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Cartagena de Indias, Colombia
25-28 August 1992

W.M. Roca and A.M.Thro, Editors

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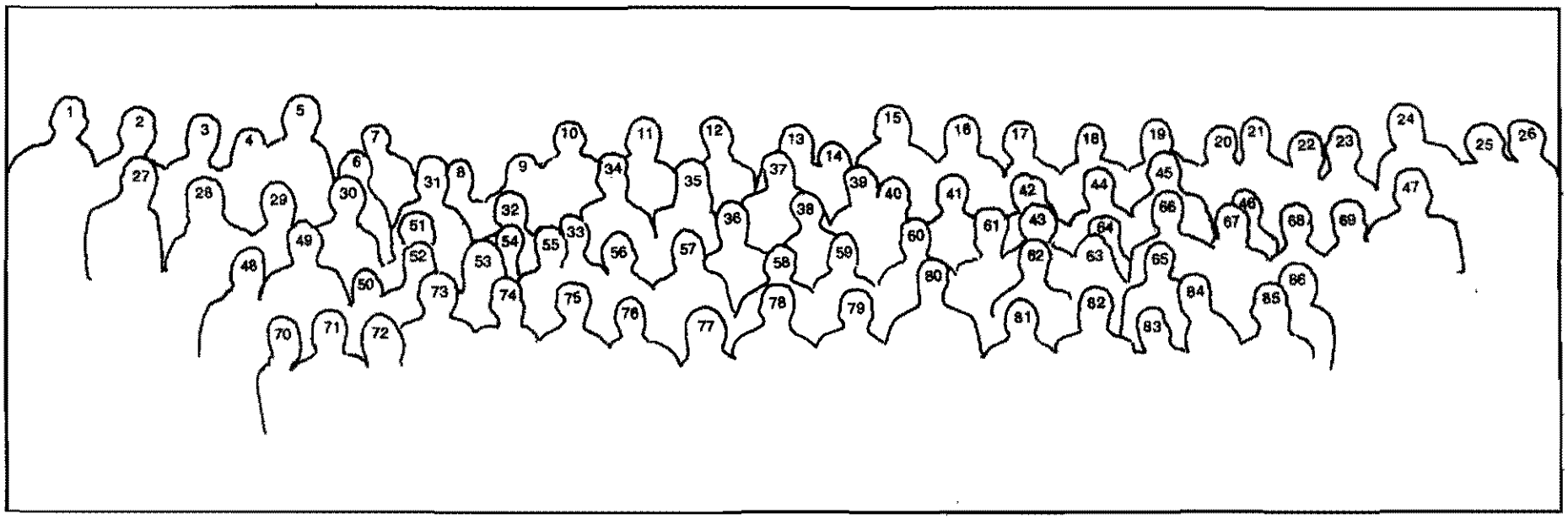
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Aug 25-28 1992 Cartagena, Colombia

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Cartagena de Indias, 25-28 August 1992

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PREFACE

Cartagena of the Indies, stronghold of the Spanish Main: here England and France challenged Spain for dominance in the New World, and here the stolen wealth of the Andes passed through the fortified harbor on its way to finance wars in Europe. In August 1992, 125 researchers from 28 countries and every continent met in this historic city to discuss contemporary research on a New World resource whose impact has spread farther than Andean gold. This resource is cassava (Manihot esculenta Crantz), the starchy root crop introduced from South America to Africa and Asia during the centuries when Cartagena guarded the Spanish treasure fleet.

The event was the first international scientific meeting of the Cassava Biotechnology Network (CBN). The meeting was organized by the International Center for Tropical Agriculture (CIAT) and sponsored by the Special Programme on Biotechnology and Development Cooperation of the Directorate General for International Cooperation (DGIS) of the Netherlands and by the Rockefeller Foundation. Cartagena was chosen as the meeting site because of its location near major cassava-growing regions of northern Colombia.

Although cassava has been called an orphan of agricultural research, in the tropics it is a mother, providing a livelihood to millions of the poorest people in the world. In recognition of its importance as a highly productive source of carbohydrates in adverse conditions where other crops often fail, the CBN concentrates on using biotechnology to enhance cassava's nutritional and economic value by solving constraints that are recalcitrant to other research approaches. In this process, the participation of developing country organizations and scientists and attention to various end-users of cassava and cassava biotechnology research receive priority attention by the network.

The CBN was founded with the collaboration of CIAT, IITA, and several national and international agricultural research institutions, in a workshop convened at CIAT in 1988. In the founding workshop, several topics for biotechnology and more basic research were identified in cassava, e.g., cyanogenesis, virus resistance, starch quality, photosynthesis under stress, post-harvest root deterioration, true seed propagation, and insect resistance. Technological constraints identified included plant transformation and regeneration, gene tagging, and molecular mapping.

In the last few years, new research priorities have entered into the network's agenda. The network approach to cassava biotechnology research has received wide acceptance by the scientific community and national an international funding agencies.

Since its foundation, the number of projects has steadily increased; currently some 25 projects are underway in developed and developing country institutions.

At the network's first scientific meeting in Cartagena, over 70 papers were presented. Perhaps the most important advances reported were those in the fundamental methodologies necessary to make possible the application of the power of biotechnology to effect specific interventions in cassava physiology, such as the progress toward a repeatable protocol for transformation and regeneration of cassava reported by several institutions. Other critical research areas reporting progress were germplasm conservation and characterization, where biotechnology can lower costs and increase effectiveness, and molecular studies of the cassava genome. The molecular genetics research will permit delineation of relationships between species, characterization of major genes to manipulate traits such as pest resistance and cooking quality, and molecular mapping to increase the efficiency of crop improvement.

We wish to thank the Special Programme on Biotechnology and Development Cooperation, DGIS, and the Rockefeller Foundation for their financial support of the meeting; the members of the CBN Steering Committee for their hard work and valued contributions to the direction of the network; conveners of the Working Groups for their leadership during the meeting; Fundiagro and Coopisan, San Caytano and the Instituto Colombian Agropecuario (ICA), Carmen de Bolivar, for organizing the field trip to visit cassava production, processing, and research; the CIAT Cassava Program for their invaluable participation and support; the CIAT Information Unit for providing the bibliographic exhibit during the week; and the personnel of the Biotechnology Research Unit at CIAT for their sustained cooperation in organizing the meeting. We are grateful for the support of M. E. Cobo of the CIAT Institutional Development Support Program for her professional assistance with logistical arrangements; P. Torres for her alert secretarial support during the meeting; the personnel of Aviatur for long hours confirming participant travel arrangements, and R. Simpson, Maria de Jesus Orozco, and Eliana Maria Gutierrez for their assistance with preparing the manuscripts for printing.

W. M. Roca
A. M. Thro
CIAT, March 1993

PROCEEDINGS OF THE FIRST INTERNATIONAL SCIENTIFIC MEETING OF THE CASSAVA BIOTECHNOLOGY NETWORK (CBN)

25-28 August 1992
Venue: Hotel Caribe, Cartagena, Colombia

CONTENTS

Section 3. Transformation, gene cloning and expression

Section 4. Quality, processing and utilization

Section 6. Cyanogenesis

[1] Authors unable to attend; papers are included for completeness of the proceedings

OPENING REMARKS

FIRST SCIENTIFIC MEETING OF THE CASSAVA BIOTECHNOLOGY NETWORK

G. A. Nores

CIAT, Cali, Colombia

First, let me welcome all of you to Colombia, and this beautiful and historical city of Cartagena. Founded 459 years ago, Cartagena is a special symbol in as much it represents the hope that the new continent brought to the rest of the world over the last half-a-millennium. As the immigrants found several indigenous crops that had been domesticated by the local cultures, the initial territorial and wealth ambitions of the colonial powers translated later in real hope, hope of life, for common people around the world.

Among the many crops that sustained the local civilizations and communities, such as maize, potatoes, tomatoes, beans, pepper and tropical fruits, there was this very peculiar root crop that natives called "yuca, mandioca, mandioc" - cassava in English. In contrast to many other crops that were highly vulnerable to environmental stresses during critical stages of the plant cycle, "cassava" proved to be both, well adapted to a wide range of tropical and subtropical environments and also very resilient to environmental stresses. Today, in terms of value of production, cassava ranks among the 10 more important crops in the developing world, and is the most important crop in Sub-Saharan Africa. But value of production is a bad proxy for the true value of this crop for millions of poor people in developing countries. As it is produced by resource poor farmers, often in poor soils that do not sustain other crops, and is consumed by low income people, the social value of cassava in the developing world is much greater than that reflected by its market value.

The fact that this First International Meeting of the Cassava Biotechnology Network is being held at Cartagena, on the occasion of the 500 year anniversary of the discovery of the Americas, is also an important symbol. It is a symbol of the hope placed by the world scientific community in the potential that the new emerging biotechniques offer to solve the problems that affect this important crop in farmers fields and to exploit more fully its potential for many end uses. It is a symbol of the hope the scientific and donor community place on science as a tool for social and economic development, for helping the poor to participate as true development actors and to share in the benefits of development. It is a symbol of our social commitment to help build a future of hope for millions of poor people in the developing world.

Nearly 130 scientists from 28 countries in the five continents are attending this meeting. More than a symbol, this is a magnificent evidence of the commitment of the international scientific community represented by you today. It is evidence of your own personal commitment to help the poor by applying science to the alleviation of the production constraints faced by millions of resource poor farmers around the

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world, and to help these farmers access expanding markets and profit on new opportunities. It is evidence of your willingness to share knowledge and research results, and to expose your research findings to the scrutiny of your peers, so as to advance faster in the important mission you have set for yourselves and for the institutions you represent. It is evidence of your conviction of the opportunities that lie ahead through collaborative research and networking. In the name of my colleagues at CIAT, I welcome all of you to Cartagena and to the better future that you will help construct.

ABOUT CIAT

Let me now turn briefly to how do we see our role at CIAT. We see our role as catalytic, facilitating and as partners in cassava research. The fact that our headquarters are strategically located near the center of origin and diversity of cassava, and in the middle of the area of domestication of cassava constitutes an asset. We are committed to share this asset and to collaborate with all of you in the search of genetic solutions to farmers problems. In spite of the reduction in financial resources for international agricultural research, CIAT remains committed to discharging its global responsibilities for cassava germplasm development in support of national programs, and to assist IITA in discharging its regional responsibilities for cassava research in Sub-Saharan Africa.

CIAT's Genetic Resources Unit is fully committed to collecting, characterizing, conserving and making available cassava germplasm to bona fide research around the world. CIAT's Information and Documentation Unit is committed to assembling and making available cassava literature in an effective manner to researchers.

The Cassava Program has redesigned its strategy in line with the long-term research challenges and opportunities emerging from farmers' fields and from advances in biotechniques. Emphasis is placed on the continuous identification of research needs with national programs, and in the definition of priorities for international agricultural research in terms of social and economic payoffs to resource poor farmers and low income consumers in major growing environments. This guides the identification, characterization and testing of source genepools for principal traits, and the development and testing of target genepools in terms of required traits for the major growing environments and the major end uses of cassava.

The Biotechnology Research Unit aims at increasing the efficiency and cost-effectiveness of research through the integration of selected biotechniques into strategic germplasm development efforts for: (i) characterization and screening of germplasm; (ii) broadening the genetic base of the crops; and (iii) identification of entry points for genome manipulation aimed at overcoming major constraints in plant-biotic/abiotic interactions. Similarly, the Virology Research Unit supports the germplasm development programs by identifying, characterizing and developing methods for controlling viruses of economic or quarantine importance. Recognizing

that the tasks are overwhelming for CIAT alone, the Units and the Cassava Program are committed to the development of effective research linkages with advanced research laboratories around the world for addressing the priority research agenda through collaborative projects, and to the testing of component technologies with partner national programs.

ABOUT THE NETWORK

In contrast with the case of crops grown in developed countries, cassava had attracted a very limited interest on the part of advanced research institutions in the industrialized world. Several recent initiatives have emphasized the need of an international cooperative effort aimed at effectively linking modern biological research with the needs of the small-scale cassava farmers and poor consumers in developing countries. In a consultation workshop on advanced cassava research, held at CIAT in 1988, the Cassava Biotechnology Network -CBN- was launched. From the beginning, the identification of research priorities and opportunities was a major objective of the Network.

Following the establishment phase initiated in 1988, the CBN enters now into a consolidation stage of its activities thanks to the generous support of the Government of The Netherlands. Under the guidance of its Steering Committee, with the scientific and logistical support of CIAT, IITA and the many research institutions that you represent, and the recent appointment of a Network Coordinator, Dr. Ann Marie Thro, based at CIAT, the CBN is now in a position to move forcefully toward achieving its objectives.

ABOUT THE MEETING

CIAT is extremely pleased to have been able to organize this first scientific meeting of the CBN. The cooperation of IITA, the support of the Biotechnology Program of the Ministry of Foreign Affairs of the Netherlands, and the support of the Rockefeller Foundation and many institutions that allowed the participation of so many scientists in this important event should be fully acknowledged.

It is important to note that the program for this meeting not only covers recent development in cassava basic research and the application of biotechnology to solve important problems of the crop, but also pays particular attention to the analysis of socio-economic aspects of cassava production and utilization. This represents the special interest we all have to continue improving in the identification and definition of research areas of high potential social payoffs.

Let me conclude by welcoming you again, and by expressing our appreciation to those of you that will present and discuss your research results. We look forward to a very productive meeting, and to the conclusions and recommendations that will derive from your deliberations. We are confident that the outcome of this meeting, particularly the future collaborative research projects among many of you, will make a reality the hopes of cassava producers and consumers around the world.

ADDRESS ON THE OCCASION OF THE SCIENTIFIC MEETING OF THE CASSAVA BIOTECHNOLOGY NETWORK (CBN)

Th. J. Wessels

DGIS, The Hague, The Netherlands

I would like to take this opportunity to share with you some of the considerations that lead us to sponsoring this workshop and more in general with the "Cassava Biotechnology Network" project. The Special Programme Biotechnology and Development Cooperation of the Netherlands' Minister for Development Cooperation, which started formally on the first of January 1992 for an initial period of 5 years, aims at improving the access of developing countries to biotechnological knowledge and applications.

The Special Programme implements these objectives in three ways:

- the integration of the development dimension in the biotechnology policy of the Netherlands;
- technical cooperation programmes;
- international coordination and collaboration.

The Special Programme focuses thematically on agriculture, health care and environmental management. Technical cooperation will be built up primarily with four countries; Kenya, Zimbabwe, Colombia and an Asian country yet to be selected. These are selected on the basis of local interest in the formulation of a national biotechnology policy, the existence of a certain local research capacity on which can be built a collaborative relation with the Netherland's bilateral programme, and the regional role the country could play.

Characteristic of the Special Programme is that the activities are identified on the basis of a participative bottom-up process. This means that needs and priorities are assessed locally with involvement of as well researchers and policy makers as NGO's and end users (or their representatives).

Apart from the country approach the Biotechnology Programme has been interested in promoting research on the so called orphan commodities, especially in root and tuber crops and leguminoses. Back in 1989 the position paper "Cassava and Biotechnology, Production Constraints and Potential Solutions" was at our request generated by the Agricultural University of Wageningen, leading into a first attempt to donor coordination in cassava research in the donor workshop of March 1990 in Amsterdam.

During that workshop, at which the leading cassava research institutes of the CGIAR - CIAT and IITA - were also represented, attempts were made towards joint priority setting among donors of cassava research. Furthermore the importance of regular information exchange on cassava projects was acknowledged and the future

role of the, at that time recently established, Cassava Biotechnology Network (CBN) in bringing together researchers and users.

It was clear that the objective to involve end users in the setting of the cassava research agenda needed special efforts. The Netherlands decided to accept CIAT's invitation to join the CBN-Steering Committee, and to help turn the CBN into a network that would be able to direct international cassava research to the farmers' needs, instead of being merely a think tank of international scientists lacking input from national scientists from developing countries and farmers' organizations.

Let me briefly give you a few characteristics of the CBN that makes it different from being just another network. The CBN will be a forum where priorities are set with regard to cassava research. Both advanced research institutes in the industrialised countries and institutes in developing countries will be represented. Moreover, local policy makers and farmer organizations will participate in the formulation of the research policy.

It is clear to you -and confirmed in the afore-mentioned Amsterdam workshop- that apart from problem-oriented cassava research, there is a need for supportive research to bring the existing biotechnological expertise to an operational level. There is also a need for upstream cassava research. Probably the most important limiting factor is the lack of a succesful protocol for the regeneration and transformation of cassava cells and tissue, fundamental to regenerate genetically engineered material into plants. In several research laboratories around the world cassava regeneration projects are taking place. However, coordination is needed.

The CBN will take up this task, including coordination of training, information supply and supportive researh.

The attention for "orphan crops" like cassava is limited to a few public research institutions, often in the margin of larger research programmes of universities. Complementarity of the research and exchange of data is therefore difficult to realise.

The CBN - and I mean the network in its totality - could function as the sounding board of the individual researcher, and stimulate the dialogue with target groups and donor agencies. It will finance small scale research and training activities in developing countries, and through the newsletter and international workshops promote the input of the target group (small scale cassava producers) in the international cassava research. In this respect the availability of CIAT's in-house expertise, facilities and contacts with national research programmes are highly appreciated.

As you can imagine, the success of the CBN depends largely on the composition and structure of the network. Highest priority should therefore be given to the formation of the network, consisting of decentralised (Asia, Africa, Latin

America) sub-units. It is the difficult, but also very challenging task of the CBN-coordinator to develop a methodology to establish the network, to organise the participation of local researchers and farmers/end users, and the linkages between the central and regional networks.

Last but not least, the composition of the Steering Committee (SC) has to reflect the composition of the network. The SC already discussed the issue at the beginning of the year, and is in the process of acquiring new members representing national scientists from developing countries, farmers' organizations, and social scientists, while at the same time cutting down the number of Western scientists.

A second issue I would like to bring forward is related to the CGIAR Taskforce on Biotechnology (BIOTASK), which I have the pleasure of chairing.

This taskforce was installed by the CGIAR-chairman during International Center Week (ICW 92) in Washington to stimulate the integration of biotechnology in the CGIAR. Members were initially USAID, Australia, FAO, IDRC, Worldbank, Rockefeller Foundation, TAC and The Netherlands, but participation grew with the invitation of the Regional Representatives and the open door policy BIOTASK adhered to.

The Taskforce acted as a pro-active group and not as a monitoring committee. The role of TAC in recommending on the support for biotechnology in the CGIAR - Centers, and its financial implications, was recognised. BIOTASK considered that there were system wide policy implications of biotechnology, which needed to be addressed by the consultative group. These included regulatory issues and the release of novel organisms, opportunities for public/private sector collaboration and related intellectual property considerations, and information supply to NARS and IARC's.

From an assessment of the activities in the period 1989-1991, it could be concluded that BIOTASK had served two important objectives: 1) become an informal platform for donors and Centers on biotechnology and related issues and 2) stimulate the discussion on biotechnology and initiation of specific activities.

With regard to the first objective, donors strongly feel the need for information on CGIAR -Centers' activities for their internal assessment, in order to secure long term commitment to the CGIAR. Especially biotechnology - given its political sensitivity characteristics like biosafety and intellectual property protection, requires donor consultation. On both issues BIOTASK has performed as a consultative forum.

Some examples of specific initiatives of the Taskforce are:

- information systems for IARC's, NARS and development agencies (Development Monitor, CABi AgBiotech Journal).

- seminar "Managing Biotechnology" at ISNAR in the Hague, addressing biosafety and intellectual property issues.
- foundation of the Intermediary Biotechnology Service (IBS), as a special project in ISNAR. The IBS would serve as a source of information and advice on biotechnology research policy, management and training for developing countries. It will function as a clearing house, linking activities in developing countries to knowledge and available technology in the industrialised world.
- RFLP workshop for scientists in NARS and IARC's.
- donor workshop Cassava and Biotechnology.

A taskforce is not established to last forever. BIOTASK concluded in her meeting during ICW-91 that, since biotechnology became more and more integrated in the CGIAR Centers, that it would ask the CGIAR for an extension of one year, to assist in further developing the CGIAR policy on biodiversity, biosafety and intellectual property. The taskforce has been working on these issues together with the TAC/Center Directors working group, with the result of the adoption by the Group at the Midterm meeting in Istanbul of a statement on intellectual property.

Although continuation of a biotechnology taskforce would not be appropriate, there might be - given the experience of BIOTASK - a need in the CGIAR for a donor-confidence-building mechanism, especially of importance to the so called "smaller donors".

There are still a few aspects related to biotechnology that will need attention in the near future and may disappear from the agendas because of the workload of TAC and CG-secretariat. Among these are: intercenter collaboration, access to new developments in biotechnology, biodiversity, and farmers' rights.

In the area of rules and regulations no particular problems are expected in the implementation of biosafety guidelines. However, in the domain of intellectual property one could question how international developments are monitored (GATT, UNCTAD, WIPO, EC etc.) and how the policy adopted in Istanbul is implemented: at Centers level or at CGIAR level?

These issues will be on the agenda at the final BIOTASK meeting at ICW-92 in Washington.

Also on that meeting I will propose to recommend that the CGIAR dissolves te BIOTASK, and to set up a Biotechnology Platform (BIOPLAT), that would have a consultative and monitoring role. It should have an open door policy and meet during the two yearly CGIAR meetings.

Finally, there is one issue that I would like to bring forward, that requires special attention of all of us. It refers to the possible dependence of CGIAR research on technology generated and owned by the private sector. As you will know it has

been a deliberate choice, on the recommendation of TAC, that the CGIAR should not build biotechnology capacity to enter in upstream research, but rather to integrate the technology as a tool in their programmes.

It implies that the CGIAR, a publicly financed international research group, will in the future dpend largely on externally (private sector) developed technology and material. Apart from secrecy restrictions imposed on the use of private sector owned material (and these will negatively affect partner relations with NAR's), it could mean that the CGIAR has to rely on technology and material that is not selected on the basis of farmers' needs and priority problems of developing countries, but rather on large scale commercial enterprises.

More than 75% of agricultural production in developing countries is small scale. The CGIAR has to be sure that the technology that the Centers generate is directed at the needs of these small scale producers and their limited ability to invest in inputs.

POLICY ISSUES IN BIOTECHNOLOGY RESEARCH IN DEVELOPING COUNTRIES

B. Nestel

ISNAR, The Hague, The Netherlands

Many developing countries have embarked upon programs of agricultural biotechnology stimulated by its potential for impact and by concerns about both maintaining product markets and obtaining access to improved genetic material protected by intellectual property rights. Whereas most advances in Third World agriculture in the past 30 years, have emanated from the public sector, modern biotechnology research with its high costs, economies of scale and opportunities for appropriating the benefits of the outcome of research is dominated by private sector companies in developed countries.

This has led to Third World governments being confronted by a whole range of new agricultural research policy issues. Some of these, such as those involving intellectual property rights and biosafety have been widely aired internationally. These are, however, only two facets in the overall policy scenario relating to how a resource poor country ensures that its (often significant) agricultural sector does not fall behind a capitalizing on the potential benefits to be derived from modern agricultural biotechnology.

The paper sicusses some of the policy issues that need to examined in addressing this question. They include policies relating to macro-economics, social development, science and technology, environmental conservation, ethics and foreign aid. A number of the relevant issues are ones for which there are few precedents. It is important that scientists involved in agricultural biotechnology should be familiar with such issues and be in a position to provide rational and appropriate advice to policy matters having to make decisions on this subject.

INTRODUCTION

The term "Biotechnology" is used to describe a spectrum of biological research ranging from "traditional" procedures in fermentation technology, practised for centuries in many developing countries, to "modern" (or new) biotechnology covering the use of more recently available techniques, particularly those based on recombinant DNA technology, cell fusion and bioprocessing.

This paper discusses some of the policy issues confronting developing countries embarking on biotechnology. A number of these issues are also relevant to developed countries, but they assume a particular importance in developing countries where resources are usually limited and the economy highly dependent on the performance of the agricultural sector.

THE PROSPECTS

In such countries the successful use of biotechnology for plant propagation and breeding could dramatically raise crop productivity and overall food production. Tissue culture techniques are already being used to produce more drought and disease-resistant varieties of cassava, oil palm, and groundnuts. Plant genetic

engineering may also result in coffee beans with less caffeine, in response to new consumer preferences, or faster growing tree species, which make reforestation easier. Better fermentation techniques in solid media, such as protein-enriched cassava flour, can improve the nutritional value of crops. Embryo transfer may raise the reproductive capacity of livestock. (National Research Council 1990)

The commercial use of new bioindustrial products could result in dramatically different patterns of agricultural production and trade. This may pose a threat to Third World export crops. Laboratory-produced vanilla and pyrethrum while helping to fill market shortfalls in the short term could, in the longer run, have an adverse effect on the income prospects of Madagascan and Kenyan farmers respectively and it is not unthinkable that consumers will soon have a choice between best Colombian arabica and biocoffee made in the North. Recent developments using gene transfer technology to redirect the innate biosynthetic pathways of plants may have major implications for the South in that they will permit the North to produce a wide range of both natural and synthetic chemicals currently based on the products of Third World Agriculture.

Another concern involves the privatization of research results. The current practice of patenting first-generation biotechnology products to cover any further use of bioengineered material will severely limit future competition. For developing countries this may also entail high licensing fees for seeds, which will make it harder to disseminate new crop varieties to smallholders. The widespread distribution of new bioengineered plant material may decrease genetic diversity and make crops increasingly vulnerable to new diseases. The positive side of this is that private companies have invested very large sums into all aspects of biotechnology and this has led to numerous advances in science and technology.

Against this background it is, perhaps, not surprising that many developing countries have embarked upon programs of biotechnology. Entry to modern biotechnology is relatively easy, particularly if efforts are focussed on tissue culture, micro-propagation and fermentation rather than molecular biology. Factors favoring entry by developing countries to biotechnology include: their growing domestic markets; their opportunities to identify niche markets of limited interest to large multi-national companies; and the fact that most of the genes likely to be of value in improving the worlds major agricultural crops are found in the Third World.

SOME CONSTRAINTS

However, barriers to entry to biotechnology are likely to become higher in the future (Persley 1988), particularly in the agro-industrial sector for the following reasons:

1) economies of scale will emerge, especially in bioprocessing, to allow the large-scale, cheap manufacture of new products;

2) complementarily with other rapidly developing technologies, such as those in information science and micro-electronics will become more important as processes become automated; and

3) access to large-scale marketing and distribution networks is becoming increasingly important in the sale of novel products.

These three factors tend to favor large companies in industrialized countries. Such companies are increasingly coming to dominate the marketing of biotechnology products. This is not surprising given that:

- biotechnology research is a risky investment,
- the payoff to biotechnology depends on adequate "conventional" research capacity and,
- agricultural biotechnology research requires a relatively large initial investment, if it is to have a high prospect of pay-off.

Developing countries can acquire new technologies in several ways. They can either make, buy, or pirate them. National programs may work directly with the private sector, through the CGIAR centers or through linkages to public-sector institutions in industrialized countries. In some countries, indigenous sources of venture capital or commercial partnerships may be available.

