santo (ES), Rio de Janeiro (RJ), Sao Paulo (SP), and Minas Gerais (MG); Midwest Region (MW)— States of Goiás (GO), Mato Grosso do Norte (MT), Mato Grasso do Sul (MS), and Distrito Federal (DF); and South Region (S) - States of Paraná (PR), Santa Catarina (SC), and Rio Grande do Soul (RS).

Results and Discussion

Agrobacterium **Conn.**

A. tumefaciens Smith & Townsend) Conn. — Crown and root galls of cultivated roses (*Rosa* spp.), peach (*Prunus persica* (L.) Batsch), raspberry (Rubus idaeus L.), chayote (*Sechium edule* (Jacq.) Sw.) (33), and lettuce (*Lactuca sativa* L.) (47). Occurs in the SE and S Regions. Strains isolated from roses, peach, chayote, and lettuce belong to biotype 1, while the raspberry strain shows characteristics common to biotypes 1 and 2 (35). On lettuce the disease was found to be severe after soil fumigation with methyl bromide (44).
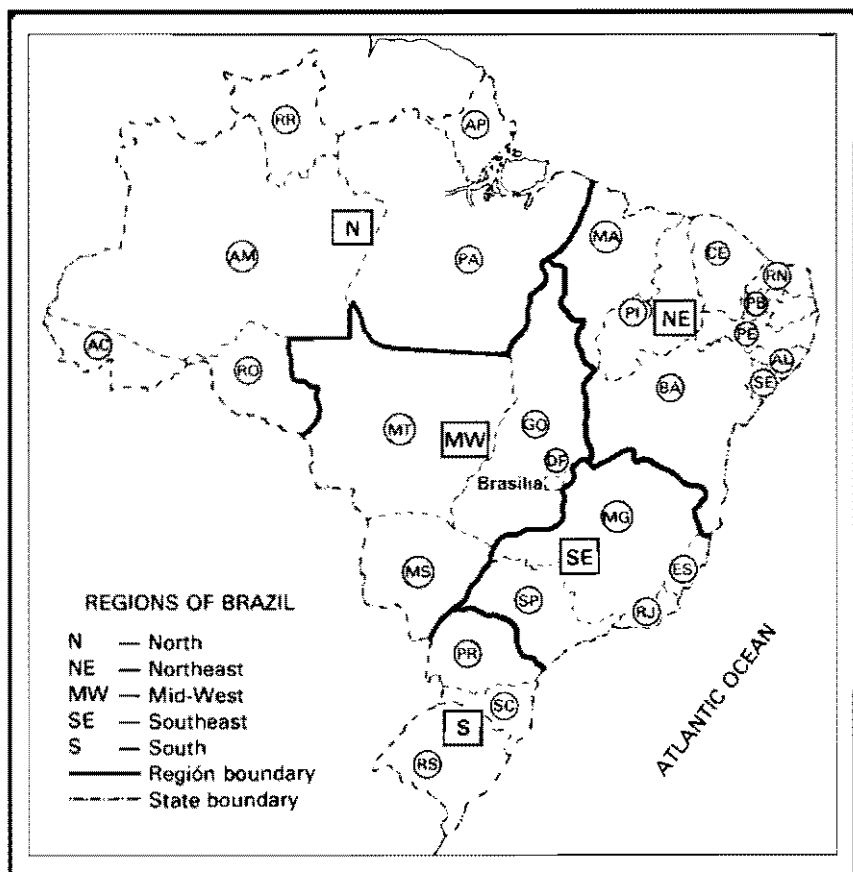
Fig. 1. Map of Brazil showing Regions, States, and Territories.

Corynebacterium **Lehman & Neumann**

C. michiganense pv. *michiganense* (Smith) Jensen.— Bacterial canker of tomato (*Lycopersicon esculentum* Mill.) Reported from the MW, SE, and S Regions. Severe outbreaks occúr. An orange-pigmented strain was isolated from diseased specimen collected in Sao Paulo (45). The common, yellow strain although pathogenic to eggplant (*Solanum melongena* L.) by artificial inoculation, has never been isolated from solanaceous species other than tomato. Deposited culture: PDDCC 2551 (orange strain).

Erwinia **Winslow** *et al.*

E. Ananas Serrano. —Bacterial watery fruit rot of pineapple (*Ananas comosus* (L.) Meir.) Reported from the NE Region. Severe outbreaks seem to be associated with stressed plants. Deposited culture: NCPPB 1845. *E. carotovora* subsp. *carotovora* (Jones) Bergey *et al.*— Bacterial soft rot and/or hollow stalk of carrot (*Daucus carota* v. *sativa* DC), tomato, sweet pepper (*Capsium annuum* L.), lettuce, brassicas (*Brassica oleracea* L.), potato (*Solanum tuberosum* L.), tobacco (*Nicotiana tabacum* L.), cucumber

603

(*Cucumis sativus* L.), muskmelon (*Cucumis melo* L.), sunflower (*Helianthus annuus* L.), and orchids (*Cattleya* spp., *Laelia* spp., and *Phalaenopsis* spp.). Occasionally found on spiny amaranth weed (*Amaranthus spinosus* L.). Reported from all regions. Heavy losses are associated with hot and rainy seasons. Infection appears to be enhanced by insect pests (borers) and also by injuries. Deposited cultures: NCPPB 1740, NCPPB 1741, NCPPB 1742, and NCPPB 1848.

E. carotovora subsp. *atroseptica* (Van Hall) Dye.— Black leg and tuber rot of potato. Reported from the S Region. The disease occurs on crops originated from seed tubers imported from Europe. It tends to disappear on crops from second or third generation seed tubers produced in Brazil. Soil survival of the organisms under Brazilian conditions seems improbable.

E. chrysanthemi Burk. *et al.*— Bacterial soft rot, black leg, and/or hollow stalk of potato, tomato, sweet pepper, welsh onion (*Allium fistulosum* L.) (46), chrysanthemum (*Chrysanthemum morifolium* (Ramat.) Hemsl.), corn (*Zea mays* L.) (37), and forage cactus (*Nopalea* sp.). Reported from the NE, MW, SE, and S Regions. Probably the pathogen has been formerly identified as *E. carotovora* and *E. aroideae*. Strains isolated from dicotyledoneous hosts did not infect either banana (*Musa* spp.), rice (*Oryza sativa* L.), sugar cane (*Saccharum officinarum* L.), or corn by artificial inoculation. Severe outbreaks occur particularly on crops subjected to sprinkle irrigation and heavy nitrogen fertilization.

Pseudomonas **Migula**

P. andropogonis (Smith Stapp).— Bacterial leaf spot or blight of sorghum (*Sorghum vulgare* Pers.), triplaris (*Triplaris filipensis* Cham.), and coffee (*Coffea arabica* L.) (53), causing diseases of minor economic importance. Reported from the MW, SE, and S Regions. Probably the organism has a wider range of natural hosts. By artificial inoculation all strains were pathogenic to velvet bean (*Mucuna* spp.), sorghum (*Sorghum* spp.), and sweet corn. However, the coffee strain did not infect triplaris or bougainvillea (*Bougainvillea spectabilis* Willd.) and the triplaris strain did not infect coffee or bougainvillea. Deposited cultures: NCPPB 3132, NCPPB 3133, PDDCC 6779, PDDCC 6780, and PDDCC 6781.

P. caricapapayae Robbs. — Bacterial leaf spot of papaw (*Carica papaya* L.). Reported from Sao Paulo, Rio de Janeiro, Minas Gerais, and Distrito Federal. Deposited cultures: NCPPB 1872, NCPPB 1877, NCPPB 3080, and NCPPB 3081.

P. caryophylli (Burk.) Starr & Burkholder. — Bacterial wilt of carnation (*Dianthus caryophyllus* L.). Reported from the SE Region. Very destructive in some growing areas.

P. cepacia Burkholder.— Sour skin and bacterial scale rot of onion (*Allium cepa* L.). Reported from the SE and S Regions. More frequently associated with a post-harvest disease of onion bulbs (51).

P. cichorii (Swingle) Stapp.— Bacterial leaf and stem blights of lettuce, endive (*Cichorium endivia* L.), tobacco, chrysanthemum, dahlia (*Dahlia variabilis* (Willd.) Desf.), sunflower, cabbage (*Brassica oleracea* var. *capitata* L.), castor bean (*Ricinus communis* L.), coffee (48), eggplant, celery (*Apium graveolens* var. *dulce* DC), and okra (*Hibiscus esculentus* L.). Widespread in Brazil. A few strains strongly reduce nitrates to nitrites (42).

Under natural conditions, infection often takes place through lesions incited by other bacterial or fungal pathogens. Deposited cultures: NCPPB 1039, NCPPB 1040, and NCPPB 3109.

P. gladioli pv. *gladioli* Severini.— Bacterial corn scab, neck rot, and leaf spot of gladiolus (*Gladiolus hortulanus* Bailey). Reported from the SE Region. Occasionally very destructive. Symptoms may be confused with those of *Fusarium* rots.

P. marginalis pv. *marginalis* (Brown) Stevens.— Bacterial blight and rot of onion, garlic (*Allium sativum* L.) chrysanthemum (20), mustard (*Brassica nigra* (L.) Koch), and lettuce. Reported from the SE and S Regions. Onion crops may be severely affected in Rio Grande do Sul, where the pathogen causes leaf scald symptoms (G. G. Luzzardi - personal communication). On chrysanthemum, the bacterium often penetrates through rust (*Puccinia hori* Henn.) sori.

P. rubrilineans (Lee *et al.*) Stapp.— Red stripe and bacterial top rot of sugar cane, bacterial leaf blight of corn, and bacterial bud rot of ornamental canna (*Canna* spp.). The corn strain was reported from Sao Paulo while the canna strain occurs in Rio de Janeiro. The sugar cane strains are widespread in Sao Paulo and Paraná. On corn, the pathogen was previously described as *P. alboprecipitans* Rosen (13). Probably the disease of ornamental canna is the same that has been ascribed to *Bacterium cannae* (2), later classified as *Xanthomonas cannae* (Bryan) Savulescu. The sugar cane strains were not pathogenic to canna by artificial inoculation. High soil fertility seems to predispose host plants to severe disease conditions. Deposited cultures: NCPBB 921, NCPPB 3029, NCPPB 3108, NCPPB 3111, NCPPB 3112, NCPPB 3113, and NCPPB 3134.

P. rubrisubalbicans (Christopher & Edgerton) Savulescu.— Mottle stripe of sugar cane and leaf spot of guaraná (*Paullinia cupana* var. *sorbilis* (Mart.) Ducke). On this latter host the bacterium occurs associated with a *Xanthomonas* sp. The sugar cane strains were reported from Pernambuco and the Guaraná strain from Bahia (50).

P. solanacearum (Smith) Smith.— This highly variable species with many strains adapted to different hosts, regions, and soils, is widespread in Brazil. The 3 known races of the pathogen occur in the country. Race 1 (Biovars II and III) - Bacterial wilt of potato, tomato, eggplant, jiló (*Solanum gilo* Raddi), tobacco, sweet pepper, dahlia, cowpea, (*Vigna unguiculata* Walp.), bean (*Phaseolus vulgaris* L.), and water cress (*Nasturtium officinale* R. Br.). Among native weeds, the host range includes: bur-marigold (*Bidens pilosa* L.), black nightshade (*Solanum nigrum* L.), castor bean, and *Blainvillea rhomboidea* Cass. The bacterium has been isolated from the rhizosphere of other weeds without inducing symptoms. Reported from all the regions, causing heavy losses to cultivated solanaceous hosts. Potato tubers affected by the pathogen often bear simultaneous infection with *E. carotovora* and *Bacillus polymyxa* (Prazmowki) Migula. On cowpea the disease is prevalent in irrigated areas of Ceará and Paraíba (36); *Race 2* (strains "SFR" and "A") - Moko disease of bananas. The pathogen was introduced and first described in the N Region (60). It represents a serious threat to banana production in other regions of Brazil. Isolates have been grouped into serovars III and IV (55). Serology was found useful for identifying strains ("SFR" or "A") under quarantine. Deposited culture:

PDDCC 6782; *Race 3* (Biovar II) - Bacterial wilt of potato. Widely distributed in the potato growing areas of the SE Region, being more prevalent in Sao Paulo.