In efforts to stay abreast of developments, several LDCs have set up national institutes or programs in biotechnology. Many simple techniques, such as tissue culture, are being widely adopted. However, faced with mounting national debts and severe budget constraints, many countries are finding it increasingly difficult to even maintain, their level of investment in conventional agricultural research, let alone embarking on new technology. Given such constraints, developing countries need to determine which biotechnology areas they should give priority to. Equally important, they need to know what training their scientists require to enable them to adapt biotechnologies for local use. In partial answer to these questions, experience shows that the first requirement is a strong program in the applied agricultural sciences involving plant breeding, plant protection, and agronomy. (Barker and Plucknett 1991)

Biotechnology research on a crop not previously subjected to biotechnological research, will be expensive, while research on a well-explored crop is likely to be much cheaper. The techniques and objectives of the biotechnology activities will also affect their costs. Developing countries, even small ones if they choose wisely, may be able to take advantage of some of the lower cost activities to produce relatively short term gains that reach down to the farmer level. For example, labor-intensive tissue culture techniques, in the hands of well trained people can produce vigorous, disease-free planting material of many species. Countries which presently have some horticultural enterprises and a functioning marketing system for such enterprises may

be able to use tissue culture to increase the efficiency or consistency of production and thereby to raise incomes. (Herdt 1991)

Such an approach has been a traditional feature of green revolution technology which has been generated mainly in the public sector with the CGIAR system playing a key role. But it is important to remember that it was conceived and implemented within the public sector. If, in a like manner, biotechnology is to service the interests of developing-country farmers, public-sector institutions in both the South and the North, may need to continue to play a central role in technology development and diffusion.

With biotechnology development, however, the private sector is already playing a much more important role than it did with the green revolution. Concern is mounting that the private-sector research agenda will become too narrowly focused on well-to-do-farmers (those who have the capacity to purchase inputs) and on well-to-do countries that have good agricultural support services.

INTELLECTUAL PROPERTY RIGHTS

In countries where property rights for genetically engineered plants or animals do not exist, or where most farmers are subsistence producers so that the market for purchased seeds or other agricultural inputs is too small to attract private firms, it is unlikely that private biotechnology research and development will be undertaken (ASA 1989). In such cases, there may be a rationale for publicly funded biotechnology research, but it still must meet the criteria discussed above, if it is to be economically justified.

These possibilities suggest that the potential exists for public sector research to play a complementary rather than competitive role to the private sector research. However, there are concerns that international differences in protection afforded by the legal system to intellectual property rights will adversely influence the respective roles of private and public R&D on biotechnology for agriculture on the world scene. (Lindner 1991).

While the products of biotechnology R&D are afforded significant patent protection in the USA, and to a similar extent in a number of other industrialized countries, the situation in the developing world is very different, with the legal systems of most countries affording very little protection to all forms of agricultural inventions, but in particular to technology embodied in living organisms. However this situation is changing rapidly and Mexico, for example, has just ratified a law giving plant breeders even stronger rights than in the U.S.A..

In the context of the international transfer of technology the significant consideration is the capacity of the national agricultural research systems of the countries to imitate, or adapt, technology generated overseas. In this regard, there are important differences between countries in the developing world, with some

scientifically advanced countries such as India and Brazil having a relatively strong capacity to copy and/or adapt biotechnologies as well as to generate them, while a range of other countries have very little capacity (Evenson and Putman, 1990). As a result, the issue of intellectual property rights is a contentious matter in the Uruguay round of the GATT negotiations. Failure to resolve this issue could widen the North-South divide.

The issue of intellectual property rights is a particularly sensitive one with many Third World countries which object to the possibility of having to purchase technology developed from germ plasm obtained free in the Third World. The availability of proprietary rights for new products and processes is, however, important to private companies investing in R&D since this is the main means by which they are able to appropriate the benefits from their substantial research investments, and to turn these to commercial advantage.

Access to protected, novel products and processes, which are likely to be useful in developing countries may be the subject of negotiations with private companies. These negotiations, could be conducted by individual countries, by regional organizations, by development agencies on behalf of one or more countries, or by an international agricultural research centre, on behalf of its clients. There are a range of opportunities for joint ventures, licensing arrangements, or other mutually useful partnerships to be developed, which would allow the use of novel products and processes in developing countries, provided that the proprietary protection was honored. (Persley 1988)

There are some products and processes which are not able to be protected by patents or other proprietary protection. These are usually generated in the public sector, and made freely available. In developing countries, many of the potential applications of biotechnology fit into this category (e.g. the introduction of disease resistance into vegetatively propagated crops such as cassava or banana).

A consideration of whether a proposed new product or process is able to be patented or otherwise protected may be a useful criterion in deciding if an activity is appropriate for support by public funds (including aid funds), and what type of support is appropriate. The likelihood of proprietary protection need not necessarily exclude an activity from public support, including support by development agencies. However, different systems are required than those used to support research from which the results are publically available.

MARKET ECONOMICS

Industrial research in developed countries is strongly market driven and designed to meet the needs of paying consumers. This research may impinge on Third World agriculture if it produces substitutes for materials currently exported from the South to North.

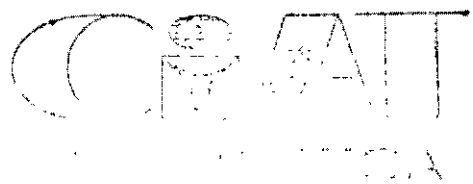
Examples are already becoming available to illustrate the loss of export markets by Third World countries. In 1986, the Sudanese gum arabic market was sharply depressed when a New York company announced the discovery of natural gum that was possibly better than the farmed product. Coconut oil that is being imported into the United States from the Philippines might be replaced by a domestic oil seed engineered to produce the shorter chain, fatty acids contained in coconut oil. (Kolattukudy 1988) Similarly enzymatic processes to produce a cocoa butter substitute from cottonseed and sunflower have been developed in the USA and could affect that countries US$ 280m a year import trade, mainly from Malaysia and Brazil (Anon 1992)

The existence of such commodity markets help to drive a fiercely competitive R&D environment in developed countries. This generates technologies which, in turn provide new products for large and affluent markets. In many Third World countries the risk entailed in product development for a limited market is seen by industrialists as being too high and an easier option is to import from abroad. However, in the case of biotechnology this poses specific problems. First government regulations concerning foreign exchange, import duties and customs formalities are often not geared to the prompt handling of perishable materials and reagents. If a country embarks on a research program in a fast moving field such as biotechnology it is essential that it has rapid access to transfers of equipment, information and supplies if it is to stay competitive. This is particularly relevant in many developing countries where the local market for sophisticated instruments, such as DNA synthesizers and protein sequencers as well as for perishable and usable materials is too small to support indigenous commercial protection.

FINANCIAL ISSUES

Funds for purchasing such equipment and supplies are also likely to be a constraint unless help is provided by international agencies. When this is done, all too often inadequate provision is made for either training support technicians or for an adequate supply of spare parts. There is an important policy issue here that is not always understood, which is the need to ensure that if "upstream" technology is to be embarked upon it is not enough just to train staff and to buy equipment, there is a major recurrent cost in having service staff and spare parts available at all times.

Another cost item that can cause problems in developing countries is that in order to put the results of biotechnology research into practice, it may be necessary to make a substantial investment in industrial plant. This can present difficulties for public sector research, for example the facilities for biotechnology based vaccine production may need to be much more sophisticated than those for traditional vaccine production if appropriate biosafety regulations are to be met. At a time when there is a global tendency to privatize many government services a very clear policy on product development may be needed before a public sector biotechnology program is begun.



EQUITY

Low rural incomes, particularly those of small farmers and landless laborers are a matter of concern to many developing country governments. Thus equity and income distribution often feature prominently as goals of national development plans. The introduction of biotechnology applications in rural areas will need to take into consideration these planning targets. (Bunders 1990) Research that leads to higher yields of subsistence food crops, reduction in the use of purchased inputs, higher cropping intensity or lower labor use could lead to a reduction in the real price of food which would have positive distributive and nutritional implications. But the use of new pest- or disease-resistant material could have a negative effect by displacing labor. There is a delicate path to be trodden here since some of the profit oriented goals of the private sector may not be compatible with the poverty alleviation goals of governments and this may mean that governments have to continue to take the main responsibility for research in those subsistence crops which are the prime products of many small farmers.

INSTITUTIONAL DEVELOPMENT

An important policy consideration for governments embarking on biotechnology relates to be location of the work. In developed countries biotechnology is a major activity in the private sector, is often prominent at universities where highly qualified academics compete for public and private sector grants, and is usually backed up by one or more publically funded institutes established to ensure that the country remains at the cutting edge of new technology.

In all but a few developing countries private sector biotechnology research is limited, if it exists at all, due to limitations of capital, skilled personnel (who can earn more in the North) and market development. University faculty in the Third World usually lack research funding from both public and private sources and adopt teaching as their primary task. Where research is undertaken it often tends to be basic rather than applied, although given the constraints faced in the fields of equipment and information it is difficult (but not impossible) to remain at the forefront of basic biotechnology research working in a Third World situation.

In such circumstances the public sector is usually called upon to take the lead where biotechnology research is seen as a national objective. This raises a number of issues:

* should the work be done in existing institutes or should a new biotechnology institute be created?

* how can the specialized shills of university faculty be optimized in terms of national policy?

* how can a national program be developed. Should there be some form of Biotechnology Research Committee at the national level?

* should the private sector be encouraged to play a larger role in biotechnology research, if so how?

HUMAN RESOURCE PLANNING

Biotechnology research requires skilled personnel. Training such people requires time and money. Usually the training takes place in well equipped laboratories of the North where trainees can compete for jobs in a fast growing and well paid market. There may be little incentive for them to return to a lowly paid public service job in a developing country laboratory with financial constraints which limit the purchase of equipment and information. This "brain-drain" question has been an issue in developing countries for many years but in the case of biotechnology it has been exacerbated by the strong market for biotechnologists in the private sector of the North.

Policy makers in the South are faced with two problems on the human resource front. First to predict the advance training needs in a rapidly advancing area where advanced training requires a commitment of time and money; and second, to derive an incentive system that will limit the "wastage" of skilled personnel in a public sector that may have little financial flexibility.

INFORMATION EXCHANGE

"Development of any technology requires availability of up-to-date information. Access to basic knowledge and to developments based on such knowledge is crucial in the newer, rapidly moving fields such as biotechnology. Such information is generated largely in the industrialized countries. The access of Third World scientists to this information base is often limited. Journals and periodicals are too expensive to afford and too slow to arrive in many Third World libraries. Even the information that arrives in central places, such as the capitals, often does not get transmitted to the relevant research laboratories without great delays" (Zilinaskas, 1988). Modern information technology makes it possible to overcome these problems, but this has a cost that policy makers must face up to if they want a viable biotechnology program.

SETTING PRIORITIES

Given the constraints indicated above, it would be unwise, except in a few larger developing countries to set aside more than a small part of research resources into agricultural biotechnology research at the present time. What is important is to determine which areas of biotechnology they wish to give priority to. Such a decision will obviously be influenced by the personnel available but will also depend on the answers to some specific questions (Herdt 1991):

1. "What are the commodities of interest on which biotechnology research might be concentrated? For each, what is the current output level and the value of that output to the nation?

2. For each, what is the state of current worldwide scientific knowledge usable in the application of biotechnologies to improve their productivity?

3. For each, what are the major problems that might be overcome through the application of biotechnologies?

4. What is the best estimate of the resources required to apply biotechnology to address specific problems, for each commodity? Given appropriate resources for each problem for each commodity, how long is it likely to take to "solve" these problems?

5. For what commodities do strong applied research and outreach capabilities now exist within the country?

6. What are appropriate national policies in science, technology and industry to facilitate the commercial use of biotechnology?"

These are complex questions that cannot be answered without a concerted effort. In the absence of answers, or at least the assembling of appropriate data, Herdt (1991) concluded that a country might be best advised to arbitrarily choose two of three important commodities and develop tissue culture and protoplast techniques for each. At the same time the nation should develop a small team on agricultural biotechnology policy, so that short, medium and long term priorities can be established within a national policy framework.

BIODIVERSITY

Conservation of genetic diversity is, like intellectual property rights, an issue that has been discussed extensively at recent meetings, sponsored by UNEP and UNCED. Various conventions have been proposed and discussed and sometimes agreed to, although disputes still exist regarding ownership rights and regarding the extent to which "life" is patentable. However, the subject has been discussed so extensively that most countries have now adopted some form of policy on this subject.

BIOSAFETY

The release of genetically engineered organisms raises a number of legal, ethical and environmental policy issues. These have been extensively studied in developed countries and have generated a mass of literature regarding procedures for the field testing and international movement of transgenic materials. This literature

is not yet consistent, with different standards being proposed in the United States and Europe.

Developing countries need to set policies and standards which fit their ow needs and to ensure that any transgenic introductions, for either testing or commercial use, take place within an acceptable risk framework.

Other considerations relating to biosafety policy relate to the establishment of national and institutional biosafety committees. Appropriate guidelines in this area have recently been set out in an ISNAR publication (Persley *et al* 1992). This is based on earlier work by OECD, IICA and others.

DONOR AGENCY LINKS

A number of donor agencies such as the Governments of the Netherlands, UK, and USA and development banks, such as the World Bank and Asian Development Bank, have provided support to developing countries in facilitating the application of biotechnology. This includes:

1. Providing support for biotechnology in association with other agricultural research support, including the provision of training and facilities in the basic biological sciences, and traditional biotechnology.

2. Providing support for biotechnology only in the public sector in developing countries, for the development of new products which are made publicly available.

   This may include support for collaborative ventures with either public or private section biotechnology groups in industrialized countries. The key criterion is that the results are made publically available.

3. Facilitating the establishment of joint ventures between public and/or private sector agencies, in both industrialized and developing countries.

Such partnerships could facilitate both the development of new products and processes, and the business systems necessary for the delivery of novel products to the users. Development agencies may be facilitators and/or investors in such ventures.

The new features of biotechnology are of sufficient importance to merit the consideration by development agencies of different systems for the delivery of effective projects in biotechnology. The design of such systems requires dialogue with individual countries and with the major players in biotechnology in the private sector. It requires both development agencies and development countries to work together to devise appropriate policies for doing business. From the developing

country standpoint this entails taking a long term perspective to ensure institutional sustainability.

An important feature of developed country support to Third World agriculture at the present time is that provided by the CGIAR system. Developing countries are represented on the governance of that system, although to date the influence of their representations has been limited. But with the CGIAR system increasingly moving upstream in its activities and becoming heavily involved in biotechnology at a number of IARC's and in networks sponsored by them (such as CBN), the opportunity to provide inputs to an important aspect of CGIAR policy exists. It may be important to explore this, given the need to ensure that biotechnological innovation meets the requirements of appropriateness, feasibility and comparative advantage for introduction and application by small farmers in the Third World.

In order to do this and also to influence policy decisions on the topics raised earlier in this paper it is important that scientists involved in agricultural biotechnology should adopt a holistic view of the subject and be in a position to offer rational and appropriate advice to policy makers with respect to all aspects of biotechnology.

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POTENTIAL IMPACT OF BIOTECHNOLOGY ON CASSAVA PRODUCTION IN THE THIRD WORLD

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Economic growth and structural change in developing economies leads to a changing role for the principal starchy staples, such as cassava, in development of the agricultural sector. Particularly, the role of the starchy staple shifts from meeting subsistence and food security objectives under situations such as in Africa where economies are still very much agrarian to meeting market and income objectives in circumstances such as Latin America where the economy has industrialized and the population is predominantly urban. The role of cassava at these various states of economic development is analyzed, especially the implications for cassava market diversification under economic growth. A case is made for very different needs for cassava development between Africa, Asia, and Latin America. The implications for technological change in the cassava crop is then evaluated at various stages of development , and particular stress is put on the impact on income distribution, as defined in a number of dimensions. Finally these various elements of market development, technological change and income distribution are evaluated in terms of the broad implications for investment in biotechnology research on the cassava crop.

INTRODUCTION

In the early phases of economic development, such as in Africa where economies are still agrarian with 70 to 90% of the population in the rural sector, rapid growth in starchy staple production is critical to meeting food security needs, keeping urban food prices and wages low, and generating purchasing power for industrial goods in the rural sector. Because food staples such as cassava bulk large in both the diet and in the cropping pattern, growth in starchy staple production is key to both development of the agricultural and industrial sectors. However,as economies diversify, the population urbanizes, and incomes increase, the relative role of starchy staple crops in direct food consumption declines. Structural transformation, which can be seen in progressive phases as one moves from Africa through South Asia onto Southeast Asia and finally to Latin America, has major implications for the relative role that the starchy staple will play in food security and rural nutrition, farmer income generation, rural employment, urban nutrition, and market development, particularly export markets. This changing role based on the structural conditions of the economy has particular implications for the types of technology, including biotechnology, required to meet these changing objectives.

CASSAVA'S CHANGING ROLE IN ECONOMIC DEVELOPMENT

The role of the starchy staple in economic development is schematically presented in Figure 1, where per capita income per country is plotted against per capita consumption of starchy staples. The first part of the graph up to $250 shows direct staple consumption increasing with income. Utilization, demand, and markets

for staples at this point are based almost solely on the use of starchy staples as direct food sources. The population will continue to increase staple consumption with increases in income up to about 90 kg per year of grain equivalent. At this stage in development protein/calorie malnutrition is still prevalent, and increased, cheap calories are the principal means to improve nutrition. At these low levels of staple consumption, income distribution and access to land resources greatly influences nutritional status and food security of the households. This situation characterizes much of Africa and South Asia at the present, and is being exacerbated by high population growth rates and high population densities in the rural areas.

At incomes above $250 economies have significantly industrialized and urbanized, diets begin to diversify away from dependence on starchy staples, and farmers' income and production is increasingly based on commercial sales. Declining demand and falling prices for staples, which still dominate land use patterns in the agricultural sector, produces stress on farm incomes and rural wages, motivating both further urban migration and , shifts to other crops being demanded in urban markets, and movement of staples into other lower cost uses. This point marks the market diversification of the starchy staple sector and the increased utilization of staples as animal feeds, industrial starches, processed foods, and most recently ethanol. Demand for such uses is income elastic, and utilization of starchy staples continues to increase with development, with no apparent upper limit having been reached. Nevertheless, rapid structural change in this period creates real dislocations and stresses in the economy. Nutritional concerns about low income households still remain, but the locus has now shifted to urban areas. Farm size structure also tends to become more unequal and small farmers and those in more marginal agricultural areas have greater difficulty taking advantage of the expanding market opportunities. This stage characterizes much of Latin America and its beginnings are apparent in much of Southeast Asia.

Cassava's changing role in economic development is a function of the processes laid out above and cassava's competitive position vis-a-vis other starchy staples, especially grains, in the agricultural economy. Although cassava is the third most important crop grown in the tropics, following rice and maize, it dominates in particular niches. These include the lowland tropical ecosystems of West and Central Africa, the more marginal soil and rainfall areas of East and Southern Africa, the Northeast of Brazil and the northern coast of Colombia and Venezuela, and the Northeast of Thailand, and the high population density areas of southeastern India, upland Java, and the Great Lakes area of Africa. Cassava is important in those ecological niches where there are major constraints on the production of other grains and root crops, especially areas marked by highly acid soils, sandy soils, and excessive or limited rainfall. Cassava also has some relatively unique social niches. The axiom is basically true that cassava is a crop grown in poor areas by poor farmers for consumption by poor consumers. While often an uninformed statement of opprobrium, these characteristics place cassava in a relatively unique position to begin to compensate for or reverse many of the unfavorable, income distributional consequences of economic growth, at the same time as contributing to the overall

development of the starchy staple sector. However, this requires more sophistication in a technology development and diffusion strategy.

Cassava's comparative role in the food economy starts with its selection as the subsistence base in rural households. Cassava's dominance in the shifting agricultural systems of the forest zones of Latin America and West and Central Africa is based on its adaptation to acidic, highly leached soils and the high returns to the very limited labor resource. Cassava's dominance in the subsistence strategies of Northeast Brazilian households and the rapid spread of cassava into savannah and semi-arid ecosystems in Africa are based more on household food security considerations. The crop's drought tolerance, its ability to yield under most disease and pest pressures, its indeterminate harvest period and ability to be stored in the ground until needed, and its yielding ability under infertile soil conditions all contribute to a steady and secure supply of calories to the household that is buffered against major climatic and biotic stresses. Cassava has also become a major staple in high population density areas, such as Rwanda and Burundi and the upland areas of Java and Kerala State in India.

In such conditions cassava's high yielding ability and its buffering capacity against stress improve the food security of households in rainfed areas with very limited land resources. The role of cassava in improving food security has fostered a particular expansion of the crop in Africa, with the one remaining question being whether it will expand into the Sahelian region (Romanoff and Lynam, 1992).

As the locus of food consumption shifts from dependence on subsistence sources to reliance on markets, the basis of the comparative advantage of cassava in the staple economy shifts from its production to its consumption characteristics. Cassava's role in the purchased food basket is founded on its price, and especially the fact that cassava is one of the cheapest of caloric sources. For low income households where calorie consumption is often below nutritional standards, cassava becomes a means of optimizing calorie consumption within the available budget. Thus, low income, urban consumers in Northeast Brazil, parts of Indonesia, and urban, anglophone West Africa depend on cassava as their basic calorie source. However, in all these cases cassava is marketed and consumed in a processed form. In order to maintain its cost advantage in urban markets, the perishability and bulkiness of cassava roots must be overcome by transformation into farinha de mandioca, gari, gaplek, or any of the numerous processed forms of cassava. Such forms are cheap, storable, and convenient, the key characteristics for an urban staple. In those areas where fresh cassava is the principal consumption form, such as Colombia or Paraguay, the crop has not been able to take advantage of quickly growing urban markets. Rather rice and wheat have become the staple, a situation occurring in francophone West Africa as well.

Cassava's role as a cheap urban staple for low income consumers carries with it a negative side. As incomes or prices increase consumers shift rapidly out of cassava into more preferred foods-- that is, dried cassava products are an inferior

good. This has several implications for farmers. First, relatively small changes in supply result in quite marked changes in prices. Such large price variability produces large variability in farmer incomes and creates little demand for new technology. Second, cassava farmers cannot take advantage of the growing markets that come with economic development, that is markets created by growing incomes and urban populations. Farmers producing cassava, usually in more marginal areas, become further marginalized, since they have few crop diversification alternatives. Thus, cassava's potential role as a cheap staple for low income consumers undercuts its possible role as a source of income growth in marginal agricultural areas. However, and very importantly, inelasticity in demand is not based on the crop per se but rather on the product form. As presented in Figure 1, demand growth under economic development for all the starchy staples depends on market, and therefore, product diversification. Interestingly, market diversification for cassava is most advanced in Southeast Asia (Table 1). Such diversification into animal feed and starch markets does not undercut cassava's role in the diet's of low income consumers, but rather brings more price stability, more robust markets, and more potential for income growth for cassava farmers, that is appropriate conditions for rapid technological change.

CASSAVA AND RURAL INCOME DISTRIBUTION

Cassava is primarily grown not only in more marginal agroclimatic zones but also by small scale producers. Given appropriate market conditions, as described above, improved cassava technology offers the possibility of increasing incomes of those rural households that are most difficult to reach by either research programs or other public policy instruments. The achievement of income distributional objectives through publicly financed agricultural research programs depends first and foremost on the choice of crop. Research on cassava can be principally justified on these grounds. Under structural change in the economy and market diversification, the comparative advantage of small scale farmers in the production of cassava has continued to hold in Latin America, Asia, and Africa. Where large scale systems have been tried, they have almost universally failed. These include the large scale farming of cassava in the Brazilian cerrado to feed the large scale ethanol plants, the plantations in Malaysia and the Philippines for starch production, and the Shell-financed plantations in West Africa for gari production. The only large scale units still in production are in the transmigration scheme on Sumatra, and these depend to a large degree on small-scale, outgrowers. Technology design decisions in cassava research programs by necessity have to be oriented to the needs of small scale production systems. Nevertheless, the question remains of identifying the basis for the comparative advantage of small scale farmers, so that it is not shifted by technology development to large-scale production and utilization systems.

The first question to ask is whether there are diseconomies in cassava root production, especially whether cassava production is suited to the relative proportions of land, labor and capital held by small farmers. Small scale farmers in general have assess to relatively more and cheaper labor, which raises the question of whether

cassava production is inherently more labor intensive than other crops grown by large scale producers. The answer to that is probably not. Land preparation, planting and weeding for cassava require no more labor than other field crops. As well, given that cassava does not have as critical requirements for time of planting or time of harvest, there are not as severe labor peaks for cassava, which could be very advantageous in large farm production systems. Harvesting does require more labor, but again this can be scheduled over a significant period. Labor for weeding can be easily substituted for by herbicides. What can be said is that cassava is more expensive to mechanize than grain crops, because both planting and harvesting require specialized machinery, which because of limited demand is relatively expensive. Nevertheless, the relative costs of labor versus capital between large and small-scale producers is probably not the major factor contributing to the comparative advantage of small-scale producers in cassava production, except in the plantation systems of Asia.

Relative location might provide a stronger explanation of cassava's predominance in small farm systems, at least in Latin America. On that continent cassava is grown in more marginal agroclimatic environments, small farmers tend to predominate in these, and for large farmers in such areas, the lack of cropping activities that are not too intensive in both labor and management for them to livestock as the principal economic activity. Such considerations do not apply so strongly in Asia and Africa.

Small-scale production patterns based on non-contiguous small plots characterized by intercropping and a fallowing regime may have major advantages over large-scale systems in reducing pest and disease severity. This is especially so since disease and pest control in cassava has not been reliant on resistance breeding. How large such an advantage may be is hard to determine and difficult to maintain, since small scale farmers would also gain from resistance breeding.

Probably the major factor underlying small farmer's comparative advantage in cassava production is the diseconomies in the transport and marketing of cassava, and the implications this has for both risk and scale of processing. These diseconomies in marketing derive partly from the bulkiness of the roots but most importantly from the roots' perishability. Perishability puts a limit on shipment radius, increases loses, reduces negotiating ability on price and thereby raises marketing risk substantially. Scale economies for bulking of cassava roots are not possible, high water content makes transport costs of roots high, and risk requires close integration of production and processing to keep marketing margins low. Processing is critical to high volume marketing of cassava but processing has no major scale economies -- except in starch and ethanol -- and because of the cost gains from links to the production unit and the high cost of root transport, all these factors lead to a very decentralized pattern of processing based on small scale units. Some specialization and a modicum of scale economies is possible in dense production areas, such as in Thailand or Northeast Brazil. Where there are processing scale economies, such as in starch production in Indonesia, these are usually balanced by the higher labor,transport, and overhead costs of plantation production. Economies

of scale in marketing do come in the stage after processing, when bulking and further weight reduction through pelleting reduce marketing transport costs. Root bulkiness and perishability lead to a comparative advantage for small-scale production units linked to small- scale processing units. Eliminating perishability, especially where demand shifts to markets such as starch and ethanol where there are processing scale economies, could shift the comparative advantage in production to large scale production units, especially given some cost savings on bulking from large scale farmers. In summary, the comparative advantage of large versus small-scale farms in cassava production is determined more by external market and processing scale economies, than the relative cost of internal factors of production.

IMPLICATIONS FOR BIOTECHNOLOGY RESEARCH

As cassava makes the transition from a food security crop to a tradeable commodity within a diversified market structure, the requirements for new technology change. However, given the role that cassava now plays throughout the tropics in small farm systems in more marginal agroclimatic zones, there is a persuasive argument for utilizing technology to improve the welfare of these particular farmers in the development process, especially since it is exactly this group of farmers that are usually marginalized in the process of rapid structural change. The questions for cassava research programs is therefore what are the requirements for improved technology for this target group of farmers, how do these requirements change with economic development and commercialization, and how might the comparative advantage of small-scale farmers be maintained under both technical change and market development.

These questions are addressed purely in relation to a more limited aspect of cassava research, namely the implications for priorities in biotechnology research. Biotechnology, at its most elemental, involves the targeting and manipulation of specific genetic characteristics. The work principally involves the cassava genome, but could involve manipulation of biological agents that either benefit or harm the cassava plant. Such research is almost always delivered through new varieties. This in turn limits the question to the role of improved cassava varieties in technological change and the characteristics that are most appropriately incorporated in those varieties.

There are two basic approaches to classifying and prioritizing such characteristics. The first is the production constraints approach and has been applied in the Rockefeller Foundation's rice biotechnology program (Herdt, 1991). In this case priorities are set by the contribution to yield of relieving the constraint and the extent of the yield constraint. Such an approach is very appropriate for rice, since in much of Asia, farmers' use of improved varieties, inputs, and cultural practices has led to a yield plateau and a very small yield gap between the research station and farmers' fields. Biotechnology offers the promise of expanding the existing yield frontier. In rice improved varieties are allowed full expression of their genetic potential because of the creation of a non-limiting agronomic environment.

Cassava, on the other hand, is grown in marginal areas, with few if any inputs, and often with very labor extensive, cultural practices. As a result there is a hugh yield gap between research station and farmer yields, often using unimproved varieties. The first, and most important, technological challenge lays outside biotechnology, and that is to improve the agronomy of cassava production, especially plant nutrition and seed quality. Without such improvements there will be little gain by focusing, as in rice, on expanding yield potential. This situation shifts the focus to areas where yield is reduced even under existing agronomic practices, namely resistance to pest and disease attack. These traits enhance the food security role of cassava and contribute to decreased costs under commercial production. Better adaptation to climatic and edaphic stresses will also further enhance the food security role and the comparative advantage of cassava in marginal areas. However, higher yields will result in a higher demand on limited soil nutrients, requiring even further emphasis on simultaneous improvements in soil fertility management. One of these interventions will appreciably shift the comparative advantage to larger scale farmers.

The other approach to prioritizing genetic characteristics is to evaluate improvements in quality and marketing characteristics. This focus results in an increased price or reduced marketing or processing costs for the commodity, resulting in potentially higher prices to farmers, independent of yield. Eliminating cyanide reduces health costs in stressed, high cassava consuming populations. It also improves the efficiency in processing where processing time, grating, and heating are important to cyanide detoxification. Finally, it improves the flexibility or uncertainty in marketing of cassava, since cyanide-free roots can go into all uses. What implications this has for the nitrogen economy of the plant remain to be seen. Improved starch quality, especially under stress, also expands the range of uses, especially for fresh use and in flours. Increasing root dry matter content would greatly lower processing costs and reduce transport costs. Given that cassava is usually consumed by the poorest, increased protein content may have a role in improved nutrition, but this has to be evaluated and this certainly should not come with an increased cost in the production of the calorie component.

Elimination of the perishability constraint is the most difficult to judge, because this might be the principal factor making cassava a small farmer crop. Making the roots less perishable would lower marketing costs, although possibly not that much, given the efficiency of existing fresh root marketing systems. Eliminating perishability does allow larger scale processing units, and less scheduling problems for large scale production units. The costs and benefits of this characteristic should be looked at in some detail, especially from the point of view of its equity implications.

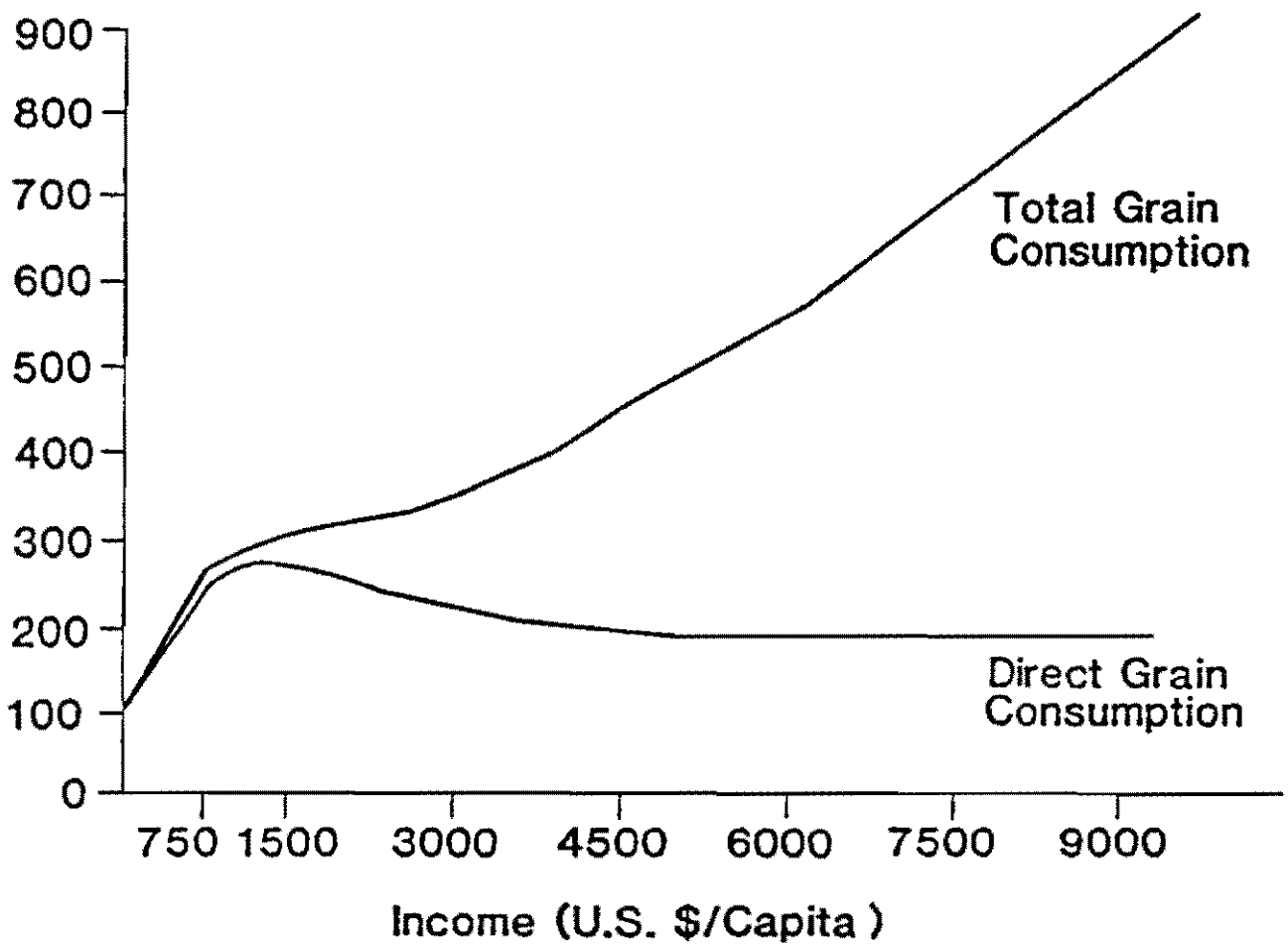
In sum, biotechnology at this point in time probably has a larger role to play in terms of improving the consumption and marketing characteristics of cassava, than in improving yields. A strategy is still needed in cassava for improving the basic agronomy practiced in the crop. The latter is partly a research question, but even more an extension and diffusion issue.

Table 1. Production and Utilization of Cassava in Principal Producing Countries in Southeast Asia.

			Domestic Utilization				
			Human Consumption			Animal	
Country	Production (000t)	Export (000t)	Fresh (000t)	Dried (000t)	Starch (000t)	Feed (000t)	Waste (000t)
India (1977)	5688	22	2610	619	1784	-	653
Kerala	4189	22	2437	619	499	-	503
Tamil Nadu	1310	-	126	-	1162	-	131
Indonesia (1976)	9686	801	3444	2212	2747	-	482
Java	6317	253	1815	1760	2134	-	355
Off-Java	3369	548	1629	452	613	-	127
Malaysia (1977)	432	66	-	-	302	43	21
Philippines (1975)	450	-	223	37	92	32	65
Thailand (1977)	13,554	9,996	-	-	745	16	2797

Source: Unnevehr, 1982; Titapiwatanakun, 1979; CIAT data files.

Figure 1.
Income and Grain Consumption, 1978



NOVEL APPROACHES TO MOLECULAR MAPPING AND FINGERPRINTING

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The polymerase chain reaction (PCR) using arbitrarily selected primers has become established as a rapid, efficient, and economical method to generate fingerprints that are useful in genetic mapping and genomic fingerprinting. The arbitrarily primed PCR (APPCR) generates reproducible fingerprints from any DNA template without the need for prior knowledge of sequences or construction of clone banks; species that have not previously been studied with molecular tools can be immediately mapped or fingerprinted. These fingerprints contain DNA fragment polymorphisms that can be (i) used for varietal identification and parentage determination, (ii) scored natural populations to study population structure and genetics, (iii) used as markers to construct genetic maps from segregating populations, and (iv) used as characters to study phylogenetic relationships, especially at the infra specific and infra generic levels. Like other molecular markers, these markers can be used to guide map-based gene cloning and in marker-aided selection. Because of the very large numbers of loci that can be screened, APPCR markers are particularly useful for rapid screening and identification of markers tightly linked to a trait of interest.

We have used the APPCR to construct a 200 marker genetic map of complex polyploid species, *Saccharum spontaneum* L., a "wild" species of sugarcane. Because of improvements to the methodology, it took only 13 weeks to generate the primary date for such a map. 280 primers (10-mers) were selected for mapping on the 88 progeny. These primers allowed for the screening of almost 1,500 loci, of which approximately 270 were polymorphic in one parent. Over 200 of these polymorphic fragments segregated 1:1 in the progeny, and therefore could be mapped using MapMaker. We are also using the APPCR to study phylogeny of polyploid species within the"*Saccharum* complex" and to study genealogy of maize inbred lines. In addition, we are comparing data from APPCR with data generated from other, more traditional approaches, such as isozymes, RFLPs, and morphophysiological characters. Such comparisons should give us a better understanding of the level of variability that the APPCR can detect.

Because of the enormous productivity of the APPCR, we are developing methods to allow automated data generation and acquisition. We have successfully tested robotic liquid handling of sample preparation and gel loading. A typical one-arm robotic liquid handling station is capable of preparing and loading about 1,300 reactions in an 8-hour day. Analysis of these data then becomes a bottleneck. We are assembling a computerized system for automated data acquisition via a CCD camera or scanner and direct digital imaging and scoring of EtBr-stained gels or autoradiographic images.

We used the polymerase chain reaction (PCR) with primers of arbitrary sequence to construct a genetic map of a complex polyploid species, *Saccharum spontaneum* L., a "wild" relative of cultivated sugarcane. We are also using the arbitrarily primed PCR to (i) study genetic relationships among polyploid species within the '*Saccharum* complex' (*Saccharinae:Andropogoneae*) and (ii) study genealogy of maize inbred lines. In the maize study, we are comparing arbitrarily primed PCR characters with other, more traditional characters, such as isozymes, restriction fragment length polymorphisms, and morphological characters. Such comparisons should give us a better understanding of the level of variability that arbitrarily primed PCR can detect and therefore allow us to further adapt its application.

Because of the high marker output of arbitrarily primed PCR, we are developing methods to allow automated data generation and computer-aided data acquisition. We have successfully tested robotic handling of sample preparation and gel loading. A typical one-arm robotic liquid handling station is capable of preparing and loading about 1,300 reactions in an 8-hour day. Analysis of these data then becomes a bottleneck.

Given the many questions about the origin, evolution, and population genetics of cassava, we think arbitrarily primed PCR offers the potential for rapidly increasing our knowledge about this socio-economically important crop species. A genetic map could be readily constructed, despite polyploidy, and genetic relationships with wild relatives could be studied. In addition, markers derived from studying progeny of appropriate crosses should be invaluable to aid and direct map-based gene cloning and marker-assisted selection. Along with ongoing coupled trans- formation/regeneration efforts, arbitrarily primed PCR constitutes a powerful tool for the application of biotechnology to cassava improvement.

INTRODUCTION

One new application of the polymerase chain reaction (PCR) combines PCR and primers of arbitrary sequence to amplify various loci from any genome, producing a "fingerprint". This method was independently and simultaneously developed by Welsh and McClelland (15) and Williams *et al.* (20) and has been called arbitrarily primed PCR (APPCR) or random amplified polymorphic DNA (RAPD) markers, respectively. For any primer, the resulting pattern of amplified genomic fragments is highly reproducible and can be used for: (i) varietal identification, parentage determination (17, 19), and studies population genetics; (ii) genetic mapping, because they are inherited in a Mendelian manner (1, 11, 18, 20) and analysis of quantitative trait loci, and (iii) for generating phylogenetic hypotheses (19). There are no conceptual differences between the two methods. We choose to use APPCR herein because we feel it is more descriptive since priming is not random but specific, using arbitrarily selected primers.

APPCR has allowed rapid construction of genetic linkage maps. Sederoff and co-workers recently constructed a 191-marker map in loblolly pine in just 2 months (11). Similar work using RFLPs might have taken at least 10-fold longer to complete. In addition, many species of pine have very large genomes, thus it is difficult to load enough DNA on the gel to allow for detection of single or low copy sequences using Southern hybridization. So, APPCR has extended the number of species amenable to genetic mapping with molecular markers, in addition to offering increased mapping output and lower cost of mapping.

Sugarcane (*Saccharum* spp.) is a complex polyploid plant with variable ploidy. In such a situation, the only markers that can be mapped are those present in one parent, absent in the other, and that segregate 1:1 in the progeny, such as in single-dose restriction fragments (21). Polymorphic fragments having other segregation ratios cannot be mapped because there is as yet no statistical method for determining their linkages. Therefore, large numbers of polymorphic fragments are required to saturate the map with those that segregate 1:1. Because of high output

and the requirement of single-dose markers, APPCR is the method of choice for genetic mapping in polyploids. We have modified and optimized the protocol to maximize the number of loci screened in each experiment, with a resulting increase in the number of polymorphisms detected with each primer (12). We have also constructed a 150-marker genetic map of the genome of a "wild" form of sugarcane, *Saccharum spontaneum*, using APPCR (1). To our knowledge this is the first genetic linkage map to be constructed directly using a polyploid species for which diploid relatives are unknown.

Others have used APPCR for sytematics of prokaryotic organisms (21). We have demonstrated that APPCR is useful in parentage determination of maize hybrids (17). Although data analysis needs to be approached cautiously, it is possible that APPCR markers can also be used to aid in systematics and, possibly, to help infer phylogenetic hypotheses about the polyploid complex known as the '*Saccharum* complex' (10), from which sugarcane is thought to have evolved. To test the usefulness of APPCR-generated characters in phylogenetic reconstruction in plant species, we are using a model system in which we are investigating genetic relationships among maize inbred lines.

MATERIALS AND METHODS

Plant materials. *Saccharum spontaneum* 'SES 208' is a 2n=64 form from Sevok, West Bengal. Fitch and Moore (4) derived haploids from SES 208 by tissue culture of anthers. *S. spontaneum* 'ADP 068' is one such haploid that underwent spontaneous doubling of chromosomes during regeneration (Paul Moore, USDA-ARS Aeia, Hawaii, personal communication). A sexual cross was made between SES 208 and ADP 068, from which over 100 progeny were derived at the Hawaiian Sugarcane Planters' Association (Aeia, Hawaii). This constituted our mapping population. Maize inbred lines were obtained from Pioneer Hi-Bred International (Des Moines, IA) and seeds were germinated and grown for 2 weeks before DNA extraction. Total genomic DNA from all plants was extracted according to Honeycutt *et al.* (5).

DNA amplification protocols. Primers were 10-mers purchased from Operon Technologies (Alameda, CA). Amplifications were done in 30-mL reaction volumes in a System 9600 cycler (Perkin-Elmer) or a PTC-100 Programmable Thermal Controller (MJ Research). Reaction components were as described by Sobral and Honeycutt (12), except amplifications done in the PTC-100 were supplemented with 100 mg mL^{-1}acetylated, nuclease-free bovine serum albumin. When amplification products were labelled for subsequent autoradiography, 1 mCi of a^{32}[P]dCTP (3,000 mCi/mmol, New England Nuclear) was included in each reaction. Unless otherwise noted, temperature profile for System 9600 reactions was as described in Sobral and Honeycutt (12). Temperature profile for PTC-100 was: 94°C/3 min, followed by 30 cycles of 94°C/1 min, 35°C/1 min, increase to 72°C at 1°C/2s, 72°C/2 min, then finished with 72°C/7 min and an indefinite 12°C soak.

Automated reaction preparation. Sobral and Honeycutt (12) described the use of a two-arm robot liquid handling station to prepare and load APPCR reactions. For primer-pair amplifications (16), the robot assembled arrays containing equimolar concentrations of 2 primers, then two reaction mixes containing the appropriate template DNAs were added.

Genetic mapping of APPCR single-dose polymorphisms. To determine linkage relationships of APPCR markers we used MapMaker II (7) using a minimum L.O.D. score of 3.00 and a maximum g value of 0.30.

Phylogenetic analyses of APPCR characters. Phylogenetic hypotheses were inferred and tested using either PHYLIP v 3.4 (3), or PAUP v 3.0 (13). In all cases, we considered the character to be the presence or absence of a DNA fragment of a particular size.

RESULTS AND DISCUSSION

Thermal profile and reaction components. Sobral and Honeycutt (12) compared output of APPCR and RAPD protocols, as reported by the authors (15, 20). In genetic mapping of sugarcane, using the temperature profile and conditions reported by Williams *et al.* (20), nearly one-half of the 10-mers they screened failed to give products (12); similar results were observed by Klein-Lankhorst *et al.* (6) in genetic mapping of tomato. This may be caused by differences in the temperature profiles of different cyclers.

Amplification of more fragments might be expected to yield more polymorphic fragments, if the ratio between total number of products and number of polymorphic products is constant for a given pair of DNA templates. Therefore, we might predict that observing more fragments would increase the output of polymorphisms in mapping experiments, or number of informative characters in phylogenetic reconstruction experiments. Surprisingly, Table 1 from Sobral and Honeycutt (12) shows that this was not true. Although more fragments were observed at higher primer concentrations and autoradiography (as in the original APPCR protocol), the number of polymorphisms per primer was very similar for both protocols.

Thermostable polymerases. A protocol that increased the total number of loci screened *and* the number of polymorphisms per primer would enhance output. Sobral and Honeycutt (12) tested alternate thermostable polymerases. They showed that the number of useful primers increased with Stoffel fragment, in relation to both AmpliTaq and *Pfu* polymerases: 30% of primers tested gave no products with AmpliTaq, 34% failed with *Pfu*, while only 2.8% failed with Stoffel fragment. More importantly, with Stoffel fragment the average number of loci screened per primer increased by 30% and the average number of polymorphisms per primer increased by 80% (Table 2) relative to AmpliTaq.

Not only is the output using Stoffel fragment and an optimized temperature profile nearly two-fold higher than that of reported protocols (15, 20), but data cost is significantly reduced because 1 U of AmpliTaq currently costs $0.62, whereas 2 U of Stoffel cost $0.39 (12). Further savings can be obtained by reducing the volume of the reaction. Successful reactions have been done in 13-mL volumes (Ron Sederoff, North Carolina State University, personal communication).

Electrophoresis and visualization of amplification products. The APPCR protocol used denaturing polyacrylamide gel electrophoresis followed by autoradiography as a suggested method of resolution and visualization of products (15), whereas the RAPD paper suggested agarose gel electrophoresis followed by EtBr staining (20). Furthermore, one can use any other variants, such as non-denaturing polyacrylamide gel electrophoresis followed by either autoradiography, silver staining, or EtBr staining.

The main goals of resolution and visualization are to allow clear and reliable detection of amplified products and be as technically simple as possible to enhance output. These goals must be weighed in each situation by pilot studies. For example, in the case of maize, a diploid species, the level of resolution offered by using agarose gel electrophoresis was sufficient (Figure 1). For studying sugarcane genetic relations, Figures 2 and 3 show that denaturing polyacrylamide gel electrophoresis followed by autoradiography was superior because in the agarose or native polyacrylamide systems it was not possible to discern well which fragments are polymorphic and, more importantly, miscoring of fragments of similar length would occur frequently. Figure 4 shows that for genetic mapping of sugarcane agarose gels followed by EtBr staining was an optimal balance between maximum output and maximum resolution.

Genetic mapping of sugarcane. Two-hundred eighty primers (10-mers) were screened against the mapping parents and approximately 90 primers were selected for mapping on 88 progeny. These 90 primers allowed for the screening of almost 1,500 loci, of which approximately 270 were polymorphic. Over 200 of these polymorphic fragments segregated 1:1 in the progeny and therefore could be mapped. Because of our improvements to the methodology (12), it would take as little as 16 person-weeks, or 12 robot-days to generate the primary data for such a map. Each amplification contained 4 repetitions each of the two parents, SES 208 and ADP 068, against which polymorphisms that were reliably observed in *all* 4 repetitions, were identified. This repetition or, better, a template titration allowed the confidence level to be increased because "sporadic" fragments were omitted from analyses. This is important because progeny are only scored once for each polymorphism in most mapping situations to allow for larger numbers of progeny to be analyzed. Figure 5 shows an example of a linkage group from the SES 208 map.

Approximately 70% of all APPCR polymorphisms observed in SES 208 were absent in ADP 068 and thereby single-dose fragments (1). This is in agreement with the ratio obtained using RFLPs and this same cross and population (2; Jorge da

Silva, Cornell University, personal communication). However, detection of single-dose RFLPs required 300-fold more DNA per lane, plus a 7-10 day exposure of autoradiograms, in addition to time spent on gel preparation, blotting, etc. (2). We have detected linkages between single-dose RFLPs (2) and APPCR fragments (1).

Phylogenetic studies, genealogical reconstruction, and population genetics. Many important tropical crop species, such as sugarcane and cassava, have been neglected by researchers in the past. In many of these cases, an understanding of the phylogenetic relationships between the crop species and its wild relatives is of primary importance to breeders. For example, identification of extended gene pools constitutes a fundamental contribution to classical breeding approaches.

APPCR fingerprints can be used in the same manner as RFLP patterns or minisatellitle hybridization patterns to generate primary data for studying genetic relationships (17), except that much more data can be produced in a much shorter time. Caution must be exercised during data analysis because the phylogenies of most plant species are reticulate, and therefore violate the assumption of a bifurcation tree that all exisiting software presupposes (14), although this fact has not kept scores of scientists from using nuclear RFLP or isozyme data to infer bifurcating trees. Given proper caution, however, APPCR fragments can be considered as discrete characters, like RFLPs, that display polarity. Polarity is important for the analysis because it begs character weighting. Character polarity is a reasonable assumption because the presence of an APPCR product of defined length is generally thought to be a function of the existence of 2 nearby target sites in which the first 7 or 8 nucleotides on the 3' end need to match almost perfectly, at least for 10-mers annealing at approximately 35°C. These sites are not unlike closely spaced restriction sites, in which the loss of a site can occur by any change in any nucleotide of the target sequence, whereas once a loss has occurred, regaining the site requires a specific modification at a specific location, a much rarer event.

To understand the capacity for bifurcating-tree-constructing-algorithms to produce useful topologies we are using maize inbred lines as a model system. Our goal is to obtain a large number of characters and blindly reconstruct the genetic relationships that these lines are known to have. A variety of different assumptions will be tested, and different tree-building algorithms will be tried.

Whenever we use APPCR to generate character data for phylogenetic studies, we find it advantageous to do experiments on *at least* 2 different template concentrations for each genotype. The main reasons for this are to have a measure of reproducibility and to allow amplification products that are template-concentration-dependent (sporadic fragments) to be eliminated from the analyses. This approach also has been advocated by others (19).

Future perspectives. We increased output of polymorphisms significantly such that the bottleneck has become data acquisition. If data acquisition were automated then using our protocol and a robot it would be possible to make a 200-

marker genetic map for a diploid organism that had a similar level of polymorphism (as sugarcane) with less than 60 useful primers. In sugarcane, 60 primers could be picked from screening approximately 100-180 primers, in less than 3 full runs of the robot (much less than one day's work). Note that in sugarcane we only map polymorphisms derived from one of the parents (SES 208) because the cross was made to a doubled haploid derived from SES 208. Furthermore, if a robot were to work at the rate of 5 runs/day (less than 5 h to setup reactions and load gels), then the primary data would be ready in 12 days, assuming our current output levels. Such productivity is especially important to those wishing to apply methodologies that have been developed to study marker-trait associations, such as bulked segregant analysis (9) and the use of near-isogenic lines (8, 22) in combination with APPCR technology. Hundreds of primers, or pairwise combinations of primers, allow the screening of thousands of loci in a large number of individuals in very few days. Marker-trait associations could be established with data from more individuals than before because of the increased output and lower cost per reaction.

ACKNOWLEDGEMENTS

We extend most grateful thanks to William Roca (CIAT, Cali, Colombia) for continued efforts to allow BWSS to attend this meeting, to John Welsh (CIBR, La Jolla, CA) for "cheap shots", to Jorge da Silva and Mark Sorrells (Cornell University, Ithaca, NY) for disclosure of unpublished data, to Ron Sederoff (North Carolina State University, Raleigh) for suggestions regarding the use of the robot (which he pioneered), to Craig Hobart (CIBR) for technical assistance, to Paul Moore (USDA-ARS, Hawaii) for harvesting plant materials, and to James Irvine (Texas Agricultural Experiment Station, Weslaco) for continued support and friendship. This work was supported by a grant from the International Consortium for Sugarcane Biotechnology to BWSS: we thank them for their commitment to molecular biology and long-term vision. Finally, we thank Pioneer Hi-Bred International (Stephen Smith, in particular) for their kind gift of the maize genotypes used in this study and for their support and interest.

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Table 1. Comparative output of AP-PCR and RAPD protocols

	AP-PCR	RAPD
Number of primers tested	18	41
Number of loci screened	482	355
Number of informative primers	12	30
Number of polymorphisms	31	67
Single-dose polymorphisms	16[a]	15[b]
Average polymorphisms per primer	2.58	2.23
Average single-dose polymorphisms per primer	1.33	1.88

[a] Number of single-dose polymorphisms was derived from the 12 informative primers, each tested on 35 progeny from the mapping population.

[b] Number of single-dose polymorphisms derived from the 8 most informative primers, each tested on 22 progeny from the mapping population.

Table 2. Comparative mapping output using AmpliTaq and Stoffel fragment

	AmpliTaq	Stoffel
Number of primers tested	7	14
Number of loci screened	80	207
Number of polymorphisms observed[a]	14	57
Average number of loci per primer	11.4	14.8
Average number of polymorphisms per primer	2.0	3.6

[a] Polymorphisms were identified by comparing the fingerprints of SES 208 with those of ADP 068, the other mapping parent. Polymorphisms that were present in ADP 068 but absent in SES 208 were not included in these totals.

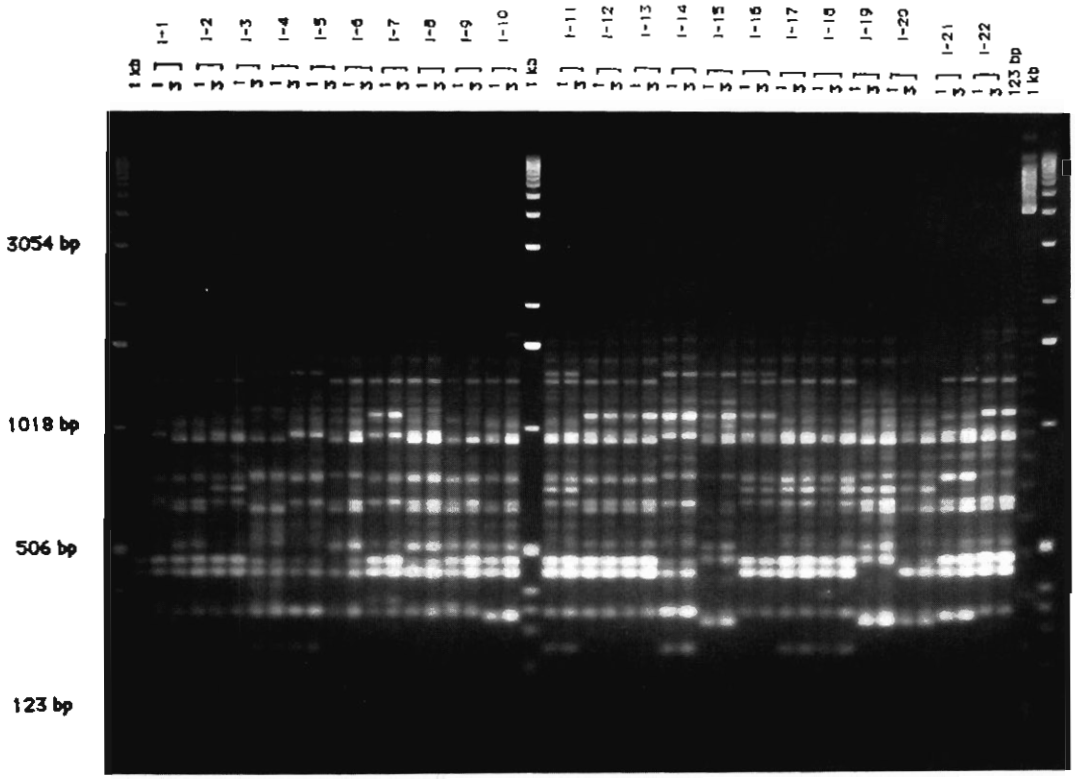

Figure 1. APPCR products generated from maize inbreds using the protocol of Sobral and Honeycutt (12) and resolved in 1.4% agarose gels run at 5-8 V cm^{-1} in a b test horizontal gel box (Stratagene Cloning Systems) for 1,100 V h. The amplification was done in a PTC-100 and a 20-mL aliquot was loaded; notice that each genotype was done twice, at two different template concentrations (1 ng $mL^{-1}1$ and 3 ng $mL^{-1}1$).