P. syringae pv. *garcae* (Amaral, Teixeira & Pinheiro) Young *et al.*— Halo blight and dieback of coffee. Occurs throughout the coffee-producing highland areas of Minas Gerais, Sao Paulo, and Paraná. All strains are "ice nucleation" positive and a few produce fluorescent pigment on King's *B.* medium. Drifting winds and low temperature seem to predispose plants to severe dieback, when in presence of epiphytic populations of the pathogen. Deposited cultures: NCPPB 512, NCPPB 588, NCPPB 589, NCPPB 3030, and NCPPB 4655.

P. syringae pv. *glycinea* (Coerper) Young *et al.*— Bacterial blight of soybean *(Glycine max* (L.) Merr.) Widely distributed in the MW, SE, and S Regions. Eleven races of the bacterium have been identified in Brazil (11).

P. syringae pv. *helianthi* (Kawamura) Young *et al.*— Bacterial spot of sunflower. Distributed througout the MW, SE and S Regions.

P. syringae pv. *lachrymans* (Smith & Bryan) Young *et al.*— Bacterial angular leaf spot and fruit spot of cucumber. Reported from the MW and SE Regions (59). Very important in certain areas.

P. syringae pv. *maculicola* (McCulloch) Young *et al.* Bacterial leaf spot of cauliflower *(Brassica olearacea* var. *botrytis* L.). Reported from Sao Paulo (31).

P. syringae pv. *mori* (Boyer & Lambert) Young *et al.*— Bacterial leaf and twig blights of mulberry *(Morus* spp.) Reported from the SE Region. Occasionally destructive.

P. syringae pv. *savastanoi* (Smith) Young *et al.*— Bacterial knot of olive *(Olea europaea* L.) Reported from Rio Grande do Sul.

P. syringae pv. *sesami* (Malkoff) Young *et al.*— Bacterial leaf spot of sesame *(Sesamum indicum* L.) Reported from Sao Paulo (30).

P. syringae pv. *syringae* (van Hall) Young *et al.*— Bacterial blight of wheat *(Triticum aestivum* L.), bacterial brown spot of *Phaseolus* beans, and fruit rot of okra. The pathogen occurs on wheat in Paraná (24) and Rio Grande do Sul (G. G. Luzzardi - personal communication). It was reported on okra and bean in Rio de Janeiro, Minas Gerais, and Distrito Federal. On okra, the bacterium also infects leaves, penetrating through lesions of *Xanthomonas campestris* pv. *esculenti* (Rangaswami & Easwaran) Dye (18). On fruits of this host, infection appears to take place through flower scars, causing an internal rot that becomes a post-harvest problem. Deposited culture: NCPPB 2596.

P. syringae pv. *tabaci* (Wolf & Foster) Young *et al.*— Wildfire of *Phaseolus* beans, soybean, tobacco, and poinsettia *(Euphorbia pulcherrima* Wild.). The non-toxicogenic strain, formerly named *P. angulata* (Fromme & Murray) Stevens, causes the blackfire disease of tobacco. There is a high degree of pathogenic specialization among strains of this pathovar isolated from different hosts (38). The bean strain has been reported from Rio de Janeiro, Sao Paulo, and Distrito Federal. It also attacks garden pea *(Pisum sativum* L.) and probably occurs in the S Region. The pathogen on beans has been misidentified as *P. syringae* pv. *phaseolicola* (Burk.) Young *et al.*, a bacterium still not characterized in Brazil. The soybean strain occurs in the NW, SE, and S Regions. The strains affecting tobacco have been reported

from the NE, SE, and S Regions. The poinsettia strain was found in Rio de Janeiro (39) and later in Rio Grande do Sul. Deposited cultures: NCPPB 2617 and NCPPB 2618 (sent as toxicogenic strains of *P. syringae* from beans).

P. syringae pv. *tomato (Okabe) Young et al.*— Bacterial speck of tomato. Distributed throughout the tomato growing areas of Sao Paulo (43).

P. woodsii (Smith) Stevens - Bacterial spot of carnation. Reported from Sao Paulo (32).

Xanthomonas Dowson

X. albilineans (Ashby) Dowson.— Leaf scald of sugar cane. Widespread in the sugar cane growing areas of Brazil. Susceptible cultivars are often seriously damaged.

X. campestris pv. *arracaciae* (Pereira *et al.*) Dye.— Bacterial blight of apio (*Arracacia xanthorrhiza* Bancr.). Reported from Sao Paulo (34).

X. campestris pv. *begoniae* (Takimoto) Dye - Bacterial spot and stem blight of begonias (*Begonia* spp.). Occurs in all the Regions of Brazil. The systemic invasion of the pathogen, inducing vascular collapse, has never been observed.

X. campestris pv. *campestris* (Pammel) Dowson.— Black rot of brassicas and radish (*Raphanus sativus* L.). Besides the vascular invasion (black rot syndrome), the pathogen also incites leaf spots and blight on kales (*Brassica oleracea* var. *acephala* L.), black mustard (*B. nigra* (L.) Koch), and radish. Widespread in Brazil. *E. carotovora* subsp. *carotovora* is commonly found in association with the back rot symptoms.

X. campestris pv. *carotae* (Kendrick) Dye.— Bacterial blight of carrot. Recently identified in Distrito Federal (58). The organism has been found associated with *Alternaria dauci* (Kuehn.) Groves & Skolko and with *Cercospora carotas* (Pass.) Sohl. Penetration of the bacterium possibly occurs through lesions of these fungal pathogens.

X. campestris pv. *cassiae* (Kulkarni *et al.*) Dye.— Bacterial leaf spot of Cassia (*Cassia macranthera* DC). Reported from Rio de Janeiro. The bacterium did not infect other species in the genus *Cassia* upon artificial inoculation, thus indicating the existence of pathogenic specialization with the pathovar.

X. campestris pv. *citri* (Hasse) Dye. Citrus canker on cultivated rutaceous hosts. In South America 3 strains of the pathogen have been described. The Asiatic strain (causing cancrosis A) which is pathogenic to virtually all cultivated rutaceous species; the South American strain (causing cancrosis B), not yet found in Brazil, which attacks mainly sweet lemon (*Citrus limon* (L.) Burm.) and limes (*C. aurantifolia* (Christm.) Swing.); and the Mexican lime strain (causing cancrosis C) on Mexican lime (*C. aurantifolia*) in the State of Sao Paulo. This latter strain differs serologically from the cancrosis A strain (26) and shows a high degree of pathogenic specialization. A comparative study of the pathogenicity of the 3 strains inciting citrus canker was carried out by Namekata & Balmer (27). The cancrosis A strain is present in non-commercial areas of Sao Paulo and also in Paraná, Mato Grosso do Norte, Mato Grosso do Sul, and Rio Grande do Sul. Eradication of the pathogen is being attempted in all these States. A recent review of the disease situation in Latin America has been published (54).

X. campestris pv. *cucurbitae* (Bryan) Dye — Bacterial leaf spot and fruit rot of winter squash (*Cucurbita maxima* Dcne.), pumpkin (*C. pepo* L.), and muskmelon. Reported from the SE Region and Distrito Federal. The pathogen has a host range among cucurbits wider than that of *P. lacrymans*. Pathogenic specialization is likely to occur. Under favorable environmental conditions the bacterium may be very destructive to muskmelon especially by impairing fruit quality.

X. campestris pv. *dieffenbachiae* (McCulloch & Pirone) Dye - Bacterial leaf spot and blight of anthurium (*Anthurium* spp.) dieffenbachia (*Dieffenbachia picta* Schott), and *Caladium bicolor* Vent. Reported from the SE Region. The bacterium is probably pathogenic to other species in *Araceae*. Deposited cultures: NCPPB 1833 and NCPPB 4656.

X. campestris pv. *esculenti* (Rangaswami & Easwaran) Dye - Bacterial angular leaf spot of okra. Occurs in the MW and SE Regions. Severe epiphytotics have been observed occasionally. In future, the pathogen may be considered as a race of *X. campestris* pv. *malvacearum*. Deposited culture: NCPPB 2190.

X. campestris pv. *glycines* (Nakano) Dye - Bacterial pustule of soybean. Widely distributed throughout all the soybean growing areas of Brazil.

X. campestris pv. *hederae* (Arnaud) Dye - Bacterial leaf spot of English ivy (*Hedera helix* L.). Reported from Minas Gerais (49).

X. campestris pv. *malvacearum* (Smith) Dye - Angular leaf spot, black arm, and boll rot of cotton (*Gossyium* spp.) Widespread in the cotton growing areas of the MW, SE, and S Regions. Three races of the pathogen have been identified in Sao Paulo (3).

X. campestris pv. *mangiferaeindicae* (Patel, Moniz & Kulkarni) Robbs *et al* — Bacterial leaf and fruit spots of mango(*Mangifera indica* L.). Less frequently observed on cashew (*Anacardium occidentale* L.). The white (apigmented) strain is prevalent in the SE Region and the yellow colony-forming strain in the NE Region. Some mango cultivars are highly susceptible whereas others show resistance to infection under natural conditions. Pathogenic to mombin (*Spondias* spp.) by artificial inoculation. Deposited cultures: NCPPB 3078 and NCPPB 3110 (yellow strains); PDDCC 4087, PDDCC 4088, and PDDCC 4089 (white strains).

X. campestris pv. *manihotis* (Berthet & Bondar) Dye - Bacterial wilt, leaf spot, and blight of cassava (*Manihot esculenta* Crantz). Although reported from all the Regions of Brazil, the pathogen is more destructive in areas of relatively low temperatures and high rainfall (57). Deposited cultures: NCPPB 1843, NCPPB 2550, PDDCC 4957, PDDCC 4958, and PDDCC 4960.

X. campestris pv. *passiflorae* (Pereira) Dye - Bacterial grease leaf and fruit spots of passion fruit (*Passiflora edulis* Sims.). Reported from Sao Paulo and Minas Gerais, causing heavy losses.

X. campestris pv. *pelargonii* (Brown) Dye - Bacterial leaf spot of geranium (*Pelargonium* spp.) Causes disease of minor economic importance in the SE and S regions.

X. campestris pv. *phaseoli* (Smith) Dye - Common bacterial blight and fuscous blight of *Phaseolus* beans. Widespread in Brazil. The pathogen is of economic importance on certain susceptible cultivars under hot and humid conditions. The brown pigment-producing strain is less prevalent (29).

X. campestris pv. *pruni* (Smith) Dye - Bacterial leaf spot, black spot, and

bacterial canker of nectarine (*Prunus persica* v. *nectarina* (Ait.) Maxim.), plum (*P. americana* Marsh.), almond (*P. amygdalus* Batsch), and peach. Reported from the SE and S regions (25). Very important on certain cultivars of nectarine and plum.

·*X. campestris* pv. *ricini* (Yoshi & Takimoto) Dye - Bacterial leaf spot of castor bean. The white (apigmented) strain of the pathogen occurs in the MW, SE, and S Regions. The yellow colony-forming strain has been isolated in Distrito Federal (A. Takatsu - personal communication).

X. campestris pv. *vesicatoria* (Doidge) Dowson - Bacterial scab, leaf and fruit spots of sweet pepper, tomato, and eggplant. The sweet pepper strain ("Rio" strain) is present in all the regions of Brazil. It becomes very destructive under high humidity and mild temperature regimes. The tomato strain has been reported from the SE Region. The eggplant strain has been found in Sao Paulo (19) and constitutes a distinct serovar shown to be related to *X. diefenbachiae* (61). Deposited cultures: NCPPB 2593, NCPPB 2594, and NCPPB 2595.

X. campestris pv. *vitians* (Brown) Dye - Bacterial leaf spot, blight, and wilt of lettuce. Distributed throughout the MW, SE, and S Regions. Extremely harmful on crops originating from contaminated seed. A dark brown pigment-producing strain has been described (43). Deposited cultures: NCPPB 1839, NCPPB 1840, and NCPPB 1841.