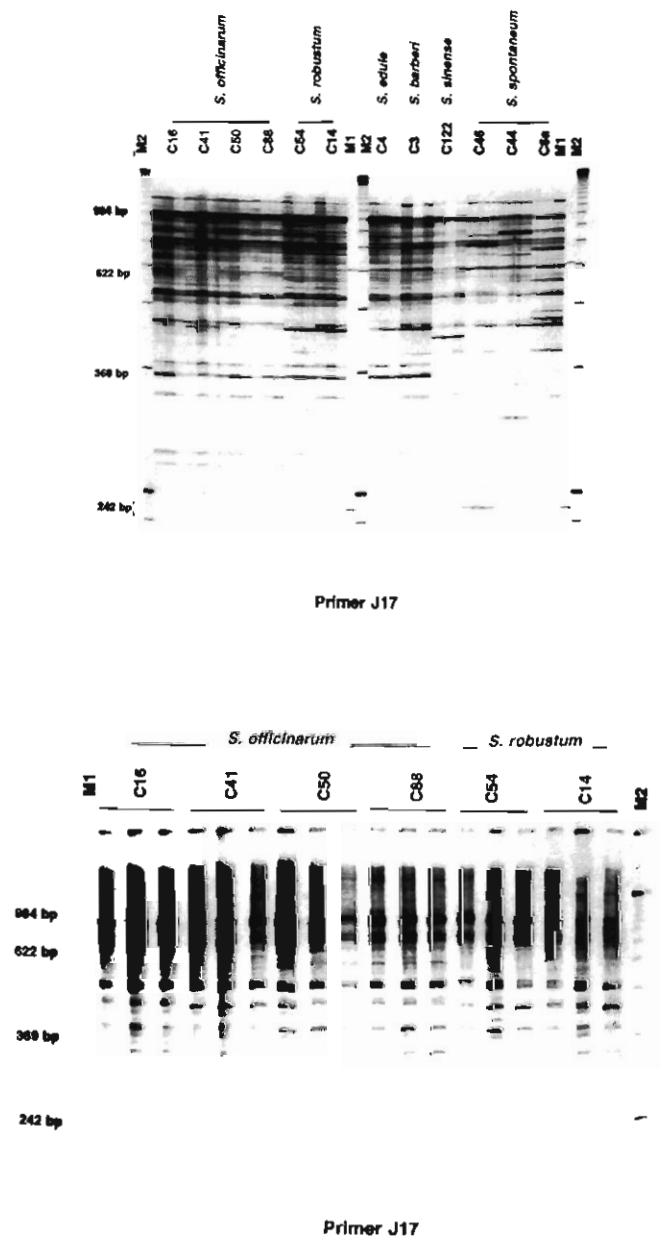

Figure 2. APPCR products generated from *Saccharum* species according to Sobral and Honeycutt (12) and resolved on a 4% denaturing polyacrylamide gel (upper panel) or a 4% non-denaturing polyacrylamide gel (lower panel), followed by autoradiography. Polyacrylamide gels were run at constant power (50 mA) for 3-5 h. Templates were used at 3 different concentrations and loaded sequentially in the following order: 0.3, 1, and 1.7 ng mL^{-1}. Molecular weight markers were as follows: M1=pBR322 digested with *Msp*I; M2=BRL 123 bp ladder.

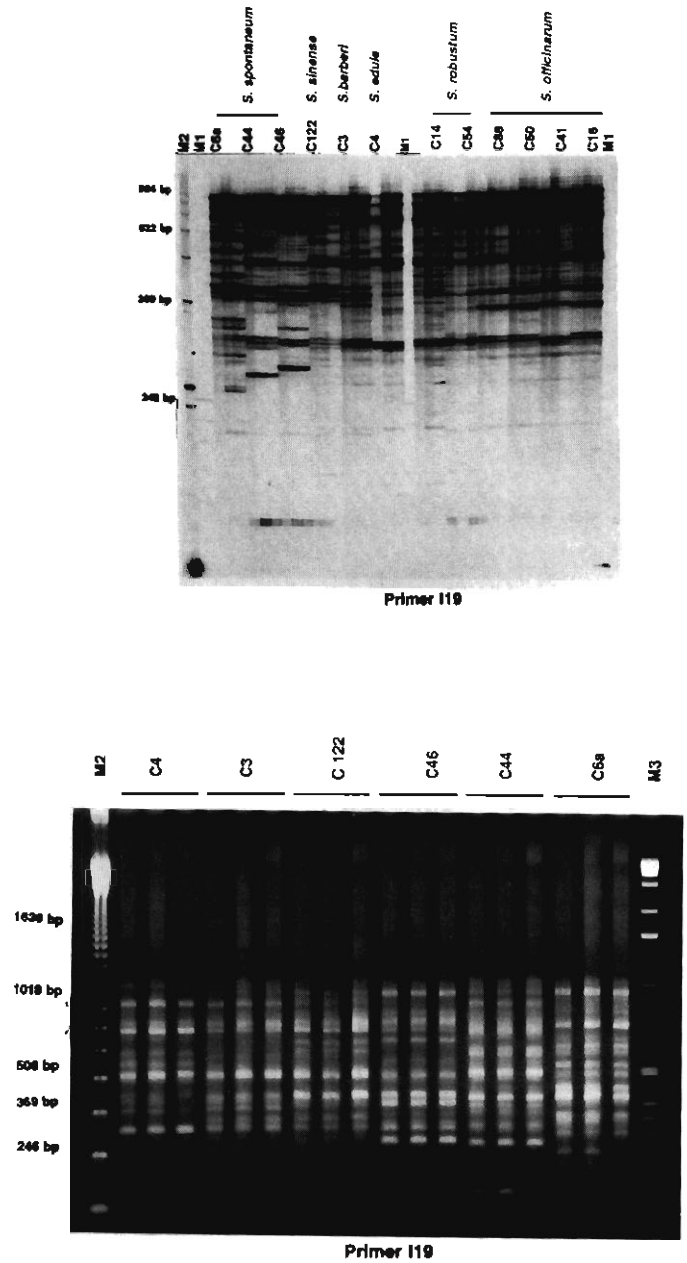

Figure 3. APPCR products generated from *Saccharum* species according to Sobral and Honeycutt (12) and resolved on a 4% denaturing polyacrylamide gel (upper panel) run at 50 mA or a 2.0% agarose gel (lower panel) run at 1,100 V h. For the polyacrylamide gel, after amplification, 4 mL of sample were taken and mixed with 14 mL of denaturing dye, and the mixture was incubated at 85°C for 5 min, put immediately in an ice bath, after which 4 mL were loaded. For agarose gels, 20 mL of the remaining reaction was loaded. C12 on lower panel should read C122. Templates were used at 3 different concentrations and loaded sequentially in the following order: 0.3, 1, and 1.7 ng mL^{-1}. Molecular weight markers were as follows: M1=pBR322 digested with *Msp*I; M2=BRL 123 bp ladder; M3=BRL 1 kb ladder.

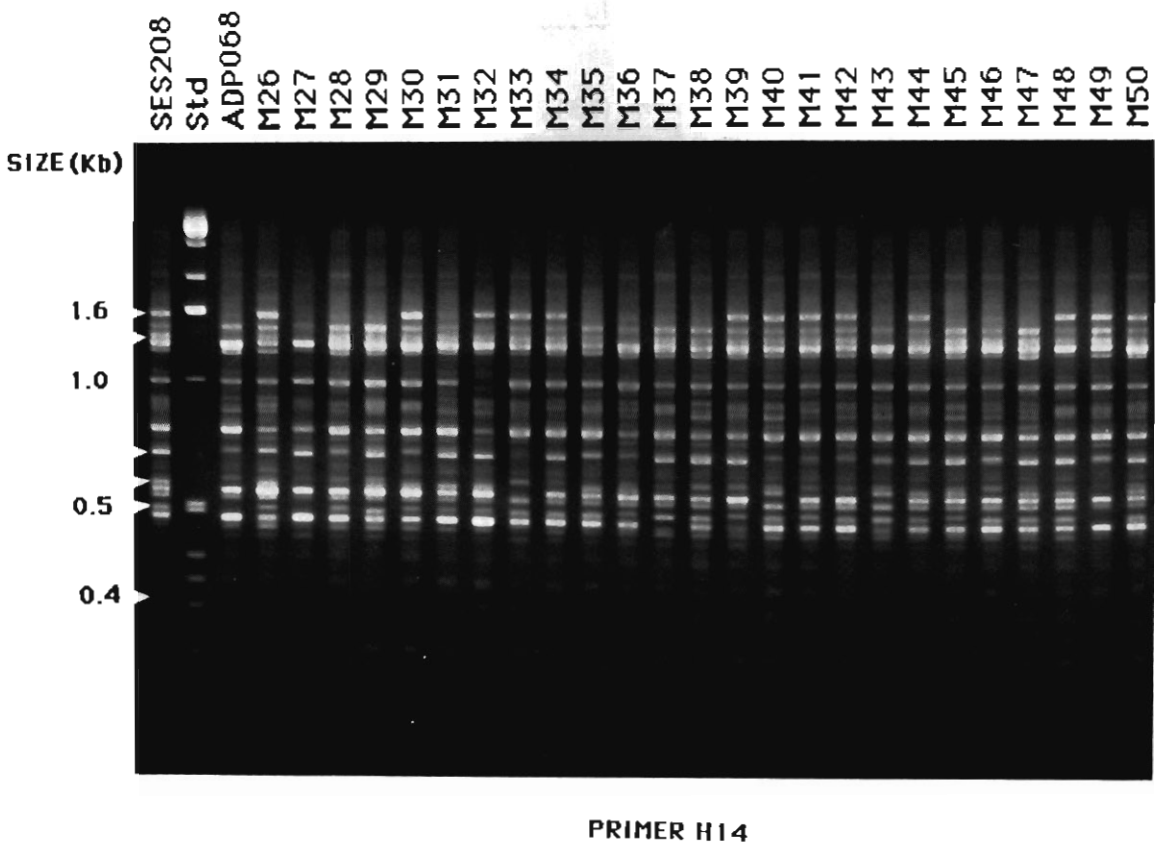

Figure 4. APPCR products generated from a segregating population of *Saccharum spontaneum*. Scored polymorphisms are indicated by arrows.

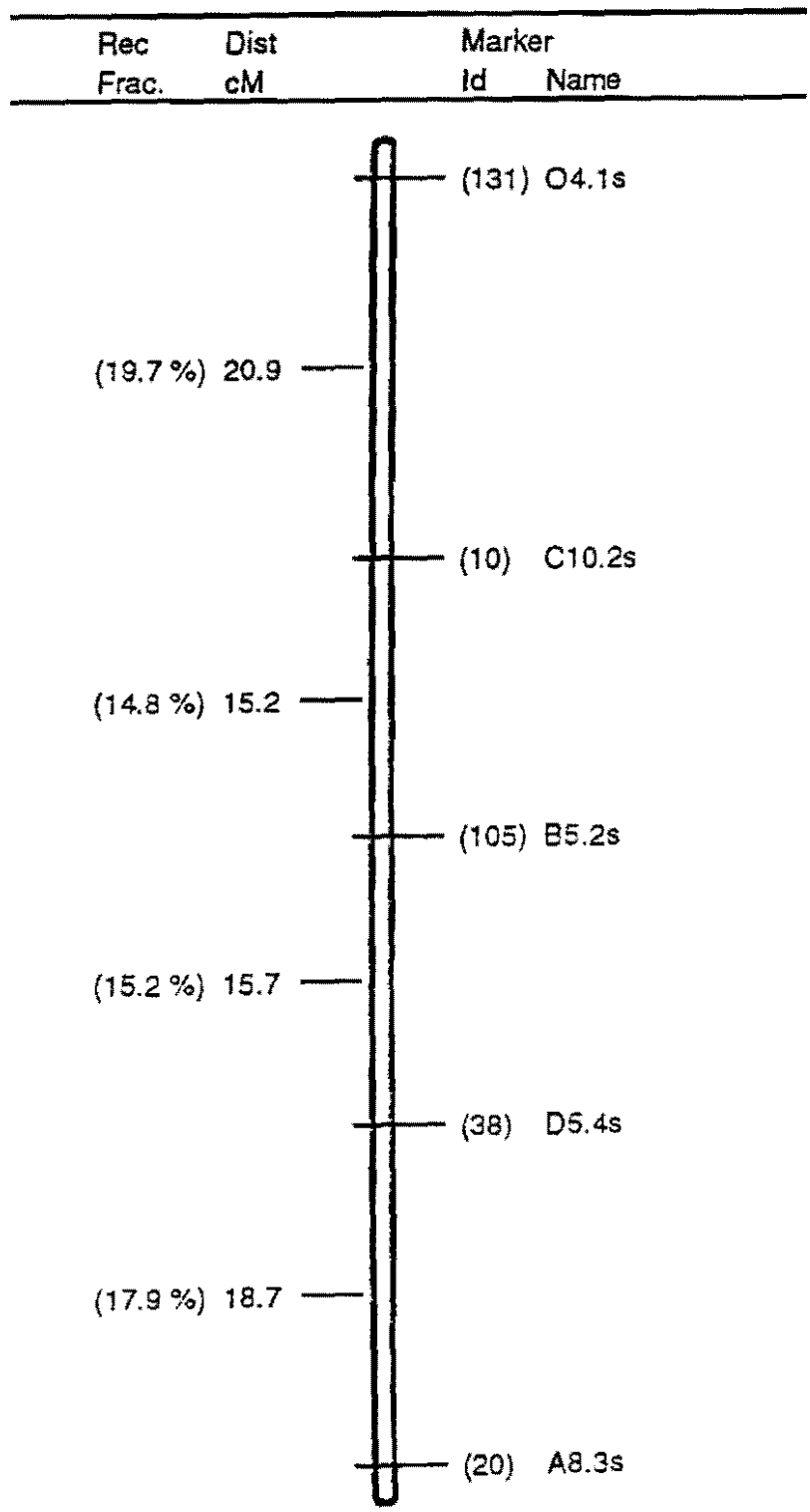

Figure 5. Sample linkage group from *Saccharum spontaneum* 'SES 208' map generated by analysis of nearly 300 APPCR polymorphisms on 88 progeny of cross described in Materials and Methods. 154 single-dose polymorphisms (c^2 at 98% level) were used to calculate this approximation of the map. Of these 133 were found to be linked in 36 linkage groups and 21 markers were unlinked.

CASSAVA GENETIC RESOURCES: A GLOBAL APPROACH FOR CONSERVATION AND USE

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An international workshop on cassava genetic resources was recently held at CIAT. The main objectives of the meeting were to assess the present status of cassava germplasm conservation and use, and to examine the possibility of establishing a global network for cassava genetic resources. A global conservation strategy was discussed, based on the present status of national and international cassava genetic resources programs. Priorities were set for future germplasm collecting expeditions and sharing of conservation workload among institutions for both cassava and wild *Manihot* germplasm. The discussion on a global strategy for conservation of genetic resources involved areas such as: studies on genetic diversity, definition of core collections, identification of duplicate accessions, in-vitro and cryopreservation techniques, duplication of germplasm collections in other institutions, sexual seed and pollen gene collections, etc. The existing mechanisms for safe germplasm exchange were evaluated for an effective movement of genetic resources within the network. Other important subjects discussed during the meeting were: the development of data bases for cassava germplasm, human resource development, and the integration of the germplasm network with other networks (i.e. CBN) and its implications.

INTRODUCTION

Most of the work being developed within the Cassava Biotechnology Network (CBN) is developed on an existing genetic base, trying to: secure its conservation, improve its characterization, facilitate the access of cassava breeders to the available variability, enhance its use and expand it. As a result of a recent international workshop held at CIAT, the Cassava Genetic Resources Network (CGRN) was formally established. Most of the conclusions from that meeting relate to the majority of the previously mentioned areas. This paper will develop the conclusions most relevant to the CBN.

GLOBAL CONSERVATION STRATEGY

Based on the available genetic diversity within the cultigen, regions of priority for future collection were detected (i.e. Bolivia, Haiti, China, Vietnam, several African countries). Major emphasis was given to coordinate the collection of a wide range of wild species in order to study their potential value for cassava breeding. After characterization and intensive evaluation, the most promising species should be thoroughly collected.

There is an urgent need to duplicate existing cassava germplasm collection in order to reduce the risk of losing valuable genetic material. To achieve that and to facilitate global germplasm interchange, the capacity at regional and national program level for testing cassava germplasm for major pathogens is going to be strengthened.

The development of molecular markers will become a valuable tool to conduct studies on the available genetic diversity and the needs for further collections either for cultivated or wild cassava relatives. Molecular markers will also take part in methodologies for the assessment of genetic erosion risk at field level.

Genetic resources represent the base upon which cassava genetic enhancement is developed. It is important to maintain that base in the most efficient way for its continuous use in the long-term. Research will be supported within the CGRN for the development of conservation methods to reduce costs of germplasm maintenance and/or to provide enhanced security. It is expected that cryopreservation will become a routine methodology for long-term conservation in few years. A back-up sample of sexual seeds and pollen should be maintained for most accessions grown in the field at the moment. National programs in regions corresponding to primary centers of diversity are going to be encouraged to set up *in-situ* conservation for areas with considerable genetic diversity of wild relatives.

GERMPLASM CHARACTERIZATION AND EVALUATION

Characterization of cassava germplasm accessions should be based on descriptors that are little influenced by the environment in their expression. In that sense, a few morphological, biochemical and molecular markers are going to be used for a more reliable description of genotypes and for duplicate identification within existing cassava germplasm collections.

Most of the time it is not possible to evaluate the whole range of available genetic diversity for certain traits of importance. Cassava breeders and scientists in related areas should develop standard, simple, reliable and non-expensive screening techniques to evaluate the existing genetic diversity and future needs. Screening methodologies are being developed for cyanide content in root parenchyma, cooking quality, starch quality, water and nutrient use efficiency, etc. For those traits where screening of large samples of genotypes remains difficult, the study on linkages with molecular markers should be emphasized. The use of molecular markers will also be considered for more efficient evaluation and selection of quantitative traits and to improve the transference of desirable qualitative traits from wild relatives.

ACCESS TO GENETIC DIVERSITY

A global database for information related to cassava genetic resources will be implemented through interlinked regional databases placed at international centers for Latin America and Africa (CIAT and IITA respectively) and at a National Program in Asia. Participants committed themselves to supplying the available information and conducting future evaluations based on standard procedures already defined (primary descriptor list and traits of importance for breeders and other scientists). This global data base will be available to network members and other related networks. Legal status will be sought for the central data base as a means of

protecting cassava germplasm from exclusive economic exploitation of landraces and wild relatives.

In order to improve the accessibility to the available genetic diversity within large germplasm collections the definition of core collections representing the range of genetic diversity has been considered as a valid approach. The use of molecular markers in genetic diversity studies will certainly improve the definition of core collections. CIAT has already defined a core collection from its germplasm collection constituted in a large proportion by Latin American accessions. It was recommended that such core collection should be: duplicated within cassava national programs in Brazil and Thailand; evaluated for relevant traits across different sites and seasons within Colombia; and the information generated should be incorporated in a special data base for the core collection which will be frequently updated and circulated among users.

Given the relevance that genetic resources for wild cassava relatives will acquire in the near future, it is important to define evaluation criteria and methodology for them, taking into account the inherent variability among and within accessions of a particular species.

BROADENING THE AVAILABLE GENETIC BASE

Cassava breeders agree that genetic diversity within the cultivated species is far from exhausted, and most of the efforts should concentrate in a better understanding of it. There are some specific traits for which there is a limited genetic diversity in existing collections (i.e. amylose, amylopectin content in root parenchyma), or other traits for which new sources of genetic information are desirable (i.e. resistance to African Cassava Mosaic Virus). In those cases, there is a need to support the development of techniques for the transfer of genetic information from distantly related species into cassava, the inclusion of regulatory gene sequences, or other possible mechanisms by means of transformation techniques.

CONCLUSIONS

A global network for cassava genetic resources has been established with strong regional components supported by International Centers. Its main functions will be: a) to promote germplasm collection in areas of major priority, including wild relatives of cassava. b) To share the workload among members in order to secure conservation, characterization, evaluation and use of cassava genetic resources. c) To coordinate training in quarantine- related aspects, pathogen testing, germplasm conservation and exchange methodologies, analysis of genetic diversity by biochemical and molecular methods, and use of software for documentation within the network's data base. d) To

make genetic resources available to users through a global data base, secure mechanisms of exchange and interaction with other networks of prime importance, such as the CBN.

Integrated use of cassava genetic resources will result from a close interaction among members of the CBN, CGRN and cassava breeders. Cassava genetic improvement should be based on a profound knowledge of what is available in nature and what could be reached through modern biotechnologies. The main result from such an integrated approach will be improved varieties, which in turn are one of the components of the cassava production system. Impact at field level will greatly depend on variety diffusion taking into consideration the complexity of the production system and the socio-economic background of cassava farmers, processors and consumers in a particular region.

CYTOGENETICS OF *MANIHOT* SPECIES AND INTERSPECIFIC HYBRIDS

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The genus *Manihot* comprises about 98 species, 22 of which are available in IITA's collection. These are being used to broaden the gene pool available to the cassava breeding unit. All the species, including cassava (*M. esculenta Crantz*), have a chromosome number of 2x = 36 and form 18 bivalents at probase-1 and metaphase-1 (M-1). Wide hybridization with cassava as well as between wild species using 12 of the available *Manihot* species was effected. The contrasting parental characters showed partial to total dominance in the F_1 hybrids. Attempts were made to identify the chromosomes individually in the species using karyomorphological criteria. Most bivalents were identifiable from characteristics such as telechromomeres, distribution of heterochromatin, association with nucleolus etc. This was especially so with about 10 bivalents. Chromosomal similarities and differences based on their karyomorphology at pachytene stage in different *Manihot* species were established. Through chromosomal pairing in the F_1 hybrids, the homoeologous partners were identified. A general decrease in the chiasma frequency at M-1 in the F_1 was observed compared to parents. The presence of chromosomal structural differences was revealed by non-pairing of chromosomes, anaphase 1 bridges and fragments etc. Pollen fertility of the F_1 hybrids varied considerably.

INTRODUCTION

The genus *Manihot* comprises about 98 species mainly confined to the American tropics. Cassava, *M. esculenta*, stands out in economic importance owing to its starchy tuberous roots which make it one of the staple foods in Africa. However, comparatively little is known about the cytogenetics of *Manihot* species. All the species so far studied have 36 somatic chromosomes (2n =36). Cassava is generally considered as a diploid but an allopolyploid origin with a basic chromosome number of x = 9 (Perry, 1943; Jennings, 1963; Umanah and Hartman, 1973) and a segmental allotetraploid origin (Magoon et al, 1969) have been postulated.

MANIHOT SPECIES AT IITA

Wild *Manihot* species are considered important sources of many economically important characters. IITA has a collection of about 22 species introduced mainly from Brazil, Colombia and Mexico which include *M. anomala, M. Brachyandra, M. caerulescens, M. carthaginesis, M. catingae, M. chlorosticta, M. dichotoma, M. epruinosa, M. flavellifolia, M. gracilis, M. leptophylla, M. neusana, M. pohlii, M. pringlii, M. reptans, M. quinquepartita, M. glaziovii* and Tree cassava. These species are being evaluated and utilized through interspecific crosses in improving the productivity, pest resistance and adaptability of cassava as a supplement to the variation available in cassava.

INTERSPECIFIC HYBRIDIZATION

To realise the potential for germplasm introgression to *M. esculenta*, biosystematic relationships among a broad spectrum of species in the genus will be very useful. The number of reported attempts to obtain interspecific hybrids of *Manihot* species are few (see for Review Bai, 1987) and not much information is available on the general cross-compatibility relationships within the genus. This may be perhaps due to the difficulty in obtaining hydrids. However, at IITA, twelve of the available species have been successfully used in crosses with cassava and hybrids have been produced. In addition to hybridising cassava with *Manihot* species, crosses have been made between wild *Manihot* species and a few hybrids have thus been produced (Table 1).

CYTOGENETICS OF SPECIES AND HYBRIDS

Meiotic studies in the *Manihot* species have revealed that normal bivalent (18II) pairing occurs in the species. Occasionally non-pairing or 1 or 2 univalents also occur (see also review, Bai, 1987). The average chiasma frequency at M-I range from 24.0 in *M. leptophylla* to 34.0 per PMC in *M. esculenta*. There was good correspondence between preponderance of rod bivalents and low chiasma frequency. In species with high chiasma frequency one or two of the ring bivalents (maximum of 13 II per PMC) frequently have 3 chiasmata. A-I disjunction and distribution of chromosomes is generally normal and subsequent divisions are also normal leading to normal tetrads and high pollen stainability (60 to 95%).

Reports on the cytogenetic analysis of interspecific hybrids with cassava (see review Bai, 1987) or between wild species are scarce. Meiosis in the hybrids presently reported was comparatively normal with 18 II at prohase-I and M-I and occasionally 2 to 4 Is or 1 IV was observed (Table 2). The chiasma frequency in the hybrids showed an overall decrease over the parents and ranged from 20 in hybrids between *M. epruinosa* x *M.leptophylla* to as high as 28 in hybrids between cassava x M. leptophylla. At A-1, though the majority of the hybrids had normal disjunction of chromosomes, in a few hybrids a bridge and a fragment indicating heterozygous inversion and one or two laggards were frequent (table 2). The average pollen stainability of interspecific hybrids of cassava ranged from 14% to 90% in hybrids with *M. tristis* and 19 to 27% in hybrids with *M. pohlii* and that of the hybrids between wild species ranged from 12 to 50% in *M. epruinosa x M. glaziovii* and 25 to 90% in *M. tristis x M. tripartita* and *M. brachyandra x M. epruinosa*.

Interspecific hybrids of cassava with species like *M. glaziovii, M. epruinosa, M. leptophylla, M. brachyandra* as well as the hybrid between *M. tristis and M. leptophylla* produce small percentages of 2n or unreduced gametes as a result of 1st division or 2nd division restitution. Such 2n gametes were instrumental in the origin of the spontaneous sexual polyploids in cassava (Hahn *et al*, 1990).

PACHYTENE CHROMOSOMES

The somatic chromosomes of *Manihot* species studied so far are small and difficult to karyotype. However, the chromosomes at pachytene stage of meiosis are amenable to karyological analysis and had been karyotyped in cassava (Magoon *et al.* 1969) and *M. glaziovii* (Krishnan *et al.* 1970).

Through the importance of studying pachytene chromosome morphology in problems of species inter-relationships and chromosome structural changes has been well realised, the difficulties associated with it have discouraged such cytogenetic analyses. In a genus such as *Manihot* where the somatic chromosomes are too small, pachytene chromosome studies should be helpful in evaluating species relationships.

In seven of the *Manihot* species reported here,the pachytene chromosomes are being studied and are found as differentiated, each chromosome consisting of a centromere with proximal heterochromatic and distal euchromatic regions and metacentric, submetacentric or acrocentric. These species have 3 chromosomes associated with the nucleolus. The morphological characters that were used to identify the chromosomes include (i) chromomeric pattern, (ii) position of centromere, (iii) presence of deeply stained knobs, (iv) presence of telechromomeres, (v) relative lengths of hetero- and eu-chromatic regions in the arms, (vi) presence of accessory nucleolus and (vii) association with the nucleolus. Total length and relative length are other criteria used to distinguish the chromosomes which cannot be easily distinguised based on the other 7 criteria. As such at least 10 out of 18 chromosomes could be distinguised using these criteria. These chromosomes can be considered as specialised chromosomes and can be readily identified from the morphological features without measuring the total length or arm ratios. There are generally 6 such types viz; (i) chromosomes associated with the nucleolus (ii) chromosomes with an intercalary accessory nucleolus (iii) chromosomes with telochromomeres in either of the arms (iv) chromosome with telochromomere on both arms and a promient intercalary knob, (v) chromosomes with completely heterochromatic short arm and (vi) chromosome with no distinct chromomeres at all.

Preliminary observations on the pachytene chromosomes of *M. brachyandra, M. catingae, M. epruinosa, M. glaziovii, M. tristis, M. esculenta and M. leptophylla* show that they differ with respect to the number of chromosomes with telomeres and the number of chromosomes with total heterochromatic short arm. *M. glaziovii* and *M. esculenta* have 7 chromosomes with telomeres, *M. tristis* and *M. catingae* have 6 with telomeres; *M. epruinosa* has 5 and, *M. leptophylla* has 8.

The pachytene chromosomes in the interspecific hybrids also showed total synapsis with occasional terminal or interstitial non-pairing or occasionally two univalents. Even when they pair completely, small structural differences in the length, number of chromomeres, size of telochromomeres etc. of the homoeologous partners

could be discerned when the chromosomes were observed at late pachytene stage. However, they have general homology and can be considered as closely related.

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Table 1. Crosses Between Cassava and Wild *Manihot* spp.

Female parent	Male parent	No. of Fls. pollinate	Fruit set (%)	Seed set (%)	No. seeds/ fruit
TMS 30555	*M. anomala* -7	115	18.26	17.97	2.95
TMS 42025	*M. anomala* -7	133	16.54	14.28	2.59
TMS 30555	*M. epruinosa* -46	338	11.24	9.17	2.44
TMS 42025	*M. epruinosa* -34	228	21.05	7.90	1.12
TMS 30555	*M. tristis* -39-3	878	32.46	27.26	2.52
TMS 42025	*M. tristis* -39-3	1870	14.06	7.50	1.60
TMS 30555	*M. gracilis* -26	93	21.50	17.92	2.50
TMS 30555	*M. tripartita* -24	50	26.00	21.33	2.46
TMS 42025	*M. leptohylla* -1	667	1.94	1.44	2.23
TMS 42025	*M. pohlii* -1	161	9.32	8.28	2.66

Table 2. Chromosome Behaviour at M-I and A-I in Interspecific Hybrids.

Parents Female	x	Male	Chromosome pairing at M-I	Chromosome separation at A-I
M. esculenta	x	*M. glaziovii*	18 II	Normal
M. esculenta	x	*M. epruinosa*	18 II	Normal/1 bridge + 1 fragment
M. esculenta	x	*M. leptophylla*	18 II	Normal
M. esculenta	x	*M. brachyandra*	18 II	Normal
M. esculenta	x	*M. gracilis*	18 II/17 II + 2 I	Normal/1 bridge + 1 fragment
M. esculenta	x	*M. anomala*	18 II	Normal
M. esculenta	x	*M. tristis*	18 II	Normal/1 laggard
M. esculenta	x	*M. pohlii*	18 II	Normal
M. esculenta	x	*M. tripartita*	18 II	Normal
M. esculenta	x	*M. flavellifolia*	18 II	Normal/1 bridge + 1 fragment/laggard
M. tristis	x	*M. glaziovii*	18 II	Normal/1 bridge + 1 fragment
M. tristis	x	*M. brachyandra*	18 II	Normal/1 bridge + 1 fragment
M. tristis	x	*M. leptophylla*	18 II/17 II + 2 I	Normal
M. tristis	x	*M. tripartita*	18 II	Normal
M. epruinosa	x	*M. leptophylla*	18 II	Normal
M. epruinosa	x	*M. brachyandra*	18 II/17 II + 2 I /1 IV + 16 II	Normal/1 bridge + 1 fragment
M. catingae	x	*M. glaziovii*	18 II	Normal
M. catingae	x	*M. epruinosa*	18 II/17 II + 2 I	Normal
M. catingae	x	*M. tripartita*	18 II/17 II + 2 I	Normal/2-3 laggards
M. brachyandra	x	*M. glaziovii*	18 II/17 II + 2 I	Normal

STUDY OF DNA POLYMORPHISM IN *MANIHOT ESCULENTA* Crantz AND RELATED SPECIES

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Phylogeny and cultivar differentiation studies were undertaken in cassava using molecular markers via recombinant DNA (RFLP) and via polymerase chain reaction (RAPD). Mitochondria from field grown cassava plants were isolated and purified in a 29% percoll solution and the organella DNA (mtDNA) extracted. The mtdna was digested with a set of restriction enzymes, blotted in a filter paper, and the mtdna polymorphism revealed using probes of genes coded in the mitochondria of a heterologous species. Total DNA from leaf of cassava was extracted by a modified method using CTAB detergent. A high molecular weight, and a high quality DNA was used in a polymerase chain reaction with an arbitrary primer (5' -TCCCAGTCACGACGT) to reveal RAPD markers for differentiating cassava cultivars. The polymorphic pattern of restricted digested mtDNA as well as random amplified nuclear DNA fragments were used to establish the species relationship and to distinguish particular individuals in a germplasm bank.

INTRODUCTION

Cassava is an outbreeding species with 2n=36 chromosomes and is considered to be of tetraploid origin. Research on the origin of cassava pool has been undertaken via species relationship studies based on morphological characteristics, cytogenetic description, natural occurrence of interspecific hybrids, facilitated gene exchange among species of *Manihot*, as well as categorization of individuals based on computer aid analysis (Rogers and Appan, 1973; Allem, 1991; Nassar, 1985). A biosystematic analysis of the Brazilian species using seed protein profile in gels reflected a wide morphological and geographical diversity of the genus (Grattapaglia et al, 1987). However, further research on the characterization of the cassava gene pool is needed. The advance on the development of methods using DNA polymorphism as molecular markers is making significant impact on studies such as species evolution, molecular taxonomy, genetic diagnostics, as well as breeding (Tanksley et al, 1989; Watkins, 1988). In order to better understand *Manihot* species relationship, our laboratory is conducting studies on the DNA polymorphism of mitochondria DNA (mtDNA) via recombinant DNA (RFLP), as well as on polymorphism of nuclear DNA via Polymerase Chain Reaction (PCR).

Breeding in this crop has been largely based on mass selection of bulk segregating population of germplasm originated from collection of plants naturally occurring in the wild. Traits such as starch quality have been difficult to access. Therefore, our project is also oriented to find a molecular marker to differentiate cultivars for starch quality variation in cassava root tuber.

METHODS

Young leaves of *Manihot* (Table 1) were collected in the field, washed, and used for total and mtDNA extraction. Two hundred to 300 g F.Wt. were gently ground in a polytron with an osmotically buffered medium, filtered and mitochondria were isolated by differential centrifugation and purified in a discontinuous sucrose density gradient followed by a continuous percoll gradient. Purified organelles were lysed in a SDS solution and mtDNA alcohol precipitated. Up to 5.0 g F.Wt. was homogenized in a buffered solution containing CTAB pre-heated at 65°C and total DNA isolated by differential centrifugation and alcohol precipitated. The total DNA extraction was used as template (5 ng) in 100 μl amplification reaction. Random primers (OPERON Technologies, Alameda, CA.), dNTPs and Taq polymerase (Stratagene, La Jolla, CA.) were used under amplification cycle conditions of 1 min at 90°C, 1 min 42°C, and 2 min 72°C, using the fastest temperature transition between cycles during 45 cycles. Amplification products were analyzed in a 1.4% agarose gel stained with ethidium bromide. Bands were scored for presence and absence from the results of six preselected primers and the genetic variability analyzed by NTSYS-pc (Rohlf, 1988).

RESULTS AND DISCUSSION

It is observed in Table 1 that the method of DNA extraction used can produce a high quality DNA of OD_{260}/OD_{280} ratio greater than 1.8, as well as a yield of up to 730.0 μg/g F.Wt. of leaf tissue. It is of particular importance since cassava leaves have a high content of latex with complex carbohydrate content. This method can also produce restriction enzyme digested DNA (Figure 1) as well as DNA of enough quality to be used in PCR.

Isolated mtDNA (Table 2) through organelle separation from green leaves tissue yielded up to 66 ng/g F.Wt., which is very low when compared with other similar methods in other species (Skubats and Bendich, 1991). The DNA polymorphism via RFLP could not yet be performed under the available conditions in our laboratory.

Screening of 20 primers of arbitrary sequence with two wide distant species of *Manihot* indicated six primers with more than six polymorphic amplified products. Those six primers were used to study genetic variability among species of *Manihot*, as shown by a sample of three primers in Figure 2. A great DNA polymorphism among species is observed under the conditions of the amplification reaction cycles used in this experiment. While the pattern of amplified products using primer OPC-14 showed presence and absence of varying size bands, the other primers showed more similarly bands of high molecular weight (Primer OPC-16) and low molecular weight (Primer OPC-09) of amplified products. Statistical analysis of the pattern of amplified products considering all bands, indicated high similarity between *Manihot esculenta* Crantz cultivars and *M. pilosa*, followed by *M. esculenta* ssp *peruviana* and *M. esculenta* ssp *flabelifolia*.

Further research is underway with more representative species of Manihot as well as more primers to better understand the species relationship in this genus.

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Table 1. DNA content in leaf tissue of cassava.

FW	A_{260}/A_{280}	µg/µl	µg/g
Manihot esculenta			
" cv. Amazonas	2.08	8.66	346.4
" cv. IAC 14-16-7	2.04	3.40	272.0
" cv. Sutinga	2.02	2.34	187.2
" cv. CM 425-6	2.04	3.60	288.0
" cv. CM 430-37	2.03	2.95	236.0
" cv. Abacate	2.06	3.10	248.0
" cv. IAC 5-36	2.05	4.30	344.0
" cv. CM 507-37	1.98	1.90	152.0
" cv. IAC 531	2.04	7.40	296.0
" cv. Sacri I	1.98	6.76	270.4
" cv. CM 525-7	1.99	4.11	164.4
" cv. Imbroxeiro	1.88	6.07	242.8
" cv. IAC 12-829	1.84	4.98	199.2
" cv. Riqueza 3	2.80	18.23	729.2
" cv. Jaçanã	1.86	5.35	214.0
" cv. Pioneira	1.86	5.20	208.0

Table 2. Mitochondrial DNA extracted from green leaves of cassava.

Sample	F.Wt.(g)	Yield(ng/g)
Manihot esculenta		
cv SUTINGA	250	59,8
cv CM 507-37	120	19,0
cv IAC 531	125	51,2
cv CLONE 83	100	65,6
cv RIQUEZA 3	156	30,7
cv PIONEIRA	150	15,0
cv IMBRUXEIRO	125	3,84

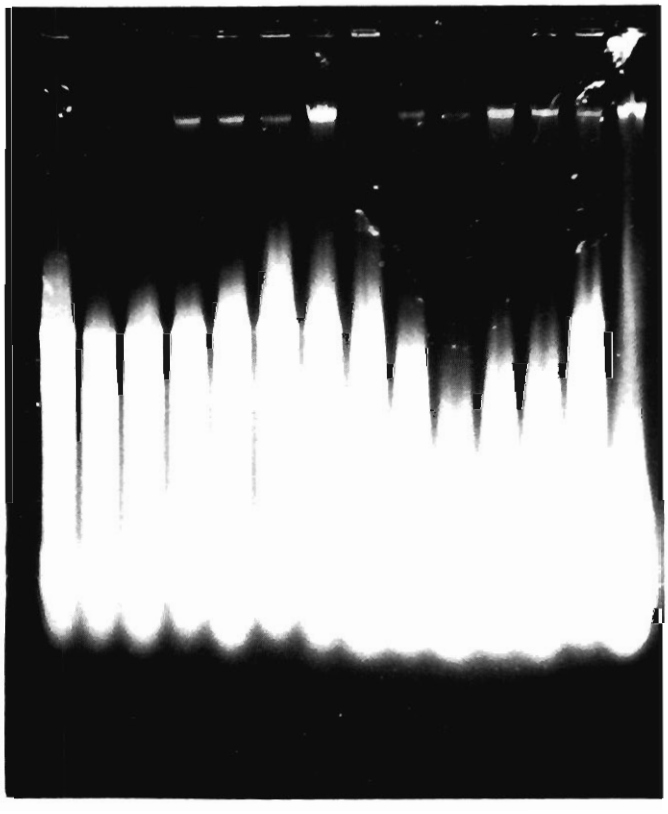

Figure 1. Total DNA from leaves of different cassava cultivars digested with restriction endonuclease.

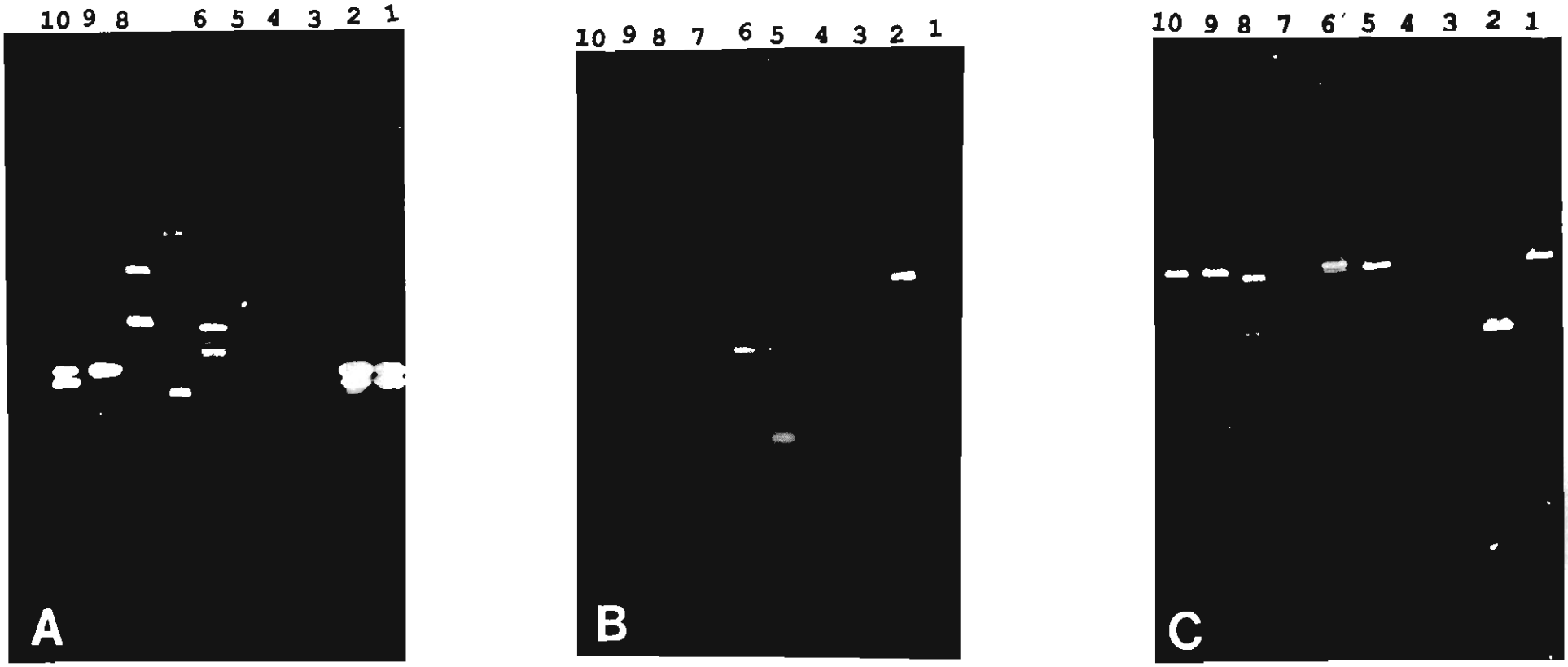

Figure 2. Pattern of amplified products of DNA extract from leaves of different species of *Manihot* using primer OPC-09 (a), OPC-14 (b) and OPC-16 (c) of arbitrary nucleotide sequence. Especies tested are *M. esculenta* cv. Abacate (1); cv. IAC 5-36 (2); cv IAC 12-829 (3); cv. Pioneira (4); *M. pilose* (5); *M. quinquepartite* (6); *M. glasiovii* (7); *M. irwinii* (8); *M. esculenta* spp. *peruviana* (9) and spp. *flabelifolia* (10).

USE OF RFLPs AND RAPDs IN CASSAVA GENOMIC STUDIES.

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The construction of a detailed genetic map of cassava (Manihot esculenta Crantz) will be useful to tag agronomically important traits as well as to isolate and to clone cassava genes in the future. This map will also contribute to the understanding of cassava genomic structure and evolution.

As a first step in developing the cassava molecular map, cloned nuclear sequences and several oligodeoxy- nucleotides primers were used to study different crosses in Manihot. Polymorphisms and segregations were detected in the parental lines and their offspring, respectively. Cloned nuclear sequences and different primers used, each 10 nucleotides in length, were selected previously on the basis of their ability to detect polymorphism in the parental lines (Angel et al., 1991).

An interspecific cross (MCol 1505 x M. aesculifolia) and an intraspecific cross (MCol 1522 x CM2772-3) were evaluated by both RFLPs and RAPDs. Segregation of the markers used here in the two crosses will be presented and discussed.

INTRODUCTION

We have initiated a research project to construct a molecular map of cassava using Restriction Fragment Length Polymorphism (RFLPs) and Random Amplified DNA (RAPDs) markers. The construction of a detailed genetic map of cassava will contribute significantly to the understanding of cassava genetics. It will be useful to analyze the genomic structure of cassava and its wild relatives, facilitate introgression from wild species for targeted traits and to tag agronomically important traits, simply and quantitatively inherited. Eventually the map will be useful to isolate and clone cassava genes.

METHODOLOGY

As a first step in the construction of a detailed genetic map of cassava we evaluated cloned nuclear sequences from five different genomic libraries generated, at the BRU, with different restriction enzymes. Different random genomic probes were compared in their ability to detect polymorphism in several cultivated lines and in one wild *Manihot* species, *M. aesculifolia*. Cloned nuclear sequences and different primers selected previously on the basis of their ability to detect polymorphism (Angel et al. 1991), are being screened in the offspring from different controlled crosses. DNA polymorphism in a wild *Manihot* species and some cassava cultivars as well as segregation of markers using RFLPs and RAPDs were evaluated.

Nuclei from green leaves were isolated as described (Vayda et al. 1986). Nuclear and genomic DNA isolation procedures were similar to that reported previously (Dellaporta, S.L. et al. 1983). Nuclear DNA was divided in five fractions

and each one was digested with one of the following restriction endonucleases (Pst I, Eco RI, Bam HI, Hind III, Xba I). Fragments between 0.5 and 3.0 Kb were cloned into the polylinker site in the pUC 19 plasmid and recombinant colonies were selected based on X-gal and IPTG screening procedures. Low copy number inserts were preselected by hybridization in dot-blot of each insert with ^{32}P-labeled total genomic DNA.

DNAs of eleven cassava genotypes from different geographical origins (three from Colombia, two from Brazil, one from Argentina, two from Thailand, one from Nigeria and two hybrids) were digested with ten different enzymes and probed with the whole plasmid including cassava inserts. A wild *Manihot* species from Mexico, *M. aesculifolia*, was included in this study.

Amplification reactions for the RAPDs analysis were performed in a Perkin Elmer Cetus DNA thermocycler programmed for 40 cycles of 1 min at 94°, 1 min at 36°, 2 min at 72° (Williams et al. 1991).

RESULTS

The polymorphism among cultivated varieties evaluated was extremely low, except for MCol 22. Nevertheless, the higher polymorphism was found when MCol 22 was compared with the other ten genotypes evaluated. The percentage of polymorphic probes in intraspecific comparison is presented between MCol 1505, which represents the group of ten cultivated varieties and M Col 22 (Table 1, Fig. 1).

In interspecific polymorphism, when patterns of hybridization between M Col 1505 and *M. aesculifolia* were compared, the percentage of polymorphic probes were, in all cases, higher than those detected among cultivated genotypes (Table 1, Fig. 2). If we compare the ability of the probes to detect polymorphism in both cases, intraspecific and interspecific, we can classify Pst I, Xba I and Hind III genomic probes in a first group, detecting higher polymorphism levels, and Eco RI and Bam HI genomic probes in a second group, detecting low polymorphism levels.

Furthermore, different restriction enzymes were compared in their ability to detect polymorphism among the same genotypes (Table 2). Eco RI was the best for displaying polymorphism in both cases. Eco RV and Hae III also detected high polymorphism levels. Bam HI in intraspecific but not in interspecific and Xba I in interspecific but not in intraspecific groups also detected high polymorphism levels (Table 2).

F1 progenies from an intraspecific cross are being evaluated in order to observe the segregation of genomic probes and polymorphic primers. Autoradiograph (Fig. 3) shows RFLP segregation displayed by a Pst I genomic probe after hybridization with Eco RV digested DNA from an F1 population. 43 plants showed the female parent pattern and 40 plants the male parent pattern. This probe detect two loci, one with a double banded allele and the second single banded with two

alleles, the second locus is heterozygous in the female parent and homozygous in the male parent (Fig. 3).

Several primers have been screened in the same cross. Segregation of two primers is shown (Fig. 4), J12 detecting one locus, and B11 detecting two loci in the parental lines (Fig. 4, see arrows). Those bands segregate close to the expected ratio 1:1.

SUMMARY

- Polymorphism detected between cultivated genotypes and *M. aesculifolia* was higher than that among cultivated genotypes.

- Hind III, Pst I and Xba I probes detected higher polymorphism than Bam HI and Eco RI probes.

- Cassava DNA digested with Eco RI, Eco RV and Hind III displayed more polymorphism.

- Four cutter restriction enzymes displayed less frequency of polymorphism when compared with six cutter restriction enzymes among cultivated genotypes.

- Polymorphism displayed by Dra I was extremely low indicating that regions rich in adenine and thymine may not be hot spots for mutations in cassava.

- RFLPs and RAPDs segregation indicated that those markers will be very useful in the construction of a molecular map of cassava.

RFLPs and RAPDs studies will be continued for the cross shown here and other crosses generated by the cassava program at CIAT, in order to construct a molecular map of cassava.

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Table 1. Genomic probes compared for their ability to detect polymorphism with at least one restriction enzyme.

INTRASPECIFIC		INTERSPECIFIC	
Probe	% Polymorphism	Probe	% Polymorphism
Pst I	60	Hind III	95
Xba I	60	Pst I	85
Hind III	55	Xba I	85
Eco RI	40	Eco RI	60
Bam HI	30	Bam HI	45

Table 2. Comparison of different restriction enzymes for their ability to detect polymorphism for all probes tested.

INTRASPECIFIC		INTERSPECIFIC	
Restriction Enzyme	% Polymorphism	Restriction Enzyme	% Polymorphism
Eco RI*	34	Eco RI*	69
Eco RV*	29	Xba I	61
Bam HI	22	Eco RV*	53
Hind III*	20	Hae III	51
Pst I	20	Hind III*	49
Xba I	16	Bam HI	44
Hae III	15	Pst I	44
Taq I	6	Taq I	N.D.
Dra I	3	Dra I	N.D.
Hpa II	1	Hpa II	N.D.

N.D.= Not determined.
* Enzymes detecting high polymorphism levels in both cases.

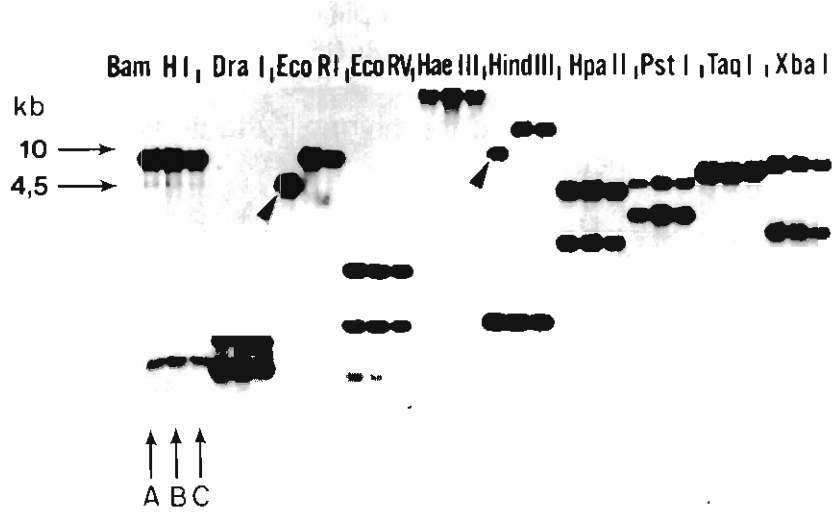

Figure 1. Polymorphism detected with a Pst I probe in three cultivated genotypes. Restriction endonucleases Eco RI and Hind III detect DNA variation in M Col 22.
A= M Col 22, B= M Col 1505, C= CM 507-37.

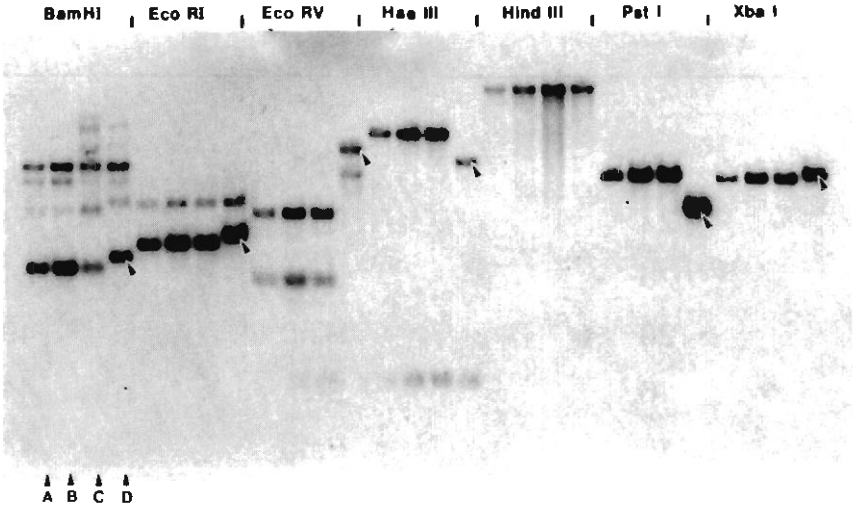

Figure 2. Polymorphism detected between three cultivated genotypes and *M. aesculifolia* with six out of seven restriction enzymes used.
A= M Thailand 8, B= M Col 1505, C= M Nigeria 5, D= *M. aesculifolia*.