X. campestris pv. *zinniae* (Hopkins & Dowson) Dye - Bacterial leaf and petal spots of zinnia (*Zinnia elegans* Jacq.). Widespread in all the regions, mostly due to the use of contaminated seed.

X. fragariae Kennedy & King - Angular leaf spot of strawberry (*Fragaria chiloensis* Duchesne var. *ananassa* Bailey). The pathogen was introduced in Sao Paulo and Minas Gerais where it causes heavy losses. Recently reported from Rio Grande do Sul. The systemic invasion of the bacterium, inducing vascular collapse, has never been observed.

Literature Cited

1. Buchanan, E. R., and N. E. Gibbons (eds.) 1974. Bergey's manual of determinative bacteriology. 8th ed. Williams & Wilkins, Baltimore, MD. 1,268 pp.

2. Bryan, M. K., 1971. A bacterial bud rot of cannas. J. Agric. Res. 21:143-152

3. Cia, E., 1977. Ocorrência e conhecimento das doencas do algodoeiro, *Gossypium hirsutum* L., no Brasil. Summa Phytopathol. 3:167-193. Piracicaba, S. Paulo, Brazil.

4. Dickey, R. S., 1979. *Erwinia chrysanthemi:* a comparative study of phenotypic properties of strains from several hosts and other Erwinia species. Phytopathology 69:324-329

5. Dye, D. W., 1962. The inadequacy of the usual determinative tests for the identification of *Xanthomonas* spp. N. Z. J. Sci. 5:393-416.

6. Dye, D. W., 1969. A taxanomic study of the genus *Erwinia*. II. The "carotovora" group. N. Z. J. Sci. 12:81-97.

7. Dye, D. W., 1969. A taxonomic study of the genus *Erwinia*, III. The "herbicola" group. N. Z. J. Sci. 12:223-236.

8. Dye, D. W., and R. A. Lelliott. 1974. Genus II. *Xanthomonas Dowson*. Pages 243-249 *in* R. E. Buchanan and N. E. Gibbons, eds. Bergey's manual of determinative bacteriology, 8th. ed. Williams & Wilkins, Baltimore, MD. 1,268 pp.

9. Dye, D. W., and W. J. Kemp. 1977. A taxonomic study of plant pathogenic *Corynebacterium* species. N. Z. J. Agric. Res. 20:563-582.

10. Dye, D. W., J. F. Bradbury, M. Goto, A. C. Hayward, R. A. Lelliott, and M. N. Schroth. 1980. International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotype strains. Rev. Plant Pathol. 59:153-168.

11. Ferreira, L. P., P. S. Lehman, and A. M. R. Almeida. 1979. Doencas da soja no Brasil. Circ. Técnica No. 1. EMBRAPA-Centro Nac. Soja, Londrina, Paraná, Brazil. 41 pp.

12. French, E. R., and L. Sequeira. 1970. Strains of *Pseudomonas solanacearum* from Central and South America: a comparative study. Phytopathol. 60:506-512.

13. Frenhani, A. A., A. P. Silveira, and B. P. Bastos Cruz. 1969. Queima bacteriana da folha, nova doença do milho no Brasil. O Biológico 35:85-87.

14. Hale, C. N., and J. P. Wilkie. 1972. A comparative study of *Pseudomonas* species pathogenic to sorghum. N. Z. J. Agric. Res. 15:448-465.

15. Hayward, A. C., 1976. Systematics and relationships of *Pseudomonas solanacearum*. Pages 4-21 *in* L. Sequeira and A. Kelman, eds. Proc. 1st. Int. Plann. Conf. Ecology and Control of Bacterial wilt caused by *Pseudomonas solanacearum*. N. C. Sta. Univ., Raleigh, NC. 166 pp.

16. Hildebrand, D. C., and M. N. Schroth. 1972. Identification of the fluorescent pseudomonads. Pages 281-287 *in* H. P. Mass Geesteranus, ed. Proc. 3rd. Int. Conf. Plant Pathogenic· Bacteria. Centre Agric. Publ. Doc. (PUDOC), Wageningen, The Netherlands. 365 pp.

17. Keane, P. J., A. Kerr, and P. B. New. 1970. Crown gall of stone fruits. II. Identification and nomenclature of *Agrobacterium* isolates. Aus. J. Biol. Sci. 23:585-595.

18. Kimura, O., R. de L. D. Ribeiro, and C. F. Robbs. 1975. Associação de bactérias envolvidas no "crestamento" das folhas do quiabeiro (*Hibiscus esculentus* L.) na Baixada Fluminense (Abstr.). Rev. Soc. Bras. Fitopat. 6-8:24-25.

19. Kimura, O., C. F. Robbs, F. Akiba, and J. Teranishi. 1976. Identificaçao de um novo grupo de *Xanthomonas vesicatoria* (Doidge) Dowson infectando a berinjela (*Solanum melongena* L.) (Abstr.). Rev. Soc. Bras. Fitopatol. 9:3.

20. Kimura, O., C. F. Robbs, and O. C. de Almeida. 1977. Identificação de *Pseudomonas marginalis* em crisantemo (Abstr.). Fitopatol. Bras. 2:83.

21. Klement, Z. 1968. Pathogenicity factors in regard to relationships of phytopathogenic bacteria. Phytopath. 58:1218-1221.

22. Lelliott, R. A., E. Billing, and A. C. Hayward. 1966. A determinative scheme for the fluorescent plant pathogenic pseudomonads. J. Appl. Bacteriol. 29:470-489.

23. Lozano, J. C., and L. Sequeira. 1970. Differentiation of races of *Pseudomonas solanacearum* by a leaf infiltration technique. Phytopathol. 60:833-838.

24. Mehta, Y. R. 1978. Doenças do trigo e seu controle. Summa Phytopathol. & Ed. Agron, Ceres, eds., S. Paulo, Brazil. 190 pp.

25. Mohan. S. K., E. D. do Nascimento, A. Y. Kishino, and S. L. C. de Carvalho. 1978. Observações sobre a occorrência de bacteriose incitada por *Xanthomonas pruni* (E. F. Smith) Dowson no Estado do Paraná. Pages 417-419 *in* Soc. Bras. Fruticult., ed. Ann. IV. Cong. Bras. Fruticult., Cruz das Almas, Bahia, Brazil. 424 pp.

26. Namekata, T., and A. R. Oliveira. 1972. Comparative serological studies between *Xanthomonas citri* (Hasse) Dowson, causal agent of citrus canker, and a bacterium causing canker on Mexican lime. Pages 151-152 *in* H. P. Mass. Geesteranus, ed. Proc. 3rd. Int. Conf. Plant Pathogenic Bacteria. Centre Agric. Publ. Doc. (PUDOC). Wageningen, The Netherlands. 365 pp.

27. Namekata, T., and E. Balmer. 1973. Comparative studies of pathogenicity among causal agents of three citrus cancrosis (Abstr.). Page 398 *in* Int. Soc. Citricult., ed. Ann. 1st. Inst. Citrus Conf., Murcia, Spain.

28. Panagopoulos, C. G., and P. G. Psallidas. 1973. Characteristics of Greek isolates of *Agrobacterium tumefaciens*. J. Appl. Bacteriol. 36:236-240.

29. Paradela Filho, O., and A. M. B. Carvalho. 1967. Ocorrencia de *Xanthomonas phaseoli* v. *fuscans* Starr & Burk. em feijoeiro no Estado de São Paulo. Rev. Soc. Bras. Fitopatol. 1: 73-74.

30. Pereira, A. L. G. 1967. Estudo do organism *Pseudomonas sesami* causador da bacterioso do gergelim. Arq. Inst. Biol. S. Paulo 34:113-125.

31. Pereira, A. L. G., and A. G. Zagatto. 1970. Mancha bacteriana na folha de couve-flor (*Brassica oleracea* var. *botrytis* L.) causada por *Pseudomonas maculicola*, ocorrendo no Estado de São Paulo. Arq. Inst. Biol. S.Paulo. 37:59-66.

32. Pereira, A. L. G., and A. G. Zagatto. 1970. Mancha aquosa do craveiro (*Dianthus caryophyllus* L.) causada por *Pseudomonas woodsii* (Smith) Stevens. Arq. Inst. Biol. S. Paulo 37:157-162.

33. Pereira, A. L. G., and A. G. Zagatto. 1973. Ocorrência de galhas em cuchu (*Sechium edule* SW) causadas por *Agrobacterium tumefaciens* (Smith & Townsend) Conn. no Estado de São Paulo. O Biológico 39:17.

34. Pereira, A. L. G., O. Paradela Filho, and A. G. Zagatto. 1971. Uma nova doenca bacteriana da mandioquinha salsa (*Arracacia xanthorrhiza*) casada por *Xanthomonas arracaciae* n. sp. Arq. Inst. Biol. S. Paulo 38:99-108.

35. Pereira, A. L. G., J. Rodrigues Neto, and A. G. Zagatto. 1975. Ocorrência de galha bacteriana em framboesa (Rubus idaeus L.) no Estado de São Paulo,provocada por *Agrobacterium radiobacter* var. *tumefaciens*. (*Smith & Townsend*) Keane *et al.* O Biológico 41:144-148.

36. Ponte, J. J., da, and A. A Santos. 1973. A murcha bacteriana do feijão macassar (*Vigna sinensis* Endl.). Bol. Cearense Agron. 14:23-27. Fortaleza, Ceará, Brazil.

37. Reifschneider, F. J. B. and C. A. Lopes. 1980. Ocorrência da podridao bacteriana do topo e do colmo do milho no Distrito Federal (Abstr.). Fitopatol. Bras. 5:447.

38. Ribeiro, R. de L. D., D. J. Hagedorn, R. D. Durbin, and T. F. Uchytil. 1979. Characterization of the bacterium inciting bean wildfire in Brazil. Phytopathol. 69:208-212.

39. Ribeiro, R. de L. D., J. P. Pimentel, O. Kimura, C. F. Robbs, and F. Akiba. 1980. Caracterizaçao da bactéria incitante do "fogo selvagem" da poinsettia (*Euphorbia pulcherrima*) no Estado do Rio de Janeiro (Abstr.) Fitopatol. Bras. 5:450-451.

40. Robbs, C. F. 1946. Contribuiçao ao estudo das bactérias que atacam plantas no Brasil. Rev. Soc. Bras. Agron. 9:195-221. Rio de Janeiro, Brazil.

41. Robbs, C. F. 1954. Bactérias fitopatogenicas do Brasil. Agronomia 13:265-282. Univ. Fed. Rural do Rio de Janeiro, Itaguaí, Rio de Janeiro, Brasil.

42. Robbs, C. F. 1960. Um estudo comparativo de cepas de *Pseudomonas cichorii* (Swingle) Stapp isoladas de hospedeiros diversos. Pages 28-30 *in* Inst. Econ. Rural, Univ. Rural, eds. Bacterioses fitopatogenicas do Brasil. Sér. Divulg. Pesq. No. 2, Itaguaí, Rio de Janeiro, Brazil. 63 pp.

43. Robbs, C. F. 1962. Relaçao de bactérias patogênicas a hortalicas observadas no Brasil. Olericultura 2:140-145. Viçosa, Minas Gerais, Brazil.

44. Robbs, C. F. 1962. Uma doença bacteriana da alface (*Lactuca sativa* L.) nova para o Brasil. Olericultura 2:150-153. Vicosa, Minas Gerais, Brazil.

45. Robbs, C. F. 1967. Estudo comparativo de algumas variantes de *Corynebacterium michiganense* (Smith). D. Sc. Thesis. Univ. Fed. Rural do Rio de Janeiro, Itaguaí, Rio de Janeiro, Brazil. 43 pp.

46. Robbs, C. F. 1980. *Erwinia chrysanthemi:* agente de uma podridao mole de cebolinha (Abstr.) Fitopatol. Bras. 2:99.

47. Robbs, C. F., O. Kimura, and G. A. A. Barbosa. 1971. Ocorrencia de galha bacteriana (*Agrobacterium tumefaciens*) em alface (*Lactuca sativa*) no Estado da Guanabara. Arq. Univ. Fed. Rur. Rio de J. 1:15-17. Itaguaí, Rio de Janeiro, Brazil.

48. Robbs, D. F., O. Kimura, R. de L. D. Ribeiro, and L. C. Oyadomari. 1974. "Crestamento bacteriano" das folhas: nova enfermidade do cafeeiro (*Coffea arabica* L.) incitada por *Pseudomonas cichorii* (Single) Stapp. Arq. Univ. Fed. Rur. Rio de J. 4:1-5. Itaguaí, Rio de Janeiro, Brazil.

49. Robbs, C. F., O. Kimura, R. de L. D. Ribeiro, and G. A. A. Barbosa. 1975. "Mancha bacteriana" das folhas da hera (*Hedera helix* L.) incitada por *Xanthomonas hederae* (Arnaud) Dowson (Abstr.) Rev. Soc. Bras. Fitopatol. 6-8.172.

50. Robbs, C. F., and O. Kimura. 1977. Uma doença bacteriana do guaranazeiro (Abstr.). Fitopatol. Bras. 2:99.

51. Robbs, C. F., O. Kimura, and F. Akiba. 1977. Duas enfermidades bacterianas da cebolla, novas para o Brasil (Abstr.). Fitopatol. Bras. 2:100.

52. Rodrigues Neto, J., M. H. Sugimori, and O. Paradela Filho. 1978. *Xanthomonas fragariae* Kennedy and King in Brazil. Summa Phytopathol. 4:101-104. Piracicaba, S. Paulo, Brazil.

53. Rodrigues Neto, J., P. Figueiredo, P. R. Mariotto, and C. F. Robbs. 1981. *Pseudomonas andropogonis* (Smith) Stapp, agente causal da mancha escura bacteriana em folhas de cafeeiro (*Coffea arabica* L.) Arq. Inst. Biol. S. Paulo (in press).

54. Rossetti, V. V. 1977. Citrus canker in Latin America: a review. Proc. Int. Soc. Citricult. 3 918-924.

55. Schaad, N. W., A. Takatsu, and J. C. Dianese. 1978. Serological identification of strains of *Pseudomonas solanacearum* in Brazil. Pages 295-300 *in* Sta. Pathol. Vég. Phytobactériol., ed. Proc. 4th Int. Conf. Plant Path. Bact. Angers, France.

56. Skerman, V. B. D., V. McGown, and P. H. A. Sneath (eds.). 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.

57. Takatsu, A., S. Fukuda, and S. Perin. 1978. Epidemiological aspects of bacterial blight of cassava in Brazil. Pages 141-150 *in* H. Maraite and J. A. Meyer, eds. Diseases of Tropical Crops. U. C. L., Louvain-la-Neuve, Belgium. 316 pp.

58. Takatsu, A., and F. J. B. Reifschneider. 1980. Ocorrencia de crestamento bacteriano da cenoura (*Daucus carota* L.) no Distrito Federal (Abstr.) Fitopatol. Bras. 5:461-462.

59. Tokeshi, H., and F. Galli. 1962. Mancha angular do pepino (*Cucumis sativus*) causada por *Pseudomonas lachrymans* (E. F. Smith & Bryan) Carsner. Olericultura 2:118-121. Viçosa, Minas Gerais, Brazil.

60. Tokeshi, H., and M. L. R. Duarte. 1976. Moko da bananeira no Território Federal do Amapá. Summa Phytopathol. 2:224-229. Piracicaba, S. Paulo, Brazil.

61. Yano, T. 1976. Estudo bacteriológico e serológico de algumas amostras pertencentes a vários patotipos de *Xanthomonas campestris* (Pammel) Dowson. M. Sc. Thesis. Univ. Est. Campinas (UNICAMP), Campinas, S. Paulo, Brazil. 65 pp.

62. Young, J. M., D. W. Dye, J. F. Bradbury, C. G. Panagopoulos, and C. F. Robbs. 1978. A proposed nomenclature and classification for plant pathogenic bacteria. N. Z. J. Agric. Res. 21:153-177.

63. Young, J. M., D. W. Dye, J. F. Bradbury, C. G. Panagopoulos, and C. F. Robbs. 1978. The use of the term "pathovar" in the classification of plant pathogenic bacteria. Pages 359-363 *in* Sta. Pathol. Vég. Phytobactériol., ed. Proc. 4th Int. Conf. Plant Path. Bact. Angers, France.

Acknowledgment

Contamination of Cassava Flowers, Fruits and Seeds by *Xanthomonas campestris* **pv.** *manihotis*

J. F. Daniel, B. Boher

Office de la Recherche Scientifique et Technique
Outre-Mer Centre de Brazzaville
Republique du Congo

Abstract

The epiphytic survival of *Xanthomonas manihotis* on aerial parts of cassava plant has been reported previously (1978). This study of bacterial microflora of flowers, fruits, and sexual seeds from cassava plants in diseased fields provides demonstration of the pathogen's presence on these organs throughout the growing cycle of cassava plants.

The bacteria was consistently found on symptomless flowers, green mature fruits, and seeds and in seed tissues (testa, caruncle, endosperm, cotyledon and embryo.) Histological studies (paraffin embedding and serial sections) using immunofluorescence to detect the pathogen confirmed these results.

The level of populations present on and within the seed may be high (10^6 bacteria/seed). The strains of *X. manihotis* present internally are viable and pathogenic. This result indicates that *X. manihotis* is a seed-borne pathogen. The seed may assume its transport, its dissemination, and its conservation.

However, in the case of low level of seed contamination, the classical technique of isolation on nutrient broth and the immunofluorescence method are unable to detect the pathogen. So, we propose a method of concentration associated with fluorescent antibody technique to increase the threshold of detection. The schedule of operations for the detection of *X. manihotis* in seed is presented and illustrated.

Introduction

Cassava bacterial blight caused by *Xanthomonas campestris* pv. *manihotis* is a widespread and damaging disease of cassava in Africa, Asia, and South America. Most of available information in epidemiology of this disease has been adquately defined, except the contamination of flowers, fruits and seeds, by the cassava blight bacteria. The presence of *X. c.* pv. *manihotis* on cassava seed has been reported previously (5), but no direct evidence has been presented to establish the internally borne bacterial infection of seed.

This paper reports the results of investigation of the presence of cassava blight bacteria in association with flowers, fruits, and seeds.

Materials and Methods

The experiments were conducted at the varietal collection of Odziba (Highlands region), where the cassava bacterial blight is endemic. Four susceptible cultivars were chosen (MB 17, MB 41, Mamanba 1, ODZ 12) and two analyses of the bacterial microflora were made at one month intervals during the flowering and fruit production periods.

Flower and Fruit Samples

For each analysis, freshly harvested flowers and mature fruits were collected from each cultivar that showed systemic symptoms of cassava bacterial blight. Samples of 100 flowers (male and female mixed) and 50 fruits were washed in sterile deionized water (500 ml for fruits, 100 ml for flowers) with one drop of Tween 80 (Difco) during 3 hours at 25°C. The resulting suspensions were assayed for the detection of *X. c.* pv. *manihotis.*

Seed Samples

For each cultivar, samples of 100 seeds were taken from fruits previously washed and from seeds which had been stored at 25°C and 60% r.h. for periods of 8 months.

Detection of Surface-borne Bacteria

Each sample of 100 seeds was washed as described above in 100 ml of sterile deionized water. The washing solution is tested for the presence of cassava blight bacteria.

Detection of Internal-borne Bacteria

The samples previously washed were immersed in 0.1% mercuric chloride solution for 20 minutes, and washed three times in sterile deionized water. The last washing water was assayed for *X. c.* pv *manihotis* as a control to ensure the reliability of the surface sterilization technique. The seeds were dried at room temperature (25°C). Samples of 100 seeds were ground in 200 ml of sterile deionized water with a Waring Blender for 2 minutes. The resulting suspensions were allowed to stand overnight at 5°C. Aliquots of the supernatant were assayed directly for the presence of *X. c.* pv. *manihotis.* The remaining suspension was clarified by centrifugation at low speed for 10 minutes. The supernatant was centrifuged at 15000 g for 30 minutes. The pellet was resuspended in 5 ml of sterile deionized water. The resulting suspension was precipitated with acetone (50%, $^V/_V$) and centrifuged at 15000 g. The pellet was resuspended in 5 ml of sterile deionized water and passed through a 0.45 nm nonfluorescent black Millipore filter (HABG).

Detection Methods

Each suspension resulting from washing and grinding was checked for the presence of *X. c.* pv. *manihotis* by the dilution plate methods of Y. P. D. A. medium (yeast extract (Difco) 5 g, bacto peptone (Difco) (g, dextrose 3 g, agar 15 g, H_2O 1000 ml, pH 7.2) with cycloheximide (50 mg/ml), leaf infiltration method (5) on two months, susceptible cassava plant, and by the fluorescent antibody technique. This last technique used the indirect method and was carried out on glass for aliquots from washing solution and on filters by the method of Bohlool *et al.* (1) for grinding suspension.

Pathogenicity Tests

After being counted any isolates of similar appearance were purified and tested for pathogenicity by leaf infiltration technique (5) and checked by indirect immunofluorence method.

Histological Methods

Seeds without testa were fixed in formalin/acetic/ethanol solution (FAA) and dehydrated in an n-butyl alcohol series (4) and embedded in paraffin. Sections (8 to 10 nm) of the seed were made with a rotary microtoms. Transverse and longitudinal sections were mounted on cleaned slides with adhesive (3) and stained by the indirect immunofluorescence method (1).

Results

Results obtained by the dilution plate method, leaf infiltration technique, and fluorescent antibody technique, demonstrated that during the flowering and fruit production period, *X. c.* pv. *manihotis* could be detected from the mixed microflora of cassava flowers, fruits, and seed. Levels of population found during these analyses are listed in Table 1. The levels of population might be high, especially on fruits (10^7-10^6 bacteria per fruit). Populations of *X. c.* pv. *manihotis* recorded by the dilution plate and leaf infiltration method indicated external and internal presence of viable and pathogenic cassava blight bacteria in freshly harvested seed and in seeds stored 8 months. In our analysis, the pathogen was more often detected on the seed surface than internally. Levels of internal population of *X. c.* pv. *manihotis* varied between 2.0×10^2 and 2.1×10^5 bacteria per seed and were lower than those monitored on the seed surface (3×10^3 to 3.3×10^6).

The fluorescent antibody technique (1) applied to the detection of internal seed-borne bacteria allowed us to detect the pathogen more frequently than the routine laboratory methods (isolation of agar medium leaf infiltration). (Table 2). During analysis, the fluorescent antibody technique could detect levels as low as 10^2 bacteria per seed.

The histological observations in epifluorescence after antibody staining of transverse and longitudinal sections of seeds of the cultivar MB 27 have shown that the pathogen might be found in embryo, cotyledon, endosperm and caruncle. However, the bacteria seemed to be preferentially localized in caruncle and in seed tissues localized under the testa.

Discussion

This study indicates that the cassava bacterial blight agent can contaminate flowers, fruits, and seeds with high levels of population. Most of the *Xanthomonad* are known to be seed-borne and to survive in or on seed for varying periods of time. Previously, Persley (5) demonstrated that *X. c.* pv. *manihotis* could be carried on the seed surface and suggested its internal presence in seed. Our study confirms these results and gives direct evidence of internally borne bacterial infection of cassava seed. The finding of viable and pathogenic strains of *X. c.* pv. *manihotis* on and within seed

616

suggests that this bacteria is a seed-borne pathogen. The seed may sustain the diseases's transport, its dissemination, and also its conservation, since the pathogen could be detected after 8 months of storage. The presence of high levels of population of the pathogen on flowers might partially explain the contamination of fruits and seeds originating from these contaminated flowers.

The histological observations confirm that *X. c.* pv. *manihotis* may occur within the cassava seed and suggest a preferential localization of the pathogen in caruncle and in tissues contiguous to testa.

The fluorescent antibody technique on black nonfluorescent filter (1) applied to the detection of *X. c.* pv *manihotis* allows detection within seed at a low level of 10^2-10^3 bacteria per seed. This technique may be useful for controlling seed stocks suspected of harboring the cassava blight organisms and in establishing the relationship between seed infection and plant infection.