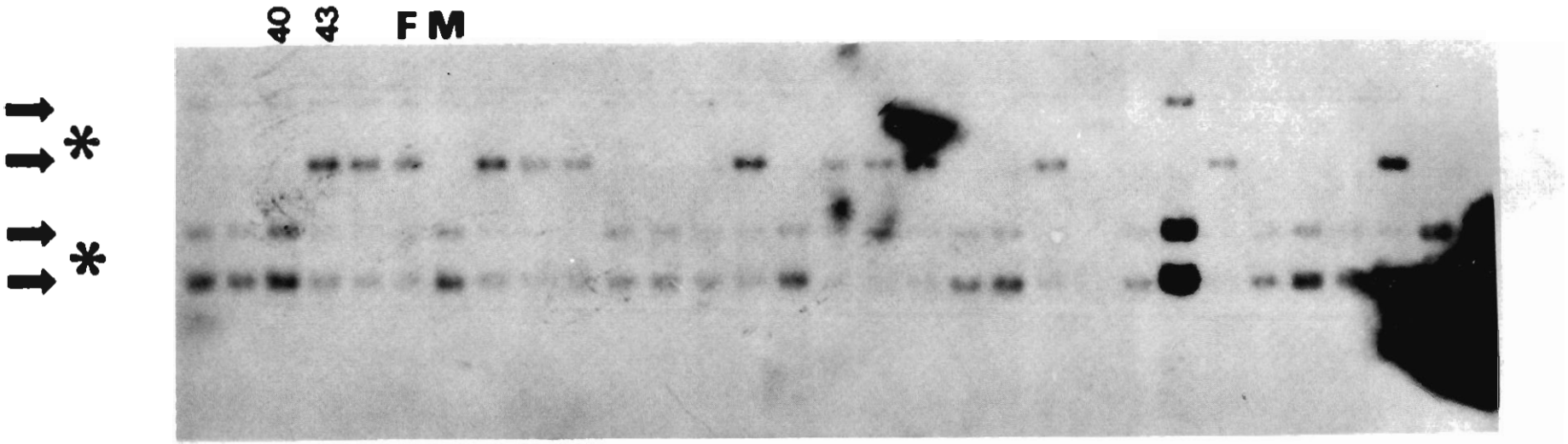

Figure 3. RFLP pattern for a segregating probe that hybridizes to two loci, one of them homozygous in the male parent (M) and heterozygous in the female parent (F). Segregation of this locus in the F1 population is evident.

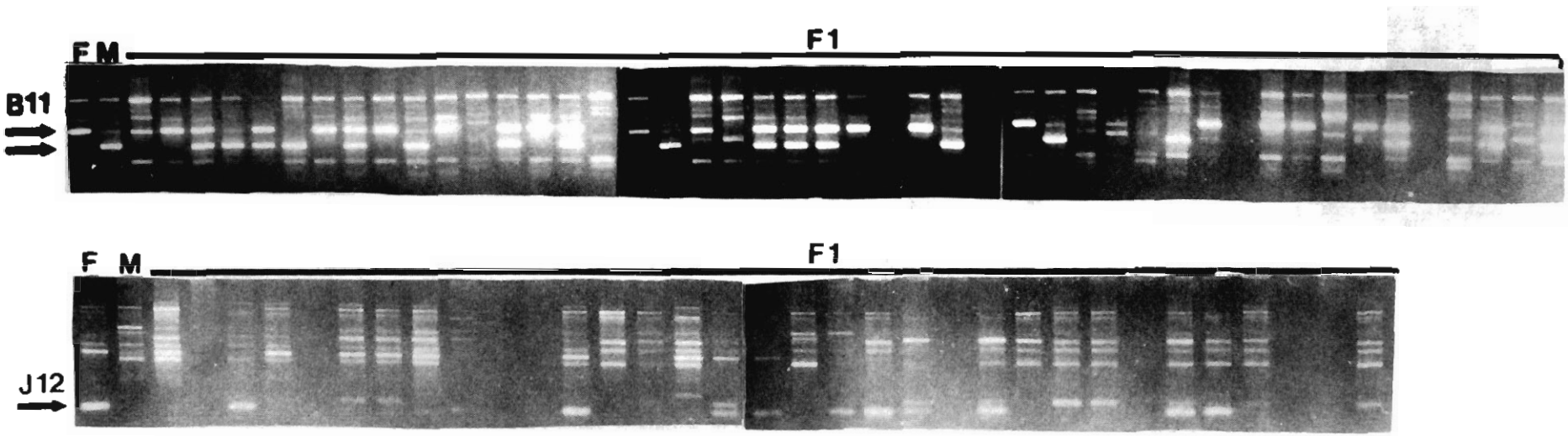

Figure 4. RAPD segregation of two polymorphic primers in the parental lines. B11 primer detecting two segregating loci (see arrows) and J12 detecting one segregating locus.

GENETIC DIVERSITY AMONG XANTHOMONAS CAMPESTRIS PV. MANIHOTIS STRAINS, CAUSAL AGENT OF CASSAVA BACTERIAL BLIGHT.

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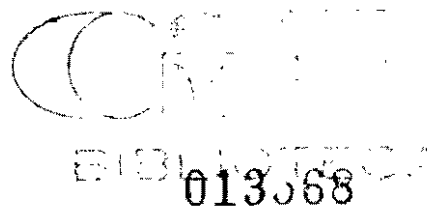

Cassava bacterial blight was first reported in South America in 1912 and has recently been detected in Africa (1972). Now it has been shown to have a worldwide distribution. To be able to detect and assess evolutionary relationships among pathovar *manihotis*, a comparison of strains from different geographical origin, was developed using a range of assays. These assays included plant pathogenicity, phenotypic features and most significantly restriction fragment length polymorphism (RFLP) analysis. The probes used were: 16+23SrRNA genes from *E.coli* and three restriction fragments from the chromosomal or plasmid DNA.of X.c pv. *manihotis* (strain CIAT1111). The distinction of four RFLP groups could be possible by using the rRNA probe. Strains from South America were heterogenous and gave different patterns. On the contrary no polymorphism was detected from among African strains. Subgroups were identified based on hybridization profiles with the three other probes. Biochemical and pathogenic variations within the strains studied were reported. Genetic variability of pv. *manihotis* was more extensive in strains from the area of origin of the host plant and more limited elsewhere. These results agree with the hypothesis of the recent introduction of the pathogen to the other countries and suggests that African strains are not already diversified at the chromosomal level. Our results indicate that probes developed in this study are useful tools for epidemiological studies and in following the genetic evolution of strains.

INTRODUCTION

Cassava bacterial blight (C.B.B) caused *by Xanthomonas* campestris pv *manihotis* is one of the most important diseases of cassava. Symptoms of the disease are characterized by angular leaf spot which appears initially as watersoaked angular areas. Bacterium multiplied extensively at the leaf surfaces and normally penetrates the host via stomatal openings or through epidermal wounds. The organism invades and destroys the mesophyll and then enters the vascular tissues. Then bacterial cells are able to move systemically through xylem vessels which cause wilting and die back.

The disease was first reported in Brazil in 1912 (Bondar, 1912) but has also been observed in Colombia and Venezuela (Lozano & Sequeira, 1974), as well as in most of African countries as Nigeria (Williams et al 1973), Zaire in 1973 (Maraite & Meyer, 1975), Congo (1976), Tanzania (1979). In Asian countries it was first described in 1974 in Indonesia, 1975 in India, West Malaysia, Taiwan and Thailand in 1978.

Cassava is multiplied by cuttings from the stem and infected ones are the most important source of inoculum in new plantations. Other inoculum sources such as seed, insects in association with the epiphytic phase have been reported earlier (Daniel & Boher, 1985).

The purposes of this study were to detect and assess the evolutionary relationship among strains of pv *manihotis* from various geographical areas and to evaluate the usefulness of plasmid and genomic probes in epidemiological studies.

MATERIAL AND METHODS.

X.c. pv. manihotis **collection.** Geographical origin of bacterial strains used in this study are listed in Table 1. Strains from two African countries have been more extensively studied: 29 strains from Togo and 198 strains from Congo, where they were isolated from samples obtained in different ecological zones.

Physiological characteristics. Different phenotypic features were examined: the in vitro susceptibility versus 20 antibiotics was determined, as utilization of carbon sources (19 tested), and amylose activity .

Phytopathogenicity test. Pathogenicity of all strains was tested on cassava plants, Congo's cultivar PMB multiplied from cuttings. The stem was inoculated by a puncture with a needle contaminated by passing it through a bacterial culture (Maraite et al, 1981). The evolution of the symptoms was monitored for one month, and the reactions were ranged from 0 to 5.

RFLP analysis. Total genomic DNA was extracted from bacterial cultures from the middle to the late logarithmic log phase (Boucher et al, 1987). Approximately 5g of DNA was digested completely with EcoRI whether by BamHI or by *HindIII* (37°c for 8 hr in buffers provided by Boehringer) and the DNA fragments separated in 0,7% agarose gels by the Southern method (Southern, 1975). Hybridization was made with different probes.

Acetyl amino fluorene labeled ribosomal 16+23S RNA genes from *E.coli* (Eurogentec, Liege, Belgium) hybridized with the genomic DNA of bacteria. The rRNA - rDNA duplexes were detected using the anti-AAF monoclonal antibody (Grimont et al 1989).

The DNA probes used in this study were: BS6 and BS8, two restricted fragments *EcoRI* from the chromosomal DNA (X.c. pv *manihotis* strain CNBP1851-CIATIIII) and pBsF2 a derivate from the 13kb-HindIII fragment of plasmid DNA. DNA probes were labeled *in vitro* by using a random priming kit with 32 p deoxycytidine triphosphate (Multiprime Amersham). Southern blots were hybridized according to previously described methods (Berthier et al 1992, Multiprime, Amersham).

RESULTS

RFLP patterns.

Using the rRNA probe, the distinction of 4 RFLP groups among the 290 strains tested could be possible. Three of the hybridizing fragments (1.Skb, 3.6kb, 10kb) were common to all four groups. The other specific fragments (12, 7.4, 5.6, 2, 1.8 kb) allow the distinction of the four groups. Strains from South America were heterogenous and gave the four different patterns, on the contrary no polymorphism was noticed in African strains.

Hybridization profiles with DNA probes could differentiate 6 groups with BS8 probe and 8 groups with BS6 probe, each group representing strains with identical RFLP pattern. Polymorphism could be noticed in South American strains which are represented in groups mentioned above. In contrast, no polymorphism was observed in African strain with BS8 and BS6 probes.

Variability among RFLP patterns of African strains was only noticed with the plasmid DNA probe pSF2.

Pathogenic characteristics.

Variability among pathogenic characteristics exists but was not related to the geographical origin of strains.

Phenotypic features.

The same results were obtained for two of the three phenotypic features tested (sensitivity to antibiotics and utilization of carbon sources). Starch hydrolysis was observed for all strains. Two groups were differentiated. all African strains, Reunion, Malaysian, 3 Brazilian and Colombian strains showed a low amylase activity, on the contrary the other strains showed a strong amylase activity.

DISCUSSION

Disease control is based on the use of resistant cultivars and thus it is important to know the limits of variation of the pathogen.

Thus variability among strains of X.c. pv manihotis have been extensively studied (Alves & Takatsu, 1984, Maraite et al, 1981, van den Mooter et al, 1987, Verdier, 1988, Grousson et al, 1991). Biochemical and physiological variations within the pathovar have been reported earlier. (Maraite et al, 1981, Grousson et al, l991) but no relation was found between features studied and its geographical origin. Van den Mooter et al (1987) and Vauterin et al (1991) indicate that the pv *manihotis* strains constitute a phenotypically and genetically homogeneous group.

In our previous data based on plasmid DNA study we have indicated the hypothesis of one common geographic origin within strains of X.c. pv *manihotis* (Verdier, 1988). In this study, using the RFLP analysis, small changes in DNA organization could be detected and the previous hypothesis confirmed.

Genetic variability of pv *manihotis* was more extensive in strains from the area of origin of the host plant (South America) and more limited in those coming from elsewhere (Africa). Among African strains homogeneity was observed with the probe corresponding to the rRNA genes and was confirmed with genomic probes used in this study.

These results agree with the hypothesis of the recent introduction of this pathogen from South America to the other countries, and suggest that African strains are not already diversified at the chromosomal level.

Using the DNA plasmid fragment as a probe, this study revealed that DNA polymorphisms exist in African strains. this is obvious since plasmids are mobile elements and may easily genetic exchange in bacterial strains (Coplin, 1989; Eberhard, 1990).

RNA and DNA probes used here were particularly useful in our epidemiological studies, providing information on the genetic population structure of these pathogens and its ability to identify clonally related individuals. Moreover no hybridization was found in total genomic DNA from non pathogenic bacterial species isolated from cassava stem or leaves. Those probes are retained for further diagnostics studies of this pathogen.

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Table 1: *Xanthomonas campestris* pv. *manihotis* strains used and information on their origin and isolation.

Strain no * and in other collection	Place and year of isolation		Isolated by
CFBP1851,CIAT1111	Colombia	1974	Lozano.J
LMG 776,NCPPB2443,HMB72,CFBP2603		1970	Lozano.J
ORST1, CIAT1060, CFBP1849		1970	Lozano.J
ORST2, CIAT1061, CFBP1850	Venezuela	1971	Lozano
ATCC 23380,HMB68,NCPPB1159	Brazil	1941	Burkholder
HMB 70, NCPBB1160, LMG5273		1941	Drumond Hipolito
HMB 55a, NCPBB1834*, LMG784		1965	Robbs C.
ORST7, CFBP1854		1973	Neto J.R.
HMB23, LMG770		1973	Pereira A.
ORST3, CIAT 1120,CFBP1852		1974	Lozano.J
ORST5, CFBP1855		1974	Neto J.R.
ORST6, CFBP1856		1976	Neto J.R.
HMB79, LMG778		1978	Takatsu A.
LMG777, HMB78		1978	Takatsu A.
LMG779, HMB80		1978	Takatsu A.
HMB25, NCPBB3060, LMG 771	Nigeria	1976	Maraite H.
ORST42		1978	Daniel J.F
ORST43		"	"
CFBP1857, ORSTOM A202.1		"	"
CFBP1858, ORSTOM A203.1		"	"
CFBP1859, ORSTOM A205.1		"	"
CFBP1860, ORSTOM A207		"	"
ORST34	Benin	1982	Daniel J.F
ORST35		"	"
ORST36		"	"
ORST37		"	"
ORST38		"	"
CFBP1944	Ivory Coast	1979	Ridé M.
LMG5249, HMB203		1981	Maraite H.
ORST55		1984	Daniel J.F
ORST56		"	"
ORST (198 strains)	Congo	1977-1991	Daniel J.F Boher B. Verdier V.
ORST (29 strains)	Togo	1987-1991	Boher.B
HMB6, LMG 767	Zaïre	1973	Maraite H.
HMB9, LMG 768, NCPBB3058		"	"
LMG 769, NCPPB3059, HMB10		"	"
LMG 766, HMB3		"	"
ORST44		1979	Daniel J.F
ORST45		"	"
ORST46		"	"
ORST47		"	"
ORST48		"	"
ORST49		"	"
ORST50		"	"
ORST51		"	"
ORST52		"	"
ORST53		"	"
ORST54		"	"
ORST186		1987	Boher B
ORST187		"	"
ORST39	RCA	1977	Daniel J.F
ORST40		"	"
ORST41		"	"
LMG 5287, NCPPB 3161	Cameroon	1976	Persley G.
HMB27, LMG629		1977	Maraite H.
LMG780, HMB81	Uganda	1979	Maraite H.
LMG782, HMB93		"	"
LMG783, HMB148	Kenya	1979	Maraite H.
LMG5288, NCPPB 3194	Niger	1978	Bradbury.J
LMG765	Malaysia	1980	Lan G.
LMG774, HMB60	Taïwan	1978	Maraite H.
HMB71, NCPBB1161, LMG775	Mauritius	1946	Orian G.
CFBP2624	Reunion	1986	Pruvost O.
CFBP2635		1987	Girard J.C

ATCC: American Type Culture Collection, Rockville, Maryland, USA.
CFBP: Collection Française de Bactéries Phytopathogènes, Angers, France.
NCPPB: National Collection of Plant Pathogenic Bacteria, Harpenden, U.K.
HMB: B.Maraite's Bacterial Collection, Louvain La Neuve, Belgium.
LMG: Laboratorium voor Microbiologie Gent Culture Collection, Gent, Belgium.
ORST: Collection du Laboratoire de Phytopathologie, ORSTOM, Brazzaville,Congo.
CIAT: Centro International de Agricultura Tropical, Cali, Colombia.
* : Pathovar reference strain.

GENETICS OF ESTERASE AND GLUTAMATE OXALOACETATE TRANSAMINASE ISOZYMES IN CASSAVA (*Manihot esculenta*, Crantz)

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Genetic studies of two isozyme loci have been carried out in root tips of eight cassava F1 progenies using polyacrylamide gel electrophoresis. Two loci, esterase-1 (East-1), the most distant cationic region, and glutamate oxaloacetate transaminase-1 (Got-1), the most proximal anionic region, were evaluated among the segregating populations.

Est-1 locus has five multiple alleles, including one null allele, and behaves as monomer, with a diploid inheritance pattern with only two alleles presenting each individual. These data were confirmed by esterase characterization of CIAT cassava germplasm bank accessions; a total of 11 different phenotypes representing 15 different genotypes for this locus were demonstrated. Got-1 locus comprises three alleles with diploid inheritance. This work provides evidence for a diploid inheritance pattern of the two isozyme loci supporting the allotetraploid nature of cassava. Many other regions of the zimograms are still to be elucidated. This information will be valuable to interpret studies on isozyme fingerprinting of cassava genetic diversity.

INTRODUCTION

Isoenzyme electrophoresis is an important tool which can provide additional data for genetic studies (Tomato: Tanksley and Rick, 1980; Potato: Quiros et al., 1985; Apple: Mangaris and Alston, 1987 and Weeden et al., 1987). The differences in electrophoretic mobility of isoenzymes are usually the result of changes in the structural genes coding for the polypeptides. Electrophoretic polymorphisms are thus the direct result of genetic differences.

Few studies on the genetics and cytogenetics of cassava have been reported. Large populations of homozygous individuals are required for useful genetic studies. However, in cassava, problems like high heterozygosity level, low seed set through controlled pollinations and strong inbreeding depression after selfing, make it quite difficult to obtain sufficient numbers of individuals that are homozygous at all loci or a given locus. As a consequence, the ploidy level is undetermined and the diploid or tetraploid status of this crop remains to be defined.

Isozyme studies could help understand cassava genetics for the selection of parental clones and to complement molecular marker analysis. The main objectives of this work were to determine the genetics of the esterase anodical region and the glutamate oxaloacetate transaminase (GOT) cathodical region in Cassava root tips.

MATERIALS AND METHODS

Plant Material

A total of nine crosses using eleven different cultivars were used for progeny studies for both esterases and GOT isozymes (Tables 1 and 2).

Preparation and Running of Samples

The methodology for determining isozyme patterns in cassava by polyacrylamide gel electrophoresis was developed by Hussain et al, 1986. Stakes from mature plants (6-9 months in age), were potted in the greenhouse in a 1:1 sand:soil mixture. After three weeks, 0.5 g of root tips were harvested and proteins extracted in 1 ml of ice cold 0.05 M Tris-Hcl buffer (pH 8.3). The crude extract was centrifuged at 27000 x g for 15 minutes. The supernatant (approx. 25 μl) was directly used for isozyme electrophoresis in a 10 % polyacrylamide gel using 0.05 M Tris-Borate (pH 9.0) as running buffer. Samples were run for six hours at 4°C and 250 Volts.

For esterases activity detection, alpha and beta naphthyl acetate diluted in acetone are used as substrates and fast blue RR salt for staining. For GOT substrates are aspartic acid, alpha ketoglutaric acid and pyridoxal-5-phosphate diluted in 1 M Tris-HCl buffer (pH 8.0) and for staining fast blue BB salt is used.

RESULTS AND DISCUSSION

Est-1 (Fig. 1) is a single locus with 5 alleles, including one null allele, and is expressed as a monomeric enzyme. For this locus, a diploid inheritance model is proposed (Table 1). None of the individuals exhibits a three band phenotype. A total of eleven different phenotypes represented by 15 different genotypes were classified from the studied progenies. These findings were supported by the esterase characterization of the cassava germplasm collection.

Got-1 (Fig. 2) is a single locus with three alleles, including one null allele. Polypeptides behave as monomers. The analysis of the two populations leads us to propose a simple diploid Mendelian inheritance for this locus (Table 2).

Although the genetics of the two isozyme loci could be interpreted, other complex regions of the zymograms could not be explained by a simple diploid inheritance and remain to be characterized, using highly contrasting parents.

For Est-1 the most complex region for analysis was the cathodical region of the zymogram, although the parents have a simple phenotype. It would be interesting to check for the occurrence of interactions between loci, as well as a possible tetraploid inheritance model.

In the case of Got-1, the analyzed anodical region has not been considered for the GOT zymogram of cassava. The central region of this zymogram is very complex with characteristics similar to the esterase loci.

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Table 1. Crosses used to study inheritance of locus EST-1 in cassava.

Cross	Parental genotype		No. of genotypes Expected	No. of Genotypes Observed	Ratio	X_2
	Female	Male				
1X1	A_oA_4	A_oA_4	A_4A_-: 22.5 A_oA_o: 7.5	A_4A_-: 23 A_oA_o: 7	3:1	0.04
1X2	A_oA_4	A_oA_3	A_3A_4: 7.25 A_oA_3: 7.25 A_oA_4: 7.25 A_oA_o: 7.25	A_3A_4: 8 A_oA_3: 6 A_oA_4: 7 A_oA_o: 8	1:1:1:1	0.4
2X1	A_oA_3	A_oA_4	A_3A_4: 7.5 A_oA_3: 7.5 A_oA_4: 7.5 A_oA_o: 7.5	A_3A_4: 10 A_oA_3: 6 A_oA_4: 8 A_oA_o: 6	1:1:1:1	1.46
3X4	A_1A_1	A_2A_3	A_1A_2: 13 A_1A_3: 13	A_1A_2: 11 A_1A_3: 15	1:1	0.62
5X6	A_2A_4	A_1A_3	A_1A_2: 6.5 A_1A_4: 6.5 A_2A_3: 6.5 A_3A_4: 6.5	A_1A_2: 7 A_1A_4: 6 A_2A_3: 5 A_3A_4: 8	1:1:1:1	0.77
7X6*	A_4A_4	A_1A_3	A_1A_4: 13 A_3A_4: 13	A_1A_4: 16 A_3A_4: 10	1:1	1.38
8X9	A_2A_4	A_4A_4	A_2A_4: 13 A_4A_4: 13	A_2A_4: 12 A_4A_4: 14	1:1	0.15
10X11	A_oA_4	A_oA_2	A_oA_o: 20.25 A_oA_2: 20.25 A_oA_4: 20.25 A_2A_4: 20.25	A_oA_o: 17 A_oA_2: 18 A_oA_4: 19 A_2A_4: 27	1:1:1:1	3.1
10X10	A_oA_4	A_oA_4	A_4A_-: 7.5 A_oA_o: 2.5	A_4A_-: 7 A_oA_o: 3	3:1	0.14

* Cross shown in Fig. 1.

1: MCOL 1505
2: MCOL 1468
3: MCOL 948C
4: CM 847-11
5: MCOL 72
6: CM 996-6
7: MCOL 1495
8: MTAI 1
9: MCR 2
10: CM 681-2
11: CM 2177-2

Table 2. Crosses used to study inheritance of locus GOT-1 in cassava.

Cross	Parental genotype		No. of genotypes Expected	No. of Genotypes Observed	Ratio	X_2
	Femal e	Male				
1X2	A_1A_2	A_1A_o	A_1A_2: 20.25 $A_1A\_$: 40.5 A_oA_2: 20.25	A_1A_2: 21 $A_1A\_$: 42 A_oA_2: 18	1:2:1	0.304
3X4*	A_1A_2	A_1A_1	A_1A_1: 13 A_1A_2: 13	A_1A_1: 12 A_1A_2: 14	1:1	0.154

* Cross showed in Fig. 2.

1: CM 681-2
2: CM 2177-2
3: MCOL 1495
4: CM 996-6

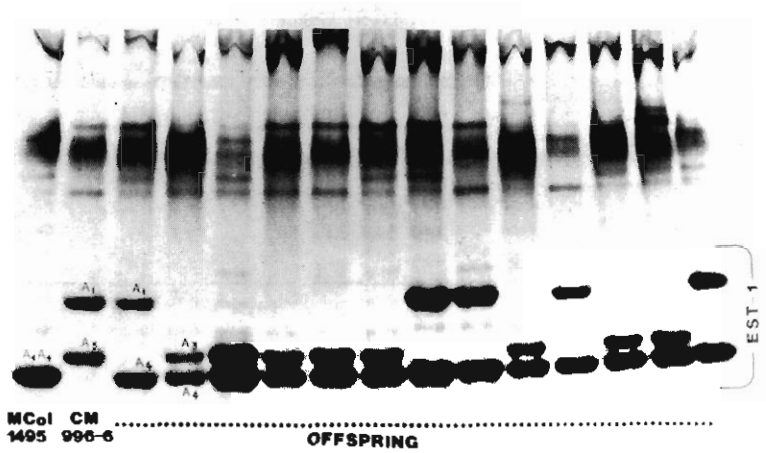

Fig. 1. Est-1 analysis in the cross MCol 1495 x CM 996-6 and its progeny. Alleles involved are A_1, A_3 and A_4. Inheritance data of this cross are shown in table 1.

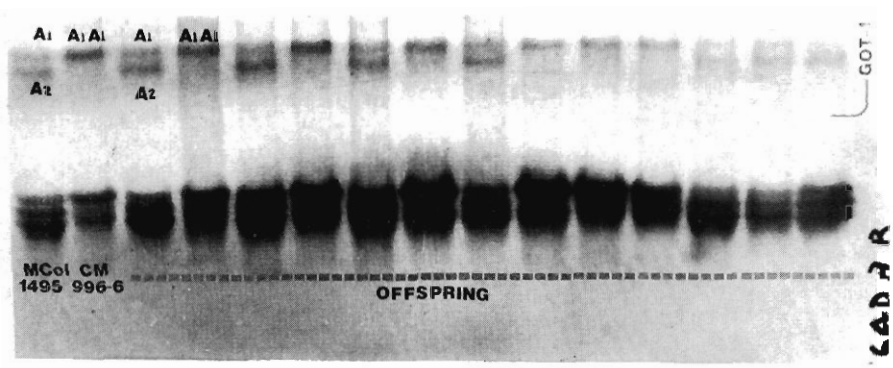

Fig. 2. Got-1 analysis in the cross MCol 1495 x CM 996-6 and its progeny. Alleles involved are A_1 and A_2. Inheritance data of this cross are shown in table 2.

ESTERASE ISOZYME FINGERPRINTING OF THE CASSAVA GERMPLASM COLLECTION HELD AT CIAT

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Esterase isozymes were chosen for fingerprinting CIAT's cassava germplasm collection, because of (1) their technical repeatability; (2) high polymorphism (i.e., many alleles per locus); and (3) high number of bands detectable in cassava clones (i.e., at least a few loci are expressed). A total of 4304 accessions (about 86% of the total collection) has been analyzed, and a total of 22 different bands were found. All bands have been coded and the existence of each band recorded in a computerized system. A total of 2146 different banding patterns were found among the 4304 accessions. A large number (1407) of patterns were represented by only one clone, while the rest had 2 to 39 clones with the same banding patterns. The former group, 1407 clones, represents unique genotypes, because different banding patterns imply genetic differences. Those clones in the latter group may represent the possibility of their being duplicates. The presence of duplicates in a germplasm collection has serious implications for germplasm conservation, as well as for use in breeding programs. A systematic procedure for duplicate identification in the collection, through the combined use of morphological and esterase isozyme descriptor, has been developed and a progressive elimination of duplicates will be carried out. A tentative list of a cassava core collection, consisting of 630 accessions, has been developed. The SAS "FASTCLUS" procedure was used to select 51 accessions, representing the range of variation of isozyme patterns, for inclusion in the core collection. Thus, data of fingerprinting has been successfully used for identification of possible duplicates and development of a core collection. Data on esterase isozyme were also used to study the distribution of genetic diversity in a subsample of the collection.

INTRODUCTION

The cassava collection maintained at CIAT, consisting of 5035 accessions from 23 countries, is the world's largest and most comprehensive germplas, collection for cassava. One of the most important activities at CIAT is through characterization of the germplasm to enhance management and use of the large collection. Biochemical markers, such as isozymes, have multiple uses for germplasm research (1). We are characterizing the whole cassava collection for αß-esterase isozyme using a methodology developed by CIAT's Biotechnology Research Unit (BRU)(3), to complement the previous work of characterization using morphological descriptors (2).

Preliminary studies indicated that αß-esterase isozyme is ideal for characterization of germplasm of cassava because of (1) their technical repeatability; (2) high polymorphism (i.e., many alleles per locus); and (3) high number of bands detectable in cassava clones (i.e., at least a few loci are expressed) (4).

In an asexually maintained germplasm collection, duplicate accessions may be common. In the case of the cassava collection at CIAT, 20% to 25% is estimated as duplicated. The presence of duplicates in a germplasm collection has serious

implication for germplasm conservation, as well as for a breeding program. The combined use of morphological and αß-esterase isozyme descriptors are expected to provide satisfactory evidence to identify possible duplicates in the collection.

To improve the efficiency and effectiveness of collection, conservation and evaluation, CIAT has established a core collection, comprising 13% of all accessions, selected to represent the total diversity of the species. Variation in αß-esterase isozyme banding patterns was used to help choose some accessions for the cassava core collection at CIAT.

OBJECTIVES

(1) To develop a description of each accession based on its banding pattern (fingerprinting);

(2) To use isozyme banding patterns as an additional criterion to identify duplicates in the collection; and

(3) To use diversity of isozyme banding patterns in the collection as one of the criteria for choosing accessions for the cassava core collection at CIAT.

MATERIALS AND METHODS

CIAT began in 1989 (GRU, and cassava program) the characterization, by means of the αß-esterase isozyme, of the entire cassava germplasm field collection maintained at CIAT, Cali, Colombia.

The electrophoretic patterns of the αß-esterase isozyme were determined using polyacrylamide gel electrophoresis of Tris-HCL 0.05 M buffer (ph 8.3) extracts of viable root tissue. The crude extract was directly used for isozyme electrophoresis. The gels contained 10% polyacrylamide and the running buffer was Tris-Borate 0.05 M (ph 9.0). Electrophoresis time was six hours at 4°C at 250 volts (3).

The gels were evaluated according to the presence or absence of each of the αß-esterase isozyme bands. These data were stored in a database, and processed by SAS to determine the isozyme patterns of each of the characterized accessions.

RESULTS AND DISCUSSION

Fingerprinting by αß-esterase isozyme

In the last three years, a total of 4304 accessions (about 86% of the collection) have been analyzed (Table 1). A total of 22 different bands were found (Figure 1). All bands have been coded and the existence of each band recorded in a computerized system. A total of 2146 different banding patterns were found among

the 4304 accessions. The number of clones for each different banding pattern was analyzed (Table 2). A large number (1407) of patterns were represented by only one clone (Figure 2), while among the remaining 739 patterns were found 2 to 39 clones with the same isozyme pattern (Figure 3). The former group, 1407 clones, represents unique genotypes, because different banding patters imply genetic differences. The clones in the latter group may be duplicates, as further discussed in the following section.

Use of fingerprinting information for duplicate identification

A two-step procedure was used whereby initial grouping is based on a subset of the most reliable morphological and isozyme descriptors (Table 3). This method identified 1282 accessions that are uniquely separated from other accessions.

On the other hand, among the rest (2978 accessions), 2 or more accessions were grouped together (Table 4). They represent possible duplicates. This is to be followed by planting those accessions in the field grouped according to tentative duplicates and reevaluation for morphological descriptors.

This process has already started in 1992. The use of DNA markers as an additional confirmation tool for duplicate identification is being investigated in collaboration with CIA's BRU.

Use of fingerprinting information for stablishing a core collection

A tentative list of 630 accessions was defined using six criteria, namely, geographic origin, morphological diversity, diversity of a αß-esterase isozyme banding patterns, most common land races, and elite breeding lines. The SAS "FASTCLUS" procedure was used to select 51 accessions, representing the range of variation of isozyme patterns, for inclusion in the core collection.

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Table 1. Number of cassava accessions of the collection at CIAT and characterized by αß-esterase isozyme electrophoretic banding patterns.

Country of Source	Accessions (No.)		
	gene bank (No.)	Fingerprinted (No.)	(%)
Argentina	16	16	100
Bolivia	3	2	66
Brazil	1085	802	74
Colombia	2010	1692	84
China	2	1	50
Costa Rica	147	138	94
Cuba	74	74	100
Dominican Republic	5	5	100
Ecuador	117	115	98
Fiji	6	6	100
Guatemala	91	87	96
Indonesia	51	51	100
Malaysia	68	66	97
Mexico	100	94	94
Nigeria	19	16	84
Panama	42	40	95
Paraguay	192	171	89
Peru	405	376	93
Philippines	6	6	100
Puerto Rico	15	15	100
Thailand	8	8	100
U.S.A.	9	8	89
Venezuela	240	237	99
Hybrids ICA	4	4	100
Hybrids CIAT	320	274	86
TOTAL	5035	4304	86

Table 2. Description of banding patterns resulting from electrophoretic analysis of αß-esterase isozyme of 4304 cassava germplasm accessions.

No. of clones for each pattern	No. of distinct esterase patterns	Total No. of clones	Percent of distinct esterase patterns
1	1407	1407	65.60
2	354	708	16.50
3	153	459	7.10
4	65	260	3.00
5	48	240	2.20
6	27	162	1.30
7	20	140	0.90
8	12	96	0.60
9	14	126	0.70
10	8	80	0.40
11	8	88	0.40
12	3	36	0.10
13	6	78	0.30
14	3	42	0.10
15	6	90	0.30
16	1	16	0.01
17	2	34	0.10
20	1	20	0.01
22	1	22	0.01
24	2	48	0.10
27	1	27	0.01
28	1	28	0.01
29	2	58	0.10
39	1	39	0.02
TOTAL	2146	4304	100.0

Table 3. Morphological descriptors and bands of αß-esterase isozyme most reliable used for identification of potential duplicates in the cassava germplasm collection maintained at CIAT.

DESCRIPTOR	PHENOTIPIC STATE OF DESCRIPTOR
Color of stem collenchyma	Light green Dark green
Color of stem epidermis	Cream Light brown Dark brown
Stem growth habit	Straight Zig-zag
Root flesh color	White or cream Light yellow Deep yellow
Electrophoretic bands 3, 4, 9, 10, 12, 13, 14, 15, 19, 20, 21 and 22	Presence = 1 Absence = 0

Table 4. Description of identical morphological/biochemical groups observed in analysis of the cassava collection at CIAT.

Clones within group (No.)	No. of groups (No.)	Total clones (No.)
1	1282	1282
2	332	664
3	183	549
4	78	312
5	52	260
6	31	186
7	35	245
8	12	96
9	18	162
10	6	60
11	5	55
12	3	36
13	5	65
14	3	42
15	5	75
16	1	16
17	1	17
18	3	54
19	2	38
21	1	21
25	1	25
TOTAL	2059	4260

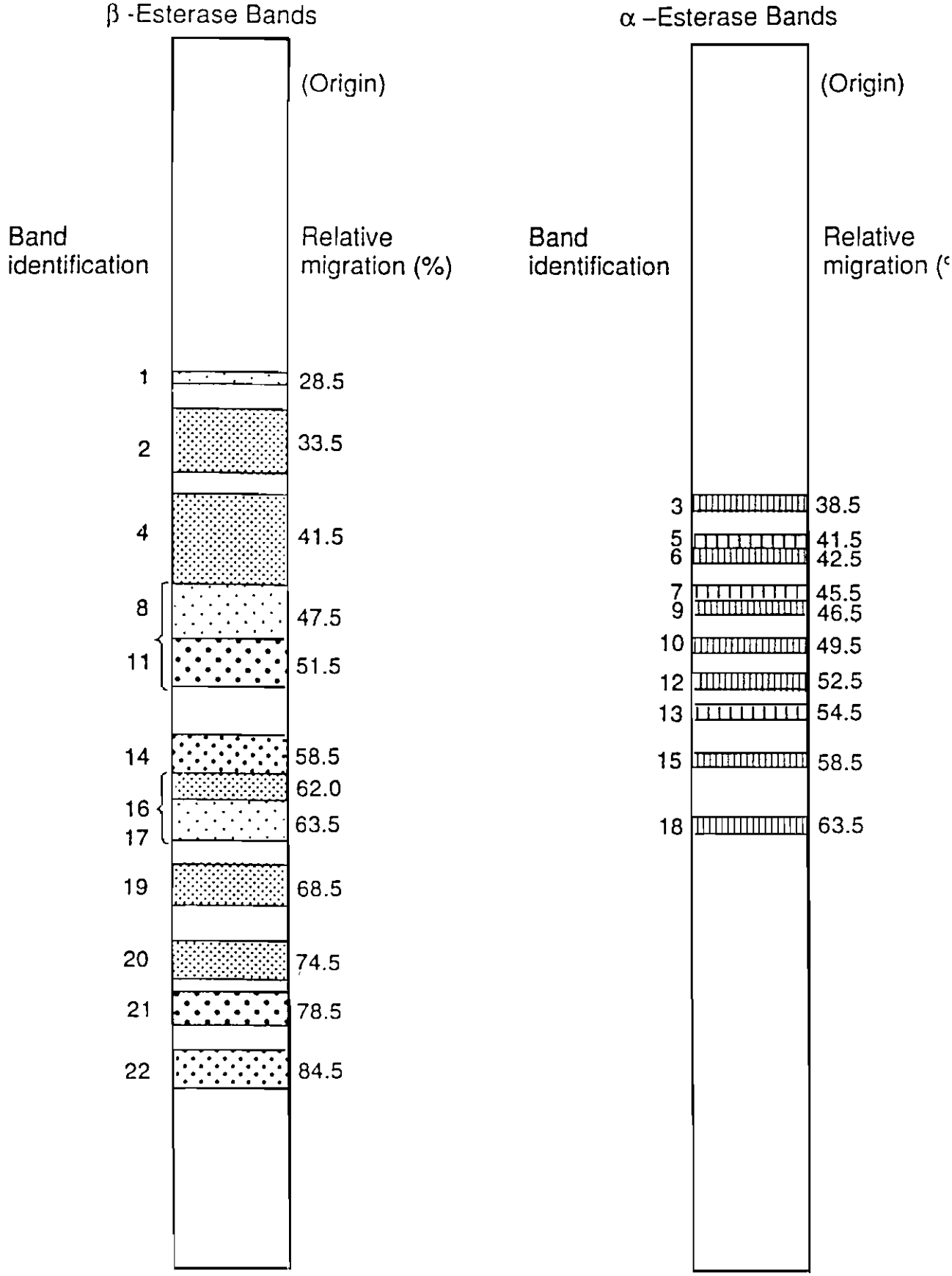

Fig. 1. Relative size and migration of bands resulting from electrophoresis on polyacrilamide gels of *aß*-esterase isozymes extracted from root tip tissue of cassava.

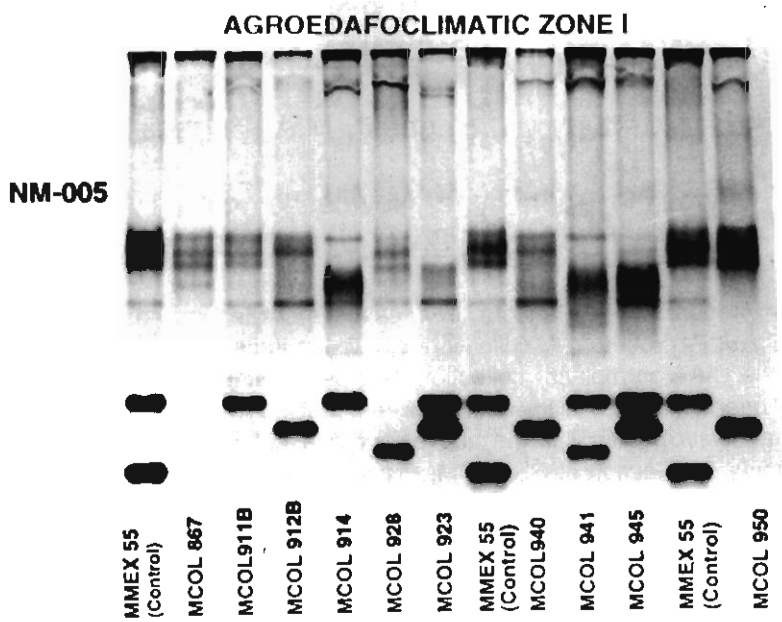

Figure 2. Polymorphism among accessions with the same geographical origin.

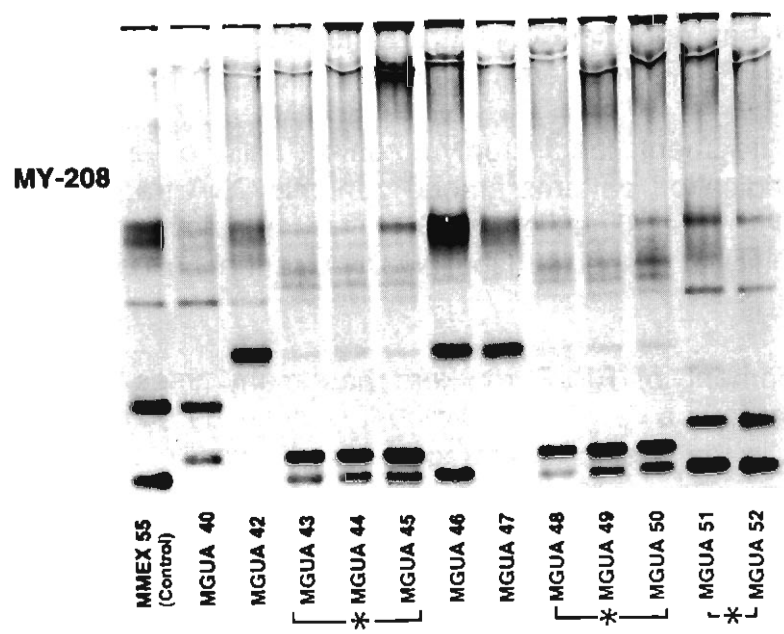

Figure 3. Identical banding patterns (*) among accessions with morphological descriptors similar (possible duplicates).

RESTRICTION FRAGMENT LENGTH POLYMORPHISM IN CASSAVA (MANIHOT ESCULENTA CRANTZ)

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A cDNA library, prepared by A. Pancoro, from cassava cotyledons has been used for the preparation of RFLP probes. A collection of 72 landrace cultivars, sent as in vitro cultivars from the CIAT germplasm collection, has been established as pot-grown plants and DNA has been extracted from all of this material. We have concentrated on the Southern blot analysis of RFLP in the above landrace collection as well as the African landrace germplasm collection at ORSTOM, France, (JB) in order to assess the potential usefulness of each cDNA clone for RFLP. All of the cDNA clones tested to date have revealed RFLP. The cyanogenic ß-glucosidase (linamarase) cDNA clone. pCAS5 (Hughes et al, 1992) reveals a large number of fragments and considerable polymorphism. This type of probe has potential ulse in DNA fingerprint type analysis where genetic relationships are to be studied. Analysis with further 7 cassava genes reveals a one or two major band pattern in each cultivar indicating that these genes are of potential use in RFLP mapping of cassava chromosomes since they probably represent single copy genes. In conjuction with these analysis we have screened our CIAT landrace cultivars for the variation in levels of cyanogenic ß-glucosidase (linamarase) and α-hydroxynitrilase activity in crude leaf extracts from 18 cultivars.

INTRODUCTION

This project has one main objective: the production of restriction fragment length polymorphic (RFLP) markers for cassava breeding and general research.

Restriction fragment length polymorphism is the term which describes differences in hybridisation patterns among genomic DNA fragments from different individuals, revealed after digestion of DNA with restriction endonucleases and subsequent probing with a specific labelled DNA sequence (usually a cloned DNA sequence).

The development of RFLP markers has many uses in a crop, such as cassava, where there is a very limited amount of genetical background information available to assist in the design of strategies for genetical crop improvement.

The potential uses of RFLP's in cassava improvement fall into the following areas:

1. They represent an easily identified set of genetic markers which could be produced in large numbers. They can be identified in one laboratory and as "tools" and be stored or sent to other laboratories/breeding stations for use. These markers can be used in crossing and selection programmes when linkage to a desired trait is established or when the RFLP is associated with the phenotype itself.

2. They will be extremely useful in establishing relationships between cultivars. There are two main centres of diversity for cassava: a major one in Brazil and a secondary one in Central America. Cassava is not known in the wild state but it can be presumed to originate in tropical South America. However it has spread throughout the Americas, Africa, India and South East Asia. RFLP analysis can be used to determine the genetical relationship between cultivars now grown on different continents as well as between local cultivars. It can thus be used to assess the genetic variation available to plant breeders.

3. All *Manihot esculenta* Crantz (cassava) cultivars so far examined have 2n=36 chromosomes. There are 99 recognised wild species of Manihot and some of these have been successfully hybridised with cassava. RFLP markers can be used to study the evolutionary relationships of these species and also to evaluate the extent of intrgression from wild relatives.

4. Cassava true seed propagation has a number of advantages and the development of true seed propagation has been give a high priority by a number of bodies. A better knowledge of the breeding system will be important in the assessment of outcrossing and in the development of parental inbred lines. RFLP analysis is an ideal tool for this research.

5. RFLP markers may be used to assist in the production of cytogenetic stocks, such as an aneuploid series, since it is possible to identify plants which have an increased copy number of a particular gene. Independently segregating RFLP probes may be used as identifying markers for particular chromosomes.

MATERIALS AND METHODS

The project has been directed towards:

a. optimising the techniques of DNA extraction, DNA digestion by restriction enzymes, electro-phoresis and Southern blotting so that RFLP can be routinely analysed,

b. optimising production of probe templates using polymerase chain reaction (PCR) so that probes can be made directly from * lysates without time consuming sub-cloning,

c. isolation of DNA from a representative selection of the plants in the CIAT germplasm collection,

d. isolation of DNA from samples of seedling material, and

e. screening of DNA clones for the use as RFLP markers by analysing both Landrace and seedlings derived from single female parents for variation.

CONSTRUCTION AND SELECTION OF cDNA CLONES

A cDNA library was constructed by Adi Pancoro from 10 day old plant cotyledons using the procedure described by Hughes et al (1992). Plant DNA preparation, restriction enzyme digestion and Southern blotting,DNA was extracted from frozen leaf material using a modification of the method of Dellaporta et al (1983). 10=| g plant DNA was digested overnight at 37 C using 30U restriction enzyme in a total volume of 100=| l restriction buffer. The DNA was precipitated by ethanol precipitation, washed with 80% (v/v)ethanol and then resuspended in 20=| l (1x) TAE electrophoresis buffer. The digested DNA was then separated, on a 1% agarose, by gel electrophoresis (2-3V cm-1 for 3-4 hrs).

The DNA in the gel was then depurinated with 0.25M; denatured with 1.5M NaCl, 0.5M NaOH and then neutralised with 1.5M NaCl, 0.5M Tris-HCl pH 8.2 as described in the Amersham protocols. The DNA was then transferred overnight onto nylon membrane (Amersham Hybond-N) by capillary blotting using 20x SSC (3M NaCl, 0.3M Na3citrate). The positions of wells were marked and the membrane washed with 2x SSC to remove any adhering agarose. The DNA was then cross-linked to the membrane by UV irradiation (Stratagene Stratalinker 120 000=| J cm-2).

Preparation of radioactive probes, prehybridisation, hybridisation, washing and exposure 25ng of insert (either cloned DNA or PCR product) was labelled using 32P dCTP (Amersham MegaPrime Kit). The resulting labelled DNA was separated from unincorporated label using a drip column (Sephadex G50 with 0.1x SSC, 0.1% sodium dodecyl sulphate). The DNA on the membrane was then prehybridised , hybridised and washed at 65 || C to 0.1x SSC stringency as described in Amersham protocols for nylon membranes. The membrane was the wrapped in Saranwrap and mounted on 3mm paper and then placed in a cassette with intensifying screens and X-ray film for 4-10 days.

RESULTS

The cDNA material which is either sub-cloned in a plasmid or a * phage lysate which has been PCR amplified for use as probes.

A collection of 72 Landrace cultivars, sent as in vitro cultures from the CIAT germplasm collection has been established as pot-grown plants. DNA has been extracted from all of this material. In addition about 300 seedlings have been grown and DNA also extracted from these plants. In Newcastle we have concentrated on the analysis of the Landrace collection in order to assess the potential usefulness of each cDNA clone for RFLP. Dr J. Beeching has used the clones to analyse variation in the collection of cassava cultivars held at ORSTOM, Montpellier, France. This collection contains a range of African material as well as 3 species and interspecific hybrids. All of the cDNA clones tested to date show RFLP in both the Newcastle and ORSTOM collections. The cyanogenic *-glucosidase (linamarase) cDNA clone,

pCAS5, reveals a large number of fragments and considerable polymorphism indicating a complex genomic organisation (Table 1).

We have also screened the landrace cultivars for variation in the levels of cyanogenic *-glucosidase (linamarase) and also *-hydroxynitrile lyase activity. Table 2 illustrates levels of linamarase and *-hydroxynitrile lyase activity recorded in crude leaf extracts from 18 cultivars.

Table 1.

Summary of cDNA clones used for RFLP analysis

clone	Size kb	PCR fragment	Subcloned	RFLP in CIAT Material	RFLP in ORSTOM Material
pCAS5	1.7	yes	yes	yes	yes
pCGT.G2	1.1	yes	yes	yes	yes
pCGT.G3	1.4	yes	yes	yes	yes
pCGT.G4	0.8	yes	yes	yes	yes
pCGT.G7	1.0	yes	yes	yes	yes
pCGT.G8	1.6	yes	yes	yes	yes
pCGT.M1	1.4	yes	yes	yes	yes
pCGT.M2	1.6	yes	yes	yes	yes
pCGT.M4	2.0	yes	yes	yes	yes
pCGT.M6	1.2	yes	yes	yes	yes

Note: A further 36 cDNA clones have been PCR amplified bult have not yet been used ro RFLP analysis.

Table 2.

Variation in levels of linamarase and α-hydroxynitrile lyase activity in cassava leaf tissue.

Cultivar	Linamarase *	α-hydroxy-nitrile lyase **
Bra12	234.67	0.86
Arg13	185.94	1.14
Ind27	162.40	2.16
Ven25	85.27	0.25
Bra383	80.57	1.32
Dom2	79.10	0.77
Fji6	68.29	1.01
Gua07	62.67	0.56
Ptr26	52.03	0.70
Col72	44.13	0.88
Col1684	37.43	0.45
Col1505	37.13	0.23
Fji4	35.17	0.64
Dom1	30.10	0.37
Nga16	29.63	0.25
Mal24	20.07	0.30
Nga1	19.87	0.30
Arg7	19.39	0.19
Sum	1283.86	12.38
Mean	71.33	0.69
S.D.	59.95	0.49

* μmoles HCN/10min/mg protein
** μmoles HCN/ 5min/mg protein

We have seen no association of linamarase activity and the CAS5 restriction fragment pattern of these plants.

IMPLICATIONS

The results indicate that our material will be of importance in the future RFLP analysis of cassava. We are currently assessing the use of the RFLP data analysed with computer programs to assess genetic distance, to study the genetic relationship between different cultivars. To date this approach looks promising and several programs which have characteristics making them suitable for this analysis have produced interesting results.

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RAPID SEEDSTOCK MULTIPLICATION OF IMPROVED CLONES OF CASSAVA (*MANIHOT ESCULENTA* Crantz) THROUGH SHOOT TIP CULTURE IN CAMEROON

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In order to improve results obtained earlier in plantlet regeneration from shoot tips of cassava (*Manihot esculenta* Crantz) improved clones (8017, 8034, 8061), the classical formulation of MS basal medium was supplemented with various concentrations of BAP (0.5; 1; 2 mg/l). Sprouting occurred in more than half of the cultures of treatment containing 2mg/l BAP. The sprouts had very short leaf petioles and stems with some having a callus-like tissue growth at the base in contact with the medium. In media supplemented with GA_3, only clone 8034 responded positively with significant changes in rates of shoot formation. Rapid clonal propagation of plantlets from subcultured nodes was obtained in MS basal medium containing 0.05 mg/l BAP and 0.01 mg/l NAA.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important staple food crop in tropical Africa (Hahn *et al*, 1979). In Cameroon, cassava roots form the basic starchy element of a diet and in some regions, the leaves are also consumed as a green vegetable providing protein, vitamins and minerals.

The Cameroon National Root Crop Improvement Program (CNRCIP) has introduced from IITA Ibadan, and tested for adaptability to local environment, many improved cultivars selected as high yielding and resistant to diseases like Cassava Mosaic Disease (CMD) and Cassava Bacterial Blight (CBB). Three of them have been well adapted to various ecological zones of Cameroon and have been identified for further release to farmers. They are clones 8017, 8034 and 8061. However a major constraint to large scale distribution of these clones to farmers is the scarcity of planting material due to slow propagation of the species by the traditional method (Cock, 1983).

Tissue culture methods have been developed for cassava. In vitro regeneration and subsequent propagation of whole plants from cassava meristems cultured in vitro is a well established technique with applications in propagation, disease elimination, in vitro conservation and international exchange of germplasm (Kartha *et al*, 1974; Mabanza and Jonard, 1981; Kartha, 1984; Roca 1979-1984; Szabados *et al*, 1987). The propagation rates achieved by in vitro meristem or shoot tip culture are considerably greater than those possible through traditional methods based on cuttings (Stamp and Henshaw, 1985).

One of the objectives of the Tropical Root and Tuber Research Project (ROTREP) is to use such techniques to develop a system of mass production of planting materials of locally available cultivars of cassava. In preliminary trials

involving cassava improved clones 8017, 8034, 8061 and local cultivars, 9 different culture media were tested and gave unsatisfactory results (ROTREP, 1989). It therefore appeared necessary to modify classical formulations of the media earlier tested in order to achieve shoots formation and elongation, and rapid clonal generation of plantlets from subcultured nodes. This paper reports some of the most important results obtained so far.

MATERIAL AND METHODS

Shoot-tips (0.5-1.0 cm long) were collected from cassava clones 8017 and 8034 preconditioned in the greenhouse. They were surface sterilized in 70% ethanol for 3 minutes, followed by dipping in 4% calcium hypochlorite for 15 minutes and rinsed three times in sterile distilled water (Roca, 1979). They were then aseptically trimmed to a size of 0.3-0.5 cm comprising the apical meristem plus 2-3 leaf primordia and a portion of the subapical axial tissue before inoculating into the culture medium. The culture media were made up of basal medium (BM), (Gibco MS mineral salts, 0.05 $mg1^{-1}$ NAA, 2% sucrose) supplemented with different levels of BAP (0.5; 1.0; 2.0 $mg1^{-1}$). Agar 8 gl^{-1} was added to each medium and the final pH was adjusted to 5.8 with 1N sodium hydroxide. Each medium treatment comprising of 21 explants was replicated twice.