Literature Cited

1. Bohlool, B. B., and E. L. Schmidt. 1973. A fluorescent antibody Technique for determination of Growth Rates of Bacteria in soil. Bull. Ecol. Res. Comm. (Stockholm) 17:336-338.

2. Dupouey, P., 1977. L'exécution des réactions d'immunofluorescence directes et indirected sur lame. Pages 67-72 in "Les techniques de l'immunofluorescence et les réactions immunoenzymatiques." Cours de l'Institute Pasteur. Maloine S. A. Editeur. 556 pp.

3. Hockenhull, J., 1978. The adaptation of the fluorescent antibody technique for "in situ" detection of bacterial antigen in serial sections of plant tissue. Proc. 4th Int. Conf. Plant Path. Bact. Angers. 1:333-336.

4. Johansen, D. A., 1940. Plant microtechnique. Mc Graw-Hill, New York. 533 pp.

5. Persley, G. J., 1979. Studies on the survival and transmission of *Xanthomonas manihotis* on cassava seed. Ann. Appl. Biol. 93:159-166.

Report on proposal for
An International Working Group

on
Bacterial Blight of Rice

Bacterial blight of rice caused by *Xanthomonas oryzae* (Uyeda et Ishiyama) Dowson, has been an important constraint to rice production in Asia. Major epidemics were reported in the 1960s in tropical Asia after introduction of improved high yielding varieties such as Taichung Native 1, IR8, and Jaya, which lacked adequate resistance. The bacterium infects rice from seedling stage to mature plants. Infection of mature plants causes severe sterility and may result in more than 50% yield reduction. The disease is most widespread and damaging during the monsoon season. season.

Bacterial blight occurs in almost all rice growing countries in Asia, northern parts of Australia, and Malagasy. The disease was reported in Latin America in 1977, and in mainland Africa in 1979, first in Mali and then in Cameroon. In Asia, the disease has increased in severity and importance in recent years, from Korea and China to Southeast Asia and the Indian subcontinent.

Despite an incredible amount of literature related to the disease and the bacterial pathogen, and considerable interest in research after the epidemics in the sixties, a gap of knowledge still exists and little success has been achieved in control of the disease. This, perhaps, reveals the complexity of the disease and the diversity of cropping systems related to rice. It also indicates a deficiency of coordinated program in research and communication among scientists. Historically, rice may be considered an Asian crop, and for years, occurrence of bacterial blight of rice was limited to Asia in economic importance. The gradual increase in rice production and research and recent report of bacterial blight in other continents may dissolve the boundary. The interest in research and in understanding of the disease may also be extended to scientists outside of Asia.

Through extensive discussion and communication among scientists on different occasions and through scientists participating in a recent monitoring tour sponsored by the International Rice Research Institute to evaluate and review the bacterial blight situation in Bangladesh, India, and Nepal, widespread interest has developed in organizing an "International Working Group on Bacterial Blight of Rice."

The objectives of this group would be:

1. To promote collaborative effort on research of the disease.
2. To strengthen communications between those interested in bacterial blight of rice.
3. To identify genetic sources of resistance to bacterial blight in screening and in breeding programs.

618

4. To establish international rice differentials to study the virulence/pathogenicity of *Xanthomonas oryzae*.

5. To organize workshops, symposia or conference to identify, and coordinate research in understanding and in developing effective controls against bacterial blight of rice.

A 10-member committee has been identified to organize the working group. Dr. T. W. Mew has been named secretary of the committee until a workshop is organized and officials are elected.

Members of the organizing committee:

Dr. A. Ezuka,	*Japan*
Dr. C. T. Fang,	*People's Republic of China*
Dr. G. S. Khush,	*The International Rice Research Institute (IRRI) Philippines*
Dr. T. W. Mew,	*IRRI, Philippines*
Dr. S. A. Miah,	*Bangladesh*
Dr. J. L. Notteghem,	*France*
Dr. A. P. K. Reddy,	*India*
Dr. D. M. Tantera,	*Indonesia*
Dr. S. Wakimoto,	*Japan*
Dr. S. Z. Wu,	*People's Republic of China*

Secretary of the committee: Dr. T. W. Mew, IRRI

General Meeting Report

The Bacteriology Section Committee of the International Society for Plant Pathology (ISPP) held its first general meeting on Friday, August 21 during the 5th International Conference on Plant Pathogenic Bacteria.

In order of business, the Chairman's report by Dr. John Young was summarized by Dr. Vidaver. She reviewed the decisions to hold the 5th International Conference in a year intermediate between the next International Congresses of Plant Pathology and to focus attention on the problems of tropical bacterial diseases. The latter decision resulted in the Conference being held at the CIAT facilities in Cali, Colombia, South America. Furthermore, the Section Committee proposed that the following resolutions be submitted to the ISPP for governance of the Section.

1. The Bacteriology Section of the International Society for Plant Pathology is constituted by those members of the Society who have an interest in the research or teaching of topics related to bacterial plant disease.
2. The Section shall meet from time-to-time in Conference.
3. (a) At the Conference, a Section Meeting shall be held at which a new committee shall be elected by a vote of those present.
   (b) The committee of six should include one member of the old committee.
   (c) The responsibility of the Section Committee is to coordinate matters relevant to that Section's interests as they arise. The Section Committee has overall responsibility for Conferences and for any subcommittees formed in the Section for specific purposes.

These resolutions were passed.

The data and venue for the 6th International Conference were discussed. It was recommended that the next Conference be in 1985. Voting for the site resulted in selection of Beltsville, MD, U.S.A.

The new Section Committee was elected by unanimous ballot. The Chairman had asked a Nominating Committee to assist in bringing nominations to the assembly. The Nominating Committee consulted with present and past Section members, and took into account nominations received by Dr. Young. The criteria for nomination included: area of expertise, geographic distribution to ensure reasonable international representation, and active interest and participation in some area of Bacteriology. Furthermore, in accordance with resolution 3 (above), a member of the immediate past Section was to be nominated to maintain continuity. The Section members elected were: Drs. Civerolo, Lozano, Perombelon, Panagopoulos, Ribeiro, and Sequeira (Chairman).

In accord with the ISPP Taxonomy Committee's recommendation, the Bacteriology Section voted affirmatively to ask that the ISPP Executive Committee transfer responsibility for the Committee on Taxonomy of Phytopathogenic Bacteria to the Bacteriology Section. The new Section

Chairman is to request that the transfer take effect at the earliest convenience of the ISPP Executive Committee.

Assuming that the transfer is endorsed, future elections of the Taxonomy Committee shall be conducted by the Bacteriology Committee and held at each International Conference on Plant Pathogenic Bacteria. Interim resignations are to be filled by a consensus of the Taxonomy Committee or at interim meetings organized by the Bacteriology Section.

Assuming favorable action by the ISPP Executive Committee, the Nominating Committee was requested to provide names for the Taxonomy Committee. Criteria were to include interest and expertise in bacterial taxonomy. In order to maintain continuity, it was suggested that four members be retained. After nominations from the floor were received, a written ballot was taken for the two open positions. The new members elected were Drs. Dickey and Vidaver. The members re-elected were Drs. Dye (Chairman), Bradbury, Goto, and Hayward.

In the absence of Dr. Dye, Dr. Bradbury reviewed the activities of the ISPP Taxonomy Committee. These activities included submission of 3 lists to the International Committee on Systematic Bacteriology (ICSB) for inclusion in the 1980 Approved Lists of Bacterial Names. These were:

Names of approved species for retention. These had been sent in and collected over the previous 2 or 3 years. This list was accepted.

Names of species that could not be adequately differentiated in laboratory tests but were essential for plant pathologists, i.e. 'nomenspecies'. This list was rejected.

Names of plant pathogenic bacteria of uncertain generic position, proposed to retain the names. This list was accepted.

It was noted that the Approved Lists contain some inadvertent omissions, but that these should be corrected in due course.

It had been decided at the 4th International Conference that should list 2 be rejected, an alternative system using 'pathovars' be proposed to retain the names of bacteria that plant pathologists wished to differentiate. This was done and published in the Review of Plant Pathology 59:153-168, 1980. The list includes numbers and locations of pathotype cultures, as well as a 'Set of Rules' called International Standards for guidance in the use of the system.

Future work of the Taxonomy Committee will include drawing up minimum standards for the description of plant pathogenic taxa. This was requested by the ICSB. Also, the Committee is to carry out suggestions from the 5th International Conference to compile lists of new pathovars as they come out, to publish them periodically in the Review of Plant Pathology or possibly elsewhere, and to study and make recommendations on the correct use of terms covering infrasubspecific taxa, e. g. pathovars, pathotype, f. sp., strain, race, cultivar, isolate, culture, etc.

In order to keep the pathovar register accurate, everyone who intends to publish a new pathovar name, is asked to advise the Chairman of the Taxonomy Committee; this should ensure that the name does not conflict with another name that has been or is being published. An example of possible duplication is *E. chrysanthemi* pv. *dianthicola* and *E. chrysanthemi* pv. *dianthi* both published in 1979.

The meeting closed with a resolution: The ISPP Bacteriology Section

expressed its profound thanks to Dr. J. Carlos Lozano, Mr. Robert Zeigler, and their colleagues and support staff at CIAT for their herculean efforts in arranging for an outstanding 5th International Conference on Plant Pathogenic Bacteria.

Respectfully submitted,
Anne Vidaver
Chairman, pro-tem.

Group Reports

GROUP I. RECENT DEVELOPMENTS IN *PSEUDOMONAS SOLANACEARUM* RESEARCH. Chairman: Carlos Martin

Originally, the discussion groups were organized in a free-speaking manner with no formal paper presentations. However, because of the limited time available for each session the discussions in this group, as in others, were based on the informal presentation of five speakers in addition to the introduction to the topic presented by the chairman.

More than explaining the recent findings on *P. solanacearum* research, the chairman focused his introduction on research problems that need prompt attention. He clearly emphasized the need for the scientists to get more involved in field-applied research. Needs were discussed for more research in areas such as plant resistance, agronomic practices, soil survival of the bacterium genetics, and better methods for screening for resistance, for example, the use of serology to determine latent infection in seed production programs in developing countries.

The first presentation, "Survey of bacterial strains from various origins for their plasmid content," was given by Dr. T. Currier (USA). From 20 strains observed, including fluid and non-fluid ones as well as strains from races 1, 2, and 3, only 6 contained plasmids. Results indicated that nonfluidal strains do not arise as the result of the loss of a plasmid from the virulent strain. The role of flagella in relation to virulence was also discussed, indicating that many strains are highly stable and they never form the flagellated avirulent type. The change from fluid to nonfluid type had been observed by several of the participants; however, the reverse process from the nonfluid to fluid type has never been observed.

The second presentation was made by Dr. N. Mukherjee (India) on "Problems of the bacterial wilt of solanaceous vegetables in West Bengal." He indicated that after sampling four places with soil pH from 4.2 to 8.0, *P. solanacearum* was found to cause wilt in tomatoes, chilli, eggplant, potatoes, and four weed species. Eggplant was the most susceptible of the plants tested.

Dr. R. Ribeiro and C. Robbs of Brazil presented some information on breeding for resistance to bacterial wilt in some crops, and the importance and occurrence of Moko disease in bananas. Dr. Ribeiro explained that all 3 races of *P. solanacearum* are present in Brazil, affecting several different hosts such as potato, banana, tobacco, eggplant, cowpea, as well as several weed species. Breeding programs have been initiated for tobacco, eggplant, and tomato, principally. The lack of quarantine regulations within Brazil was also discussed, as well as their implications in favoring the spread of the disease from one state to another.

Dr. Robbs explained that Moko disease of bananas is spreading very fast and getting very close to large plantations in the south and northeast of Brazil. He also pointed out the disease probably came from the Guyanas because the SFR strain of the bacterium was found there before it was found in Brazil. However, he also pointed out that both strains of *P. solanacearum* infecting bananas have been found in Brazil.

The fourth presentation was made by Dr. E. Echandi (USA) on "Biological Control of *P. solanacearum* in tobacco." One hundred and forty-

nine strains were screened for bacteriocin production and from these only the avirulent ones were selected. Root-dipping inoculation was used to inoculate tobacco plants before they were transplanted. By using different strains and different inoculum concentrations of a virulent strain he found that some bacteriocin-producing strains could protect tobacco plants in soils infested up to 10^7 CFU/g of soil in greenhouse experiments. However, field trials have given some conflicting results.

Dr. Echandi also explained that avirulent strains that do not produce bacteriocin also protected tobacco plants but bacteriocin-producing strains are much better. He mentioned that the ideal strain to protect plants should be one that multiplies extensively in the roots, induces resistance in the plant, and is a bacteriocin-producer. Discussion went on to the possibility of using tolerant tobacco plants together with inoculation with a bacteriocin-producing strain.

The final presentation was by Dr. L. Sequeira (USA) on recent finds on induced resistance in potato to bacterial wilt. The possible role of agglutinins (lectins) in resistance of potato clones to *P. solanacearum,* and the interactions of *P. solanacearum* cell surface polysaccharides (LPS and EPS) and potato lectins were discussed.

GROUP II. RECENT DEVELOPMENTS IN SOFT ROT BACTERIA AND POST-HARVEST DECAY RESEARCH. J. P. Mildenhall, Chairman.

Introductory Remarks by J. P. Mildenhall. Studies on the soft rot bacteria have progressed on a broad front·since the Angers Congress in 1978. The growing importance of *Erwinia* as a plant pathogen has been indicated by several reports of new diseases caused by this organism. Other areas in which studies have progressed include the genetics, ecology, host specificity, serological relationships of soft rot *Erwinias* and the mechanism of enzyme action in soft rot development. To achieve closer cooperation between workers in this broad field, the formation of an international soft rot working group is being discussed.

Comments on "Inoculation of Perishable Produce During Post-harvest Handling" by J. A. Bartz. Considerable post harvest decay of pepper and tomato fruit results from the washing process. Both the temperature of the fruit and the temperature of the wash water influence the ingress of pathogens. Warm fruits immersed into cool wash water absorb water, thereby enabling ingress of pathogens. The depth of the fruit in the wash water and the duration of washing also influence the amount of water absorbed. To limit post harvest losses, free water should not remain in contact with fruit for more than 5 minutes; fruit should not be harvested wet and the temperature of the wash water must be controlled.

Comments on "Soft Rot Problems in Potato Storage and Transit" by A. Kelman. Major losses occur in the potato industry during storage and transit. To assess the potential for soft rot development in potato tubers,

humidity was employed for inoculation experiments. Tubers harvested by hand had a 2.5% soft rot potential, whereas, in mechanically harvested tubers the soft rot potential increased up to 100%. During storage the tubers "recovered" and soft rot potential decreased. Handling associated with removal of tubers from storage caused an increase in soft rot potential.

Potato tuber tissue with a low water potential has a lower soft rot potential than potato tissue with a high water potential. In addition, the calcium level in the tuber also influences the potential for soft rot development.

Comments on "Soft Rot Problems in Selected Tropical Crops" by Jorge Victoria. A series of slides were shown on soft rot diseases affecting tropical crops.

Comments on "The Problem of Lactose Utilization by *Erwinia Chrysanthemi,* **by J. P. Mildenhall.** Lactose utilization by *E. chrysanthemi* is reported to be a variable characteristic. When *E. chrysanthemi* pv. *zeae* was streaked on a lactose mineral salts yeast extract agar, a thin film of growth developed over a 48 hour period. By 60 hours, turbid slimy colonies developed at points over the streak. Subsequent transfer of the slimy colonies showed that they were able to metabolise lactose and produce a slime. These lac+mutants retained this character upon subsequent transfer. On nutrient agar, however, colony appearance was identical to the lac⁻type.

Comments on "Future Work" by A. Chatterjee. The following areas of research in the soft rot group demand further investigation: (1) Epidemological aspects, e.g. Serotyping; (2) methods for inoculation in order to develop screening for resistance; (3) role of associated bacteria in soft rot disease especially spore forming types; (4) influence of water relationships; (5) oxygen effects on pectolytic enzymes; (6) polygalacturonate transeliminase, (a) structural differences of isozymes, and (b) mode of excretion and factors that influence excretion; (7) polygalacturonase; role of exotype in generating PATE activity; (8) role of other extracellular enzymes in pathogenicity, (a) protease, (b) cellulase, and (c) phospholipase; (9) role of cyclic AMP in pathogenicity; (10) genetics of *Erwinia carotovora;* and (11) role of plasmids in pathogenicity.

Report on International Working Group by A. Kelman. Under the aegis of ISPP, a soft rot working group is to be established. Dr. M. C. M. Perombelon was nominated as chairman.

GROUP III. RECENT DEVELOPMENTS IN PLASMIDS AND PATHOGENICITY RESEARCH. A. K. Vidaver, Chairman.

A short review of past and recent work was presented, reflecting the intense interest in plasmids, particularly of *Agrobacterium tumefaciens.*

The Cali group was interested not only in the role of plasmids in pathogenicity, but also in plasmids of unknown function (cryptic), as well as physical and genetic characterization. It was evident from the presentations that plasmids are very common in all the easily cultivable genera of plant pathogenic and related bacteria. However, plasmids are not universally present, according to current detection methods. It was emphasized that newer methods, particularly *in situ* lysis, might enable detection of very large plasmids.

Problems and pitfalls in working with plasmids were discussed; for example, the difficulty of detecting a single or small number of virulent transconjugants by bioassay in the presence of large numbers of avirulent cells. The use of transposable genetic elements (transposons) in marking and mutagenesis of plasmids and the chromosome were discussed. Some plasmids of *Pseudomonas syringae* pathovars appear to code for metal resistance and bacteriophage receptor sites. In cultivation of other strains of *P. syringae*, it was postulated that the changes observed in plasmid mass and restriction endonuclease fragments as well as plasmid homology to the chromosome might be due to a transposable element analogous to the bacteriophage Mu; no phage production by these strains has been detected, however.

Finally, it was recommended not only that work in different plasmid systems be continued, but also that the genetics of the chromosome be examined more extensively to clarify its role in pathogenicity and other properties.

GROUP IV. RECOGNITION MECHANISMS OF BACTERIAL PATHOGENS— THE CURRENT VIEW. Luis Sequeira, Chairman.

There is little question that the field of recognition, as it pertains to plant pathogenic bacteria, has finally come of age. The upsurge of interest in this field is evidenced by the packed-house attendance at recent scientific meetings in which recognition, adhesion, and host response mechanisms have been discussed. The discussion session on this topic at the 5th International Conference on Plant Pathogenic Bacteria was no exception. The large audience listened for 2 1/2 hours to a discussion of a wide range of subjects dealing with the architecture of the outer membrane of Gram-negative bacteria (A. Chatterjee), the role of extracellular polysaccharides (EPS) in virulence (D. Coplin), the induction and repression of the hypersensitive reaction (HR) (R. Stall and S. Patil), and the role of plant agglutinins in disease resistance (R. N. Goodman, presented by L. Sequeira). The final presentation dealt with possible control of recognitory structures in bacteria by phase variation in transposon insertion (D. Mills).

In the introductory remarks (L. Sequeira), an attempt was made to point out the problems involved in the study of the wide range of recognition events involved in the interaction of plant pathogenic bacteria with their hosts. This was illustrated with the *Pseudomonas solanacearum*-tobacco

system, in which some of the details are known concerning the biochemistry of the process of attachment of bacteria to host cell walls and of the ensuing response of the host cell. Other speakers provided evidence from a variety of other host-parasite systems and it became clear that it is not possible to generalize as to which structures are important in recognition.

It is difficult to provide a summary of the discussions that took place during this session because of the widely divergent topics that were covered. The following is an attempt to bring into focus some of the conclusions that were reached and some of the opinions that were expressed.

The Phenomenon of Attachment

Bacteria adhere to a wide variety of inert surfaces and, therefore, it is not surprising that they should attach to plant cell wall surfaces. Whether they do so specifically on certain tissues is the real question. There is considerable physical evidence for attachment of bacteria to leaf, mesophyll cell, and root surfaces, but (a) there is lack of quantitative evidence for possible differences in frequency and degree of attachment of homologous or heterologous strains of bacteria on a particular host, and (b) specificity in attachment has been shown only in the case of symbiotic bacteria (*Rhizobium* spp.) involved in nodulation of particular legumes, and, more recently, for certain HR-inducing, avirulent strains of *P. solanacearum*

The recent development of quantitative methods to determine the kinetics of the process of attachment, particularly in the laboratories of Huang, Sequeira, and Bauer, indicates that we are beginning to resolve the first problem. The problem of specificity, however, remains difficult to approach in most host-parasite systems. The general consensus, however, is that attachment is an essential first event in recognition and that, with the exception of rhizobia and *Agrobacterium tumefaciens,* pathogenic strains of bacteria somehow avoid or inhibit attachment to the host cell wall.

Bacterial Cell Wall Structures Involved in Recognition

There is increasing evidence that the production of EPS, either in the form of capsules or slime, is essential for virulence of plant pathogenic bacteria. The most recent evidence stems from the collaborative work in the laboratories of Coplin, Sequeira, and Kelman on the role of EPS of *Erwinia stewartii.* There is the suggestion that the presence of EPS in the form of a capsule effectively blocks recognition of other cell wall constituents by plant aglutinins. Thus, the role of EPS may be to provide a physical barrier that prevents attachment of the bacterial cells and, plus, to avoid subsequent host responses that would prevent bacterial multiplication in the intercellular spaces or in xylem vessels. That this is an oversimplified interpretation is clear from the work with rhizobia, which appear to depend on specific EPS terminal sugar residues for attachment to particular legume root surfaces.

That the outer membrane components of Gram-negative bacteria may be involved in recognition is shown by the demonstration that strains of *P.*

solanacearum with defective (rough) LPS are more rapidly attached by tobacco cell walls than those with complete (smooth) LPS (Duvick). Also, some rough LPS strains induce the HR in tobacco leaves, whereas smooth strains do not. Thus, the possibility that terminal sugar residues on LPS carry the information that is essential to firm attachment and for the induction of host responses remains an attractive possibility.

The information potential of the proteins in the outer membrane has been disregarded, although, as Chatterjee pointed out, they are of considerable significance in the pathogenicity of mammalian pathogens. In particular, the fimbriae (pili) of strains of *E. coli* have been shown to be responsible for specific attachment to the intestinal mucosa. It seems likely that similar proteins, which have lectin-like activity and project out from the outer membrane, play an important role in attaching the bacterium to the plant cell wall. The process may depend on recognition of specific saccharides on the plant cell wall, but these are probably of common occurrence and, thus, all bacteria containing the same type of pili would be expected to attach.

Plant Cell Wall Structures Involved in Recognition

Although, as indicated above, bacterial lectin-like proteins may be involved in recognition, research has centered mainly on the possibility that plant proteins play the role of the receptor. Emphasis has been placed on cell wall, seed, or tuber proteins that have the capacity to agglutinate bacteria. Interest in these proteins stems from their ability to bind differentially to avirulent, but not to virulent forms of plant pathogenic bacteria. A recent addition to a fairly long list of such proteins is the one isolated from apple seed by Goodman and collaborators, which is capable of specifically agglutinating strains of *Erwinia amylovora*. As in the case of the potato agglutinin that interacts with strains of *P. solanacearum* (Leach and Sequeira), it is proposed that the activity of the apple agglutinin is mediated by charge-charge interactions with the negatively charged LPS of the bacterial outer membrane. It is difficult to envisage a specific role of such proteins in bacterial attachment, but they are present on the cell wall (potato agglutinin) and in xylem fluids (apple agglutinin) where they are likely to interact with particular strains of the bacterial pathogens.

Induction of Hypersensitive Response

The best evidence for recognition between host and pathogen is the fact that only certain bacterial strains, on certain plant hosts, induce the HR. That very close contact (attachment?) between the host cell wall and the bacterium is essential for induction of the HR has been shown by Stall and co-workers in the *Xanthomonas vesicatoria*-pepper system. What transpires between the bacterium and the host prior to collapse of the host cells, however, is unknown. The general consensus is that more emphasis needs to be placed on the "transducing" mechanism that carried the message between the cells and the host cell plasmalemma. The fact that certain bacterial toxins interfere with the expression of the HR and, thus, allow growth of the pathogen (Patil) could be very useful in the analysis of the receptor for the inducer of the HR. By analogy with fungal pathogens which produce "elicitors" of the HR, which are components of the hyphal

cell wall or produced by degradation of the plant cell wall, it seems likely that similar "elicitors" might operate in the case of bacterial-plant cell interactions. Indeed, there is limited evidence from Keen's laboratory that bacterial cell wall proteins may play this role.

The Genetic Control of Recognition

As was pointed out by Mills, modern genetic analysis has not been applied to the problem of recognition of plant-pathogenic bacteria. Reference was made to phase variation in the insertion of transposons in *Salmonella*, in which, depending on the direction of transcription, different flagellar antigens are produced. The use of such transposable elements could be extremely important, to determine, in a critical way, whether outer membrane proteins or polysaccharides are indeed involved in recognition of bacteria by the plant. It is hoped that greater efforts to apply these and other modern genetic approaches to the problem of recognition in the next few years will provide some of the critical evidence by the time the next International Conference on Plant Pathogenic Bacteria takes place.

GROUP V. CRITERIA FOR DISTINGUISHING RACES VERSUS STRAINS OF BACTERIAL PATHOGENS. C. M. E. Garrett, Chairman.

Many people had expressed interest in this subject but mainly from the standpoint of receiving rather than imparting information as few contributions were offered beforehand.

Participants were reminded that among the many methods employed for race and strain differentiation were pathogenicity tests, colonial morphology, biochemical characteristics, phage sensitivities, and serological methods. Some of these had greater value with some genera or species than with others. The complexity of the problem facing plant bacteriologists was highlighted in four introductory talks.

Dr. French spoke of the difficulties encountered in the selection of certain potato clones where resistance to *Pseudomonas solanacearum* was not race specific but pathovar specific and, furthermore, decreased as temperature, moisture and inoculum potential increased. A rapid method of indentifying pathovars, he pointed out, is urgently needed for more efficient selection of resistant potato clones.

The epiphytic populations of *P. syringae* pv. *savastanoi* on olive were described by Dr. Ercolani as heterogeneous. Many factors, including time of year, leaf age, time of leaf formation, and such environmental factors as air temperature and rainfall etc. influence these populations. Cluster analysis of physiological and biochemical characteristics indicated that the only homogenous population was found in January. At other times a range of strains of different biochemical types have been found.

The advantages of serology for distinguishing between organisms were expounded by Dr. Boer for strains intermediate between *Erwinia carotovora* var. *carotovora* and *E. c.* var. *atroseptica*. This had proved of

value in ecological and epidemiological studies. Civerolo showed that of the three pathotypes of *Xanthomonas campestris* pv. *citri*, pathotype A was clearly serologically distinct from the others in ELISA tests.

The general discussion centered on the definition of the term 'pathovar' as set out by Dye *et al.* (1980) as the conference was obliged to consider it and this seemed an opportune time. A single amendment to the existing wording was proposed so that the definition reads: "The term pathovar is used to refer to a strain or set of strains with the same or similar characteristics, differentiated at infra subspecific level from other strains of the same species or subspecies on the basis of distinctive pathogenicity to one or more plant host species."

Dr. Bradbury reported that the ISPP Bacteriology Taxonomy Committee were drawing up minimum standards for the description of plant pathogenic bacteria. This should help those who need to describe new organisms; in the meantime any new organisms should be described as fully as possible. It was proposed that this committee might consider acting as a clearing house for names of pathovars and publish an annual list of names that have been accepted.

GROUP VI. METHODOLOGY FOR EVALUATING PLANT RESISTANCE TO BACTERIAL PATHOGENS. G. L. Ercolani, Chairman.

The discussion centered on some aspects of methodolody that require priority attention to establish acceptable procedures for assessing plant resistance to bacterial pathogens. These included the nature and composition of the inoculum, the method of inoculation, the type of host plants, and the definition of the indicating effects and the resulting estimates of resistance.

Major contributions to the discussion were made by E. R. French, Centro International de la Papa, Lima, Peru; C. M. E. Garrett, East Malling Research Station, Kent, UK; H. Maraite, Laboratoire de Phytopathologie, Université Catholique de Louvain, Louvain-la-Neuve, Belgium; K. Rudolph, Institut für Pflanzenpathologie and Pflanzenschutz, der Georg-August-Universität, Grisebachstrasse, Göttingen, Federal Republic of Germany; and J. E. Bowman and L. Sequeira, University of Wisconsin, Madison,WI, USA.

Dr. French gave an account of his work on "Selection of resistance to *Erwinia* soft rot and *Pseudomonas solanacearum* wilt of potatoes." Different approaches have been used at the International Potato Center to develop resistance to two bacterial pathogens. For tuber resistance to *Erwinia chrysanthemi*, infectivity titration inoculations were made to tubers inoculated anaerobically. Consistent results and a full range of resistance to susceptibility were found among hundreds of clones. No breeding has yet been initiated and still to be determined is the possible correlation between tuber resistance to soft rot and plant resistance to

blackleg. With bacterial wilt resistance (*Pseudomonas solanacearum*), the level of inoculum that gives the greatest differences among individuals in a segregating population bred for resistance, under standardized environmental conditions, is used. Results are not yet very consistent or always representative of field screening results. The use of infectivity titration inoculation methods would be very labor and space intensive, but current work may resort to it eventually.

Dr. Garrett reported on "Problems in Screening for Stone Fruit Resistance to Bacterial Canker. "New clonal cherry rootstocks selected at East Malling Research Station were assessed for resistance to bacterial canker. Resistance was not stable at the seedling stage, and trees should be assessed when not less than three years old. Variation in soil conditions markedly affected the response of the rootstock. When a new race of *Pseudomonas syringae* pv. *morsprynorum* was included in the tests many rootstocks were equally resistant to both this and the typical race, but a few hybrid rootstocks had very low resistance to the former. Plum trials under more uniform conditions gave less variable results, but seasonal and other factors induced some variation between inoculations of one year and another, and between inoculations and natural infections.

Dr. Maraite discussed his experience in evaluating resistance of cassava to *Xanthomonas campestris* pv. *manihotis*. Cassava bacterial blight is characterized by angular spots and blight areas on the leaves and tip die-back after systemic infection of the stem. There are differences in cultivar resistance to stem infection. In order to reveal and to study differences, Maraite tried various inoculation methods. Pricking the internode between the third and the fourth leaf from the top with a needle contaminated by passing it through a 2-day-old culture of the pathogen gave more consistent results and permitted a better study of the progress of infection and evaluation of the cultivar resistance than stem injections with known concentrations of bacterial suspensions or insertion in the node of wooden toothpick saturated with known concentrations of bacterial suspensions.

Dr. Rudolph considered the influence of inoculating bean leaves with a mixture of low and highly virulent isolates of *Pseudomonas syringae* pv. *phaseolicola* on the final reaction. When testing the degree of resistance of bush bean cultivars towards pv. *phaseolicola* he found it is better to do several inoculations with different bacterial isolates (or races) on different individual plants. A mixture of bacterial isolates had been used earlier, hoping that the most virulent ones would always dominate the reactions. This assumption was not always valid. Isolates of low virulence can favor a resistant reaction also toward the highly virulent isolates and thus lead to wrong grouping of some plant cultivars.

Dr. Sequeira spoke on "Resistance to *Pseudomonas solanacearum* in potato: infectivity titrations in relation to multiplication and spread of the pathogen." When several wilt-resistant potato clones were tested against a highly virulent strain from Mexico (No. 276) by standard stem inoculation methods, only one clone of *Solanum phureja* was resistant. When different inoculum concentrations were introduced quantitatively for infectivity titrations, however, different levels of resistance were clearly defined among clones previously rated as susceptible. Linear regression was used

to estimate the ED_{50} values (dosage required to wilt 50% of the population) for each of seven clones. These values ranged from 3 and 100 colony-forming units (CFU) for Katahdin and Russet Burbank, respectively, to 2.1 x 10^6 CFU for *S. phureja* clone 1386.15. The distribution of bacteria in stems of Russet Burbank plants inoculated with the compatible strains 276 and K-60 and the incompatible strains B1 and S-210 was very different. In all cases, incompatible bacteria were not detected 10 cm above the inoculation point in the stem, even by 12 days after inoculation, whereas compatible strains had multiplied rapidly at this site by 6 days. At the inoculation site and at sites adjacent to it, incompatible bacteria attained populations that only differed by one order of magnitude from those of compatible bacteria. Similar results were obtained when a resistant clone (MS 118.24) was inoculated with the virulent strain K-60. Thus, resistance was characterized both by reduced acropetal spread and by tolerance to large numbers of the bacterium.

GROUP VII. SEED—BORNE BACTERIAL PATHOGENS AND THEIR DETECTION. A. W. Saettler, Chairman.

Seed pathology and seed-borne bacterial diseases are becoming important subject matter areas in plant pathology. For example, the American Phytopathological Society and the Association of Official Seed Analysts recently established Seed Pathology Committees. In the U.S., two regional research projects are devoted specifically to seed-borne plant pathogenic bacteria: NC-100 is concerned solely with black rot of crucifers (*Xanthomonas campestris*), while NC-135 deals with "Detection, survival, and control of plant pathogenic bacteria on seeds and other plant parts." At the international level, the International Seed Testing Association (ISTA) Committee on Plant Disease has established a working group on seed-borne bacterial pathogens.

Our present subject of seed-born bacterial diseases and their detection is important for several reasons:

1. Infected/contaminated seed is a major source of primary inoculum in almost every important food and fiber crop routinely propagated by true (botanical) seed.

2. Recent establishment of germplasm centers for the major food crops, and the widespread exchange of seed between countries expand the opportunity for long distance dissemination of seed-borne bacteria.

3. The threat of such dissemination becomes even more meaningful due to the existence of pathogen strains/races with pathogenic potentials greater than those existing in countries receiving such strains.

4. Several recent studies have demonstrated transmission of phytopathogenic bacteria in seed of even tolerant crop cultivars,and

in seed of susceptible cultivars harvested from plants showing little or no disease symptoms. Thus, seed certification programs based on visual inspections to guarantee freedom from disease, may not be completely effective.

Discussion Group VII focused on two aspects of seed-borne phytopathogenic bacteria and their detection, *Isolation* and *Identification*.

Isolation methods include selective media/enrichment as well as direct plating. B. N. Dhanvantari, Ontario, Canada, contributed a special paper on this entitled, "Semi-selective Media for Detection and Monitoring of Some *Xanthomonas campestris Pathovars.*"

There is considerable current interest in selective media, and numerous phytobacteriologists are employing this tool in epidemiological studies.

Identification methods suggested for use with seed-borne phytopathogenic bacteria included selective media, serology, bacteriophage, pathogenicity, and growout tests. Two papers were contributed on this subject. The first, "Demonstration of Contamination of Cassava Flowers, Fruits, and Seed by *Xanthomonas manihotis,*" was by J. F. Daniel and B. Boher, Republic of the Congo. Subsequent discussion emphasized the extreme caution one should exercise in assuming that specific bacteria detected by immunofluorescence are viable. The second paper, by A. W. Saettler and G. E. Trujillo, East Lansing, Michigan, was on "Serology and Semi-selective Medium for the Detection of *Xanthomonas* Blight Bacteria in Bean Seed."

Discussion was then directed toward defining research priorities and the following were developed:

1. Little is known regarding the relationship between incidence of seed contamination, disease development, and effects on yield. This information is urgently needed to establish phytosanitary tolerance.

2. Participants could not agree on whether a zero tolerance was the best choice.

3. Techniques for sampling seed lots, and establishing confidence limits have not been satisfactorily resolved.

The discussion session participants unanimously recommended that (1) the ISPP, Bacterial Section, continue to recognize the importance of seed transmission of phytopathogenic bacteria, and that (2) a Session on Methods of Eradicating Seed-Borne Bacterial Pathogens be included in the 6th International Conference on Plant Pathogenic Bacteria.

GROUP VIII. Role, and challenges to international and national agricultural research in management of bacterial diseases. H. D. Thurston, Chairman; summary by Robert Zeigler.

The discussion group leader opened the session with a summary of the various species, international, regional, and national, involved in tropical

agriculture research and extension. A brief critique of the current coordination revealed inefficiency due to tremendous duplication of effort and imbalance in funding. National research and extension programs, which are the essential links in disseminating and implementing information developed by regional and international agencies are, almost without exception, severely underfunded and understaffed. The problems confronting national programs are compounded by their difficulty in keeping the scientists they send to developed nations for training. This leads to serious problems in terms of continuity of research.

There was general agreement among participants that bacterial diseases in the tropics should be approached from a management rather than eradication perspective. That is, learning to live with a given, predictable and stable level of disease. Within this general context a discussion on the integrated pest management (IPM) concept followed. Host-plant resistance and cultural practices were generally considered to be the components of IPM most readily available for implementation within the socio-economic constraints of the developing world. However, the group repeatedly emphasized that the challenges in the tropics, as represented by the number of crop species, and the number of pathogens attacked by them, dwarfed those encountered in temperate zones.

Official quarantine and seed certification programs, while attractive in principle, are not practical control measures in most developing nations. Chemicals, likewise, are of limited practical application, at least when considering small, low-input farming systems.

The subject then shifted to international centers. It was suggested that in these centers emphasis was placed on disease control through resistance at the expense of other methods, such as biological control and cultural practices. Staff members of several such centers agreed that there was a disproportionate breeder to pathologist ratio (given the group surrounding them, it is difficult to imagine them saying anything else). A discussion then followed on the role of the centers. It remained unresolved whether they should have a mandate weighted more toward generation of basic research, or toward technology transfer. It was suggested by one participant that basic research scientists could spend sabbatical leaves in the centers to strengthen basic research programs.

Scientists from national programs of several developing nations stressed the difficulties with which they are confronted. In terms of developing control measures through cultural practices they cautioned that measures effective against one problem may enhance another. Furthermore, cropping systems are often so complex that manipulations to control one pathogen may be impossible to define, or may perturb the system to an unacceptable degree. Each system requires study, with emphasis being placed on what might be called "holistic epidemiology and etiology." Such studies obviously require large numbers of skilled and dedicated scientists, well beyond the present capabilities of national programs.

A point was made regarding the use of chemicals in developing countries. The combination of farmer ignorance, lax government regulation, and occasional unscrupulous representatives of agricultural chemical companies can result in improper, wasteful, dangerous, and counterproductive use of chemicals in those countries. Numerous disturbing

examples were presented. The blame has been passed several times between government and industry.

A very lively discussion then developed on exactly how much small farmers know about farming. What emerged was the consensus that small farming systems which, under different population and economic conditions, were successful are now being placed under severe strain as pressure for arable land decreases rotation times, etc. In addition, as farmers and settlers move to new areas, these systems are not necessarily transferable. Most participants agreed that a considerable amount could be learned from truly traditional agriculture. Those with some experience in the field related that small farmers desperately wanted improved techniques and varieties; however, they must be relevant to their needs and capabilities.

The discussion ended with a brief exchange of ideas on how best to approach the use of host resistance in disease management. It was felt that it is best to begin with locally adapted varieties already in use and try to improve them. Programs should do their best to release more than one variety at a time, each with different characteristics and tolerant to as many different constraints as possible. A "package" of varieties approach was thought to be most appealing. Within these objectives, two major pitfalls were mentioned. The first was that resistance will certainly not solve all problems, e.g. Moko disease of bananas. The second was that we must all keep in mind that the capacity of national programs to receive and effectively utilize resistant material is seriously limited.

GROUP IX. RECENT DEVELOPMENTS IN RICE BACTERIAL BLIGHT RESEARCH. T. W. Mew, Chairman.

This discussion group met on August 16th. It was regretted that most scientists in Asia whose research has contributed significantly to our understanding of the disease did not attend the conference. In response to the discussion group chairman prior to the conference, however, they provided information of past achievements, recent progress, and future efforts concerning research and collaboration. Their comments and ideas, together with those raised during the discussion session, are summarized in this report.

Because only a few participants had conducted research in the bacterial blight of rice, format of the discussion session was organized to review the research work either achieved or being carried on in Asia. Through this brief review it was hoped that a general understanding of the disease and problems associated with its research would be apparent. It should be acknowledged, however, even though attempted, it was impossible for this brief review and discussion to cover every facet of research and achievement made by various scientists. Highlights of the discussion included:

— The bacteriophage method has been used to forecast rice bacterial blight outbreaks in Japan in rice culture under irrigation. To adapt it to rainfed rice, the method should be carefully examined and evaluated.

— Survival of the bacteria in rice stubble, straw, and in weed hosts such as *Leersia* spp. is important to perpetuate the pathogen.

— Although it has been demonstrated that the bacterial pathogen can be carried by seeds, the nature of seed transmission, and its importance, especially in the tropics, is not certain. Whether the disease recently reported in Africa and Latin America relates to seed transmission from Asia needs to be confirmed and studied.

— *Xanthomonas campestris* pv. *oryzae* has been reported to produce some phytotoxic substances. Their role in pathogenesis of the rice bacterial blight syndrome is yet to be elucidated.

— Three serovars, A, B-1, and B-11 are known among strains of the bacterium in Asia. Serovar A appears to be most widely distributed. The serological relationship of the Asian strains to those in Africa and Latin America may provide useful information as to whether they are closely related.

— Pathogenic specialization of *X. campestris* pv. *oryzae* on rice cultivars that have specific genes for resistance to bacterial blight has been reported. A set of rice differential cultivars is being developed through a collaborative effort among Asian scientists. These differential rice cultivars are useful not only to develop resistant rice varieties but also to map the virulence of the races of the bacterium in Asia and outside of Asia. With this information, the rice *X. campestris* pv. *oryzae* may provide a useful system to study the resistance mechanism, genetics of host-parasite interaction of a bacterial disease, virulence, pathogenesis, and other factors.

— Rice cultivars infected by an incompatible strain of the bacterium have revealed that the bacterial cells embedded in fibrillar materials, apparently derived from the vessel cell wall. No fibrillar material has been observed to surround the bacterial cells in rice cultivars infected by a compatible strain.

— Planting of resistant rice cultivars has been effective in keeping the disease in check in many otherwise epidemic-prone regions in Asia. Breeding rice for bacterial blight resistance is therefore successful and the effort should be continued. Scientists should be cautioned to observe and monitor whether new races of the bacterial pathogen will evolve after extensive plating of resistant cultivars.

— Cultural practices such as managing diseased straw, and using lower rates of fertilizer, have been studied and related to the lower incidence of the disease.

— Chemical control is practiced in only a few countries. A consistently effective bactericide is yet to be developed for controlling the bacterial blight of rice. There is a need for one in regions where rice production is intensified.

— In evaluation of and screening for rice cultivars/lines resistant to bacterial blight, the clipping method of inoculation is efficient and widely used. There are concerns, however, if *X. campestris* pv. *oryzae* survives as epiphyte and if infection carries through broken trichomes, the difference of varietal response to the disease due to trichome density may not be detected by the clipping method of inoculation. No evidence has been found

that the bacteria could establish in the parenthymatous tissues of rice leaves. Trichomes may provide sites for epiphytic survival before the bacterial cells make their way to the hydathodes which are the natural opening for bacterial blight infection.

— A simple and rapid means to differentiate strains of *X. campestris* pv. *oryzae* or of pv. *oryzae* from other pathovars of the species is needed. The disease, however, can be diagnosed rapidly by cutting small pieces of leaf tissues from the margins of the lesion, placing them in a drop of water on a slide, and observing them for bacterial ooze streaming from the cut end.

— Infestation of rice plants with water weevils has been demonstrated to cause severe Kresek infection, the wilting phase of the bacterial blight syndrome. The role that insects play in long distance dissemination of the bacteria is not known.

Finally a proposal to establish an international working group (or committee) on bacterial blight of rice has been submitted to the International Society for Plant Pathology. Such a working group is, no doubt, needed and will promote collaboration among scientists, and exchange useful information. Last but not least, many scientists in Asia working on bacterial blight of rice have urged the Discussion Group Chairmen to express their gratitude to Professor Arthur Kelman for his personal interest and concern in the research of this important bacterial disease of a major food crop and for this generous assistance and encouragement to many of them.

GROUP X. SPECIAL PROBLEMS OF PLANT BACTERIOLOGY IN THE TROPICS. C. F. Robbs, Chairman.

Tropical countries are suited to supplying the food needed for future generations. Opportunity for continuously increasing agricultural production in those countries is enormous, due to favorable climates and adaptability of certain crops offering great potential. A good example is cassava, reported to produce more available carbohydrates per area than any other crop in the world. However, tropical regions also provide a perfect environment for a great number of plant pathogens, like the bacteria, capable of causing considerable damage, particularly when susceptible host genotypes are grown intensively in large areas.

In developing countries, bacterial wilt caused by *Pseudomonas solanacearum* has limited to a great extent the productivity of food crops such as potato, bananas, and plantain. Epiphytotics of bacterial wilt on bananas and plantains in the Amazon basin represent a serious threat for the well being of thousands of people, since these crops constitute the major sources of energy in the diet of native populations. Cassava bacterial blight (*Xanthomonas campestris* pv. *manihotis*) often reduces yield drastically and, under certain specific conditions, may completely destroy large plantations, thus generating famine, as occurred in south Zaire.

Citrus canker (*X. c.* pv. *citri*) has limited severely the production of citrus fruits in many Asiatic countries and has been a permanent threat to South

America. *X. c.* pv. *oryzae* also has been extremely destructive in Asia and plant pathologists must remain alert in regard to phytobacteria, such as this rice pathogen, which is able to disseminate disease long distances through contaminated seed.

A constant exhange of information, on an international basis, concerning pathogenic variability of plant bacteria, their potential to induce economic losses, means of survival, and dissemination, becomes exceedingly important, particularly for the benefit of developing countries. Likewise, information on the performance of genetic materials, as well as the maintenance of the existing gene pools, under the widest possible range of conditions, is essential for the breeding of resistant cultivars. Such resistant cultivars are currently the best approach to the control of bacterial plant diseases. Useful exchange of information is achieved through the organization of international conferences and symposia. Another important aspect linked to the progress of tropical plant bacteriology is a continuous and efficient training program aimed at preparing specialists in the field. This can be successfully and more easily carried out by internationally funded institutions such as CIAT.

A paper, included in the Special Contributions section of this publication, entitled "An Annotated List of Bacterial Plant Pathogens Occurring in Brazil" was presented. Highlights of the paper and discussion following its presentation included:

(a) Citrus canker in Latin America - Characterization of strains and epidemiology of *X. c.* pv. *citri;* control strategies.
(b) Coffee bacterial diseases - Pathogens involved; role of "ice nucleation" activity of resident populations of *Pseudomonas syringae* pv. *garcae* on disease severity.
(c) Wildfire of *Phaseolus* beans, soybeans, and tobacco pathogenic specialization of the causal bacterium (*P. s.* pv. *tabaci*); epidemiological aspects.
(d) Mango leaf and fruit spots - Variability and host range of the pathogen (*X. c.* pv. *mangiferae indicae*); disease resistance.
(e) Cassava bacterial wilt - Epidemiology and control of the causal agent (*X. c.* pv. *manihotis*).
(f) Bacterial spot of sweet pepper, tomato, and eggplant - Pathogenic grouping within *X. c.* pv. *vesicatoria;* epidemiology.

Conclusions

A consensus in the group following discussion included:
(A) A great deal of research has been accomplished with respect to plant bacteriology in the tropics, but more investigation is needed in the areas of ecology, epidemiology, and assessment of crop losses.
(B) Special efforts should be placed on control strategies for bacterial plant diseases, including legal regulations, disease-free seed production and certification, biological and cultural practices etc.
(C) The shortage of well trained personnel is considered one of the major constraints to development of plant bacteriology in the tropics, where a great number of problems are to be faced and solved.