In another experiment, GA3 (0.1 mgl^{-1}) was added to each of the above media. Six treatments were then obtained as follows:

A : BM + 0.5 mg/l BAP
B : BM + 0.5 mg/l BAP + 0.1 mg/l GA_3
C : BM + 1.0 mg/l BAP
D : BM + 1.0 mg/l BAP + 0.1 mg/l GA_3
E : BM + 2.0 mg/l BAP
F : BM + 2.0 mg/l BAP + 0.1 mg/l GA_3

Data was collected on sprouting of explants and shoot formation.

RESULTS AND DISCUSSION

Forty three days after culture (Table 1), the explants sprouted in more than half of the treatment cultures containing 2 mgl^{-1} BAP. The sprouts had very short leaf petiole and stems with some having a callus-like tissue growth at the base in contact with the medium. No rooting was observed at that stage. The most developed cultures had a proliferation of green tissue loosely lying on the surface of the medium with several swarf leaves. Short stems could be identified in some recalling the "rosette type" growth (Fig. 1) reported by Roca (1979). However the rate of shoot differentiation was very low in each of the 3 treatments applied with a maximum of 46.15 in the medium supplemented with 1 mg/l BAP.

The effect of GA3 in shoot formation (Table 2) was found to be clone dependent. With cultuvar 8034 the addition of GA_3 to the initial media has contributed to a significant change in rates of shoot formation, except for treatments F which formed fewer shoots than treatment E. A contrary result was obtained with cultivar 8017. In general the addition of GA_3 was detrimental to shoot formation except for treatment B which gave quite the same number of shoots as treatment A. For all the treatments, and regardless the cultivar, the percentages of plantlet development were low, the highest one (60%) being obtained with clone 8034 in medium B. In addition the shoots formed were rosette-like in nature bearing several shoot stems as earlier observed in media devoid of GA_3. Callus could also be seen at the base of some of them. Similar growth has been reported by various authors (Mabanza and Jonard, 1981; Roca, 1984). To obtain shoot elongation (Fig. 2), each stem was isolated and cultured in fresh medium A.

Although more confirmation experiments are still to be conducted for cassava shoot tip culture, the regeneration of plantlets through subculture has been obtained in MS basal medium supplemented with 0.05 NAA and 0.5 mgl^{-1} BAP as suggested by Roca (1979). This method is routinely used for mass culture of available clones giving a multiplication rate of 1:5 with clone 8034.

Tissue culture derived cassava plantlets have been successfully acclimatized using sterilized vermiculite/soil mixture (50:50) (Fig. 3). Despite this success, vermiculite is expensive and not commonly found in the local market. This unavailability would in the long run impair the smooth running of acclimatization of cassava.

This success of the vermiculite/soil mixture can be attributed to better retention of water and aeration (vermiculite acting probably as a soil conditioner). In addition there is an appreciable quantity of nutrients (minerals) also present in the vermiculite.

Field adaptability of tissue culture derived cassava in the ROTREP experimental field (Fig. 4) is a practical reality. In a preliminary experiment set up to obtain primary observation on how the tissue-cultured cassava plant would perform in the field, very interesting results were obtained. The plants branched at two levels. The number of stems ranged from 1-3 while the highest mean height to first branching was 129.5 cm. This growth pattern is typical of the 8034 cultivar. Moreover only two plants were found to show 40-60% African Mosaic Virus disease symptoms leaving about 90% of the plants more or less symptomless. Their stakes were used directly for further multiplication.

Even though the experimental size did not permit a statistical analysis of the results, yield data obtained about 13 months after planting showed better yields (Fig. 5) than randomly harvested non-tissue culture plants of the same clone, 17 months old.

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TABLE 1: Effect of different concentrations of bap on strouting of shoot apices and shoot differentiation of clone 8034, 43 days after culture.

TREATMENT	# OF PLANTS CULTURED	SPROUTING		SHOOT DIFFERENT-IATION	
		#	%	#	%
Basal medium + 0.5 mg/l BAP	42	19	45.23	8	42.10
Basal medium + 1 mg/l BAP	42	13	30.95	6	46.15
Basal medium + 2 mg/l BAP	35	18	51.42	7	38.88

TABLE 2: Effect of different media on shoot formation in 2 improved clones of cassava.

CLONE	TREATMENT	BAP mg/l	GA_3 mg/l	% SHOOT FORMATION
8034	A	0.5	0	52.1
	B	0.5	0.1	60.0
	C	1.0	0	30.0
	D	1.0	0.1	47.8
	E	2.0	0	47.3
	F	2.0	0.1	29.4
8017	A	0.5	0	30.1
	B	0.5	0.1	33.1
	C	1.0	0	19.6
	D	1.0	0.1	15.1
	E	2.0	0	30.2
	F	2.0	0.1	18.5

However when compared to other locally available and cheap materials, the vermiculite/soil substrates gave the best survival rate (55%). The coffee parchments/soil showed the highest plant vigor but very low survival rate. Plantlets acclimatized on vermiculite/soil substrate also had the highest mean plant height and leaf number (Table 3).

TABLE 3: Effect of the different substrates on the survival rate, plant vigor, mean plant height and leaf number.

TREATMENTS	SURVIVAL RATES (%)	PLANT VIGOR	MEAN PLANT HEIGHT/PLANT	MEAN # OF LEAVES/ PLANT
Vermiculite/ Soil	55	3	6.8	4.1
Saw dust/soil	25	2	3.6	1.5
Coffee parchments/soil	25	4	2.9	2.0
Soil	5	1	0.3	0.2
Mean (m)	27.5	2.5	3.7	1.95
Standard Deviation (S)	20.0	1.3	2.7	1.6

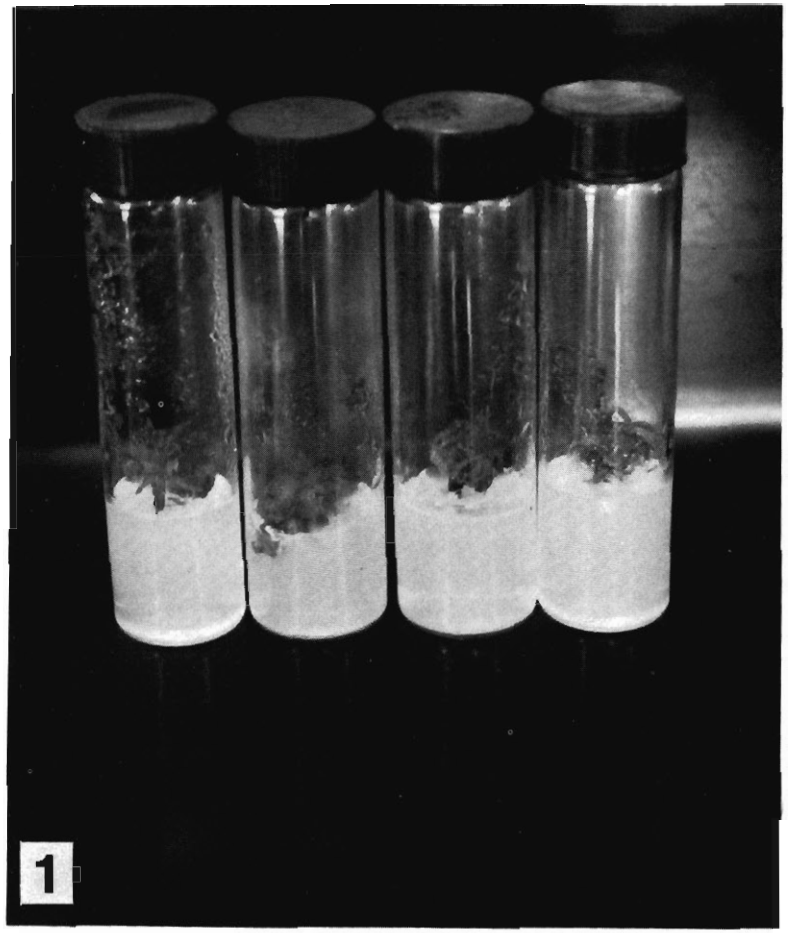

Fig.1. Typical growth response of cassava shoot tips in BM supplemented with BAP or GA_3 showing rosette-like plantlets.

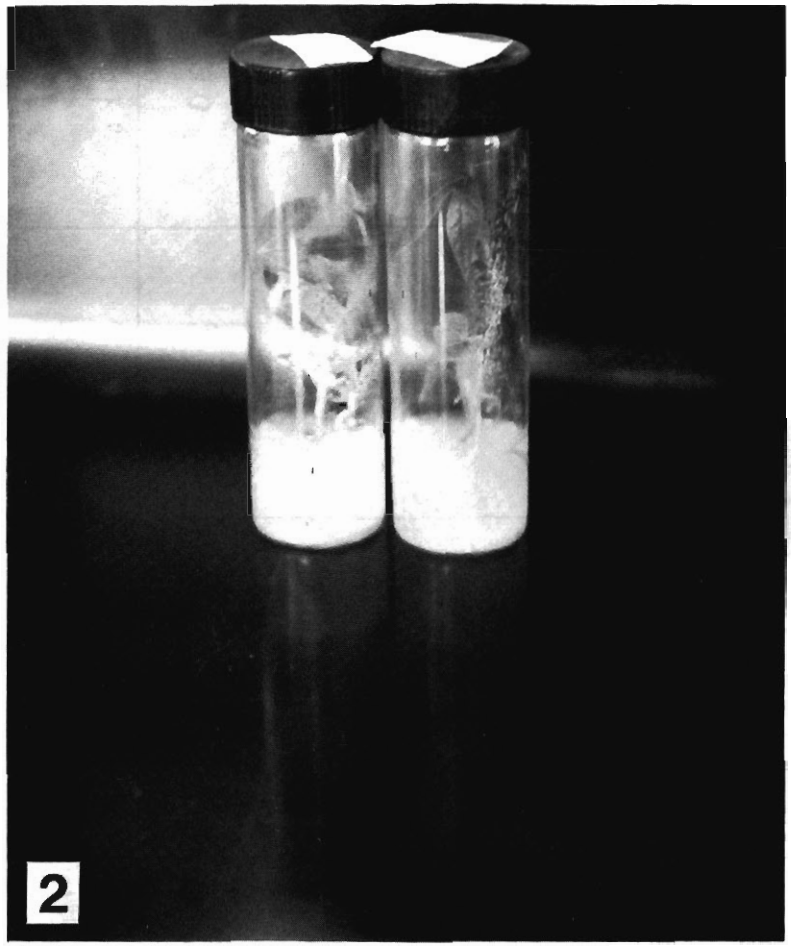

Fig. 2. Shoot elongation obtained when rosette-like stems were isolated and cultured in fresh medium A.

Fig 3. Acclimatized tissue-culture derived cassava in pot.

Fig. 4. Tissue-culture derived cassava clone 8034 in the field.

Fig. 5. Tubers harvested from clone 8034 tissue-culture derived plant, 13 months after planting.

FIELD EVALUATION OF IN VITRO PLANTS OF CASSAVA (MANIHOT ESCULENTA CRANTZ) FOR SEVERAL GENERATIONS

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Plants of forty-two cassava (Manihot esculenta Crantz) cultivars micropropagated in vitro have been introduced in Trujillo State (70°:46' LW, 09°:38'LN and 60 m.a.s.l.) for the production in large scale of cassava planting material. Under greenhouse conditions, in vitro plants were acclimatized, prior to the establishment of the field adaptation trials. From the forty-two, fifteen cultivars were selected in the first generation which were evaluated in the successive ones. A randomized block design was used for the evaluation of in vitro plants (first generation) and for the successive generations a simple random sampling method was applied. The planting material used for the evaluation of the successive generations was that obtained through multiplication of the in vitro plants. A period of harvest of eight months was established for all the evaluations. The results are presented in terms of fresh root yield and starch content. Two cultivars cv 28 and cv 29, sweet and bitter respectively, under the edaphoclimatic conditions of the zone, maintained a high and stable yield for five generations. The statistical analysis showed no significant correlation between yield vs. generations r= 0.154 , r= 0.136, respectively. For both cultivars, the mean yield of cv 28 and cv 29 were 2.52 ± 0.1 and 3.97 ± 0.2 Kg/plant, respectively. Preliminary results of the evaluations of eight sweet and five bitter cultivars for two generations are presented. These results suggest that for the selection of a cultivar, it is necessary to evaluate more than two generations prior to its establishment in a large scale production of cassava planting material.

INTRODUCTION

Alfonzo Rivas & Cia, a Venezuelan company, has developed several agricultural biotechnology projects. In 1987 this company started a large scale project on cassava production in Trujillo State. This project has two main purposes: to improve crop productivity and to produce good quality planting material all year round (Coll, et al., 1992). To insure high quality planting material in vitro plants were used. Micropropagated in vitro cultivars were introduced, evaluated and selected through field adaptation trials. The main selection criteria were high and stable yield, high starch content and high planting material production.

Previous data indicated that cassava genotypes might or might not show any decrease in yield on successive generations of planting material after in vitro culture (Cock, 1985). According to this, it was necessary to evaluate the performance of the introduced cultivars for several generations.

This paper shows the results obtained from field evaluations of two selected cultivars through five generations. Preliminary data from pre-selected cultivars through two generations is also presented.

MATERIAL AND METHODS

Field trials were performed in "Hacienda Caño Grande" located at the "Cuenca del Lago de Maracaibo" region (70°:45' LW, 09°:38' LN and 60 m.a.s.l.). Climate and soil conditions were described in a previous work (Coll et al., 1992).

The forty-two cultivars introduced to the project initially came from CIAT's in vitro Germplasm Collection and micropropagated by Laboratorio Bioplanta C. A. Some of these cultivars have been improved by CIAT while others are native from Venezuela, Brasil, Colombia and Panama.

When evaluating in vitro plants, first generation, a randomized block design with two replications was used. Thirty plants of each cultivar were planted in 5 x 6 m plots, at 1 x 1 m distance and 0.4 m ridges high. Eight months after planting, twelve central plants of each plot, were harvested and evaluated (Villegas et al., 1988).

Pre-selected cultivars from the first generation were field multiplied to obtain the planting material to establish commercial plantings. A simple random sampling method was applied to evaluate successive generations using three plots, each one with twelve competitive plants, from the commercial plantings. Population density was the same that for block design, 10,000 plants/Ha. Agronomic practices used were described previously (Coll et al., 1992).

Some traits such as establishment percentage, vigour, morphological traits, pest and disease susceptibility were evaluated six and eight months after planting. Quality and quantity of planting material, ease of harvest, position of roots, fresh root yield, starch and HCN content, ease of root peridermal removal, culinary quality and post-harvest deterioration were evaluated eight months after planting. The starch content was determined by measuring the density according to the method described by Toro and Caffa (1979). HCN content was determined through the colorimetric method (Williams and Edwards, 1980).

RESULTS

From the first evaluations, two cultivars cv 28 and cv 29, sweet and bitter respectively, were selected and established in the project. Figures 1 and 2 illustrate the relationship between fresh root yield and generations for these cultivars. Regression analysis, considering all observations, showed no significant regression and very low values for the coefficient of determination, cv 28 R^2 = 2.39% and cv 29 R^2 = 1.86% (r= 0.154 and r= 0.136 respectively). Lack of fit test (Draper and Smith, 1981) showed that lineal model used was correct. Therefore, low coefficients of determination were not caused by model inadequacy but variation in fresh root yields was very low explained by the generation variable.

The mean fresh root yields for cv 28 and cv 29 were 2.52 ± 0.1 and 3.97 ± 0.2 Kg/plant, respectively. It was observed that the mean yields per generation were maintained for the cv 29 over 3.5 Kg/plant, except for the second generation which was affected by flood. For cv 28, the mean yields were maintained over 2.0 Kg/plant. These results showed that both cultivars maintained high and stable yields during the evaluated generations. It was also observed that both cultivars have had a better performance in terms of yield, compared to local cultivar cv LL (Figure 3).

Table 1 and 2 show results of mean yield and average of starch content for two generations, obtained from the evaluations of 15 preselected cultivars, 10 sweet and 5 bitter, including cv 28 and cv 29 as controls. A significant difference was observed between yields of first and second generations for some cultivars. In order to determine their adaptation to the region, all these cultivars will be evaluated for more than two generations.

CONCLUSIONS

- For cv 28 and cv 29, there is no functional relation between fresh root yield and generations after In vitro culture. This holds for the edaphoclimatic and biotic conditions of the region and up to five generations.

- Cv 28 and cv 29 maintain high and stable fresh root yields at least up to five generations, in the edaphoclimatic and biotic conditions of the region.

- Performance of cassava cultivars for several generations after in vitro culture reflects their adaptation to edaphoclimatic and biotic conditions of specific environments. So, it is necessary to evaluate more than two generations prior to the establishment of any cultivar in large scale production of cassava planting material.

ACKNOWLEDGEMENTS

We thank Dr. Rafael Alfonzo for his interest and helpful guidance throughout the entire project, Eng. Romulo Del Valle for helpful in statistical analysis, and critical reading of the paper; technical personal of Hacienda Caño Grande for valuable collaboration in field work.

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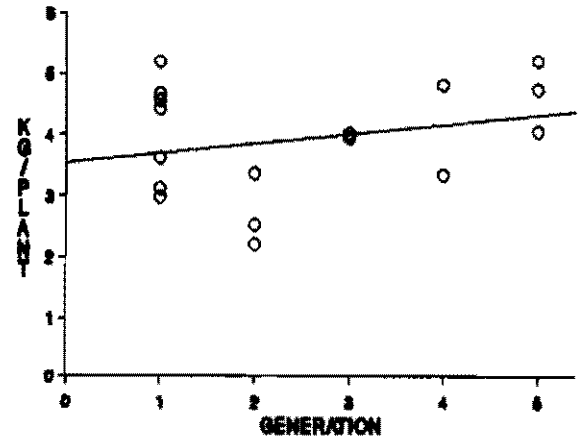

FIGURE 1. Relationship between fresh root yield and generations for cv 29.

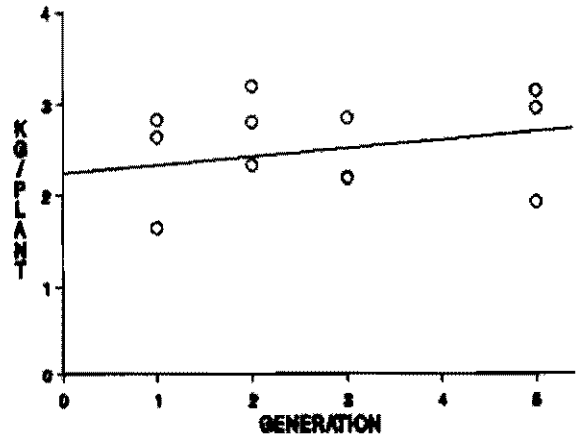

FIGURE 2. Relationship between fresh root yield and generations for cv 28.

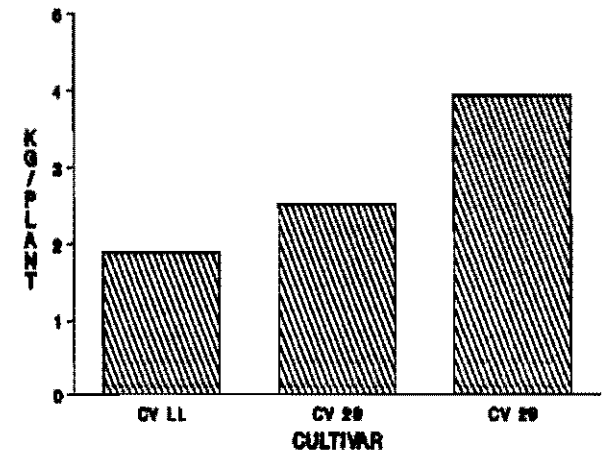

FIGURE 3. Mean fresh root yield of two selected cultivars (cv 29, cv28) and one local cultivar (cvLL).

PROGRESS ON CASSAVA IMPROVEMENT THROUGH BIOTECHNOLOGY AT THE NATIONAL ROOT CROPS RESEARCH INSTITUTE, UMUDIKE

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A plant tissue culture laboratory was set up at the National Root Crops Research Institute, Umudike in 1990 through the support of the International Atomic Energy Agency Vienna. The primary objectives are to improve root and tuber crops through mutation breeding and *in vitro* culture techniques. Rapid micropropagation is routinely carried out for cassava, to multiply irradiated plantlets through the various vegetative cycles (M_1V_0 - M_1V_3) leading subsequently to the selection of desirable mutants. Plantlets of two cassava cultivars (TMS 30572 and U/41044 raised) *in vitro* were irradiated using 20 and 25 Gy after a radiation sensitivity test. Up to five thousand M_1V_3 plantlets at the first instance, will be hardened, transplanted to the field and screened for early maturing types, reduced cyanide levels, resistance to pests (green spider mites and mealy bug) as well as the African Mosaic Virus. Some observable characters in the M_1V_2 population *in vitro* include very vigorous growth and stunting with pronounced tuberlet formation.

Up to 58.96% loss was sustained in the Institute's cassava germplasm collections between 1980-1990 due to pests, diseases and adverse weather conditions. Consequently, duplication of collections *in vitro* for safer preservation is in progress.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is a major root crop cultivated extensively in the hotter lowland tropics as a carbohydrate-rich staple. Current world production is about 157 million tons from 15.6 million hectares of land. Nigeria is a leading world producer, accounting for 16% of the total world production (FAO, 1990).

In Nigeria, cassava is grown by 70% of farmers and is important in the domestic economy of the country such that most adverse food situations are largely and directly linked to a low supply of cassava roots. Over 90% of an estimated 26 million tons of cassava currently produced in Nigeria (FAO, 1991) is by peasant technology, characterised by inefficient mixed cropping patterns, low inputs and poor cultural and soil management systems. Yields are usually 6-8 t/ha in mixed cropping and 10-12 t/ha in sole cropping against experimental yields of 15-20 t/ha and 25-30 t/ha in the former and latter cropping systems respectively.

The National Root Crops Research Institute (NRCRI), Umudike, has the mandate to conduct research leading to improvement in the production, processing, utilization and storage of root and tuber crops of economic importance in Nigeria and to integrate them into improved and viable farming systems. So far research efforts through conventional breeding for cassava has resulted in the production of high yielding and disease-tolerant genotypes with desirable food qualities, for example NR/8082, NR/8083, NR/8230 and NR/83107. However, genotypes that combine many desirable characters are rare and yet other traits of extreme value in cassava

agriculture such as earliness to mature and acyanogenesis remain to be introduced into existing germplasm collections. Furthermore, certain well adapted cultivars with high consumer acceptability but low yielding and susceptible to biotic stresses cannot be improved conventionally or integrated into breeding programmes due to acute deficiency in flowering.

Hence, the use of mutation techniques was incorporated to complement the existing breeding programme, the aim being to improve well-adapted cultivars by the alteration of one or two deficient traits only and without significantly altering the remaining genotype (Broertjes and Van Harten, 1988).

THE BIOTECHNOLOGY APPROACH

A plant tissue culture laboratory was set up at NRCRI, Umudike in 1990 through the support of the International Atomic Energy Agency (IAEA), Vienna, Austria. The primary objectives are to improve the Institute's mandate crops through mutation breeding and *in vitro* culture techniques. For cassava, an *in vitro* mutation system using gamma radiation for mutation induction has been initiated (Mbanaso, 1991). Defined objectives are to select early maturing, pest and disease resistant mutant lines with extremely low or nil cyanide content. Parameters guiding selection following screening procedures would include: for resistance to disease (African cassava mosaic virus) and pests (cassava mealybug and cassava green mite), zero infection in high pressure areas for three seasons plus hairiness and thickened cuticle. For earliness to maturity, early bulking and initiation of starch accumulation linked to dry matter build-up within a specified period. Tests to determine HCN content of roots will be carried out during the same period.

ASSESSMENT OF RADIATION SENSITIVITY

Two well-adapted cultivars, TMS 30572 and U/41044, were chosen for further improvement in line with the specified objectives. A basic radiation sensitivity study to determine the dose range of gamma radiation for effective mutation induction was carried out (Ene *et al.*, 1991).

Exposure of *in vitro* grown plantlets of TMS 30572 and U/41044 to 0, 10, 20, 30 and 40 Gy gamma radiation did not hinder breaking of buds in subcultured single nodes although increasingly higher doses decreasingly reduced both the total number of buds sprouted and the number developing into plantlets subsequently for both cultivars (Table 1). ALthough 50% growth reduction (GR_{50}) was obtained between 10 and 20 Gy irrespective of cultivar, 4-5 nodes and 2-4 nodes for TMS 30572 and U/41044 respectively were produced at 20-30 Gy (Table 2), a dose range considered suitable for *in vitro* mutation induction for somatic mutagenesis in cassava (Mbanaso and Novak, 1989). Within this range plantlet recovery was over 50% in both cultivars (Table 1). Post irradiation micropropagation is also applicable to enhance the chances of mutant isolation, since a number of vegetative cycles (M_1V_1-

M_1V_n) are necessary to allow the mutated sector to grow into non-chimeric shoots (Broertjes and Van Harten, 1988).

CURRENT SITUATION

Based on the radiation sensitivity study a massive irradiation of *in vitro* plantlets of TMS 30572 and U/41044 was carried out using 20 Gy for both as well as 25 Gy for U/41044. The M_1V_0 -M_1V_2 generations were multiplied through single nodal culture resulting in M_1V_2 plantlet populations of 2,212 (20Gy) and 706 (25Gy) for U/41044 and 1,654 (20Gy) for TMS 30572. These are at present being subcultured to raise the M_1V_3 generation. Due to constraints in handling this process will be carried out in three batches. At this first instance up to five thousand M_1V_3 plantlets will be hardened and transplanted to the field for screening. At least 8000 M_1V_3 plantlets per cultivar per dose is the target if any desirable mutant selection is to be achieved.

Morphological characters observed in the M_1V_2 populations include very vigorous growth, stunted plantlets with pronounced tuberlet, elongated internodes and variation in leaf sizes. These are being followed up to the field stage.

GERMPLASM CONSERVATION

Currently, NRCRI has 417 cassava collections maintained under field conditions. Up to 58.96% of the Institute's original collection was lost between 1980 and 1990 due to pests and diseases, fire hazards as well as adverse weather conditions. Duplication of collections *in vitro* for safer preservation is in progress, while further collections are being made to build up and expand the germplasm.

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Table 1. Percentage sprouted and percentage developed in irradiated axillary buds of two cassava cultivars in culture.

	TMS 30572			U/41044		
Dose (Gy)	% Sprouted 1 WIC*	2 WIC	% Explant Developed 7 WIC	% Sprouted 1 WIC	2 WIC	% Explant Developed 7 WIC
0	96.3	100.0	77.8	63.0	76.0	76.0
10	51.0	72.2	54.1	64.0	84.0	88.0
20	42.9	52.3	52.4	64.0	69.2	64.4
30	25.0	31.3	20.0	13.8	39.3	10.0
40	10.0	10.0	0.0	6.1	8.0	0.0

* Weeks in culture

Table 2. Organ development in irradiated axillary buds of two cassava cultivars in culture (7WIC).

	TMS 30572			U/41044		
Dose (Gy)	Mean plant Ht. (cm)	Mean No. Node/ Plant	Mean No. Expd. Leaves/Plant	Mean Plant Ht. (cm)	Mean No. Node/ Plant	Mean No. Expd. Leaves/Plant
0	5.3	6.1	6.0	3.2	4.6	4.8
10	4.3	5.6	5.3	2.4	4.6	4.6
20	2.1	4.9	4.9	1.4	3.5	3.9
30	1.9	4.4	3.2	0.8	2.0	1.0
40	0.0	0.0	0.0	0.0	0.0	0.0

CRYOPRESERVATION OF CASSAVA SHOOT TIPS

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CIAT, Cali, Colombia

Currently, ex-situ conservation of cassava germplasm at CIAT is carried out both in the field and in the laboratory as shoot tip cultures. The *in vitro* conservation constitutes an active collection and clones are sub-cultured and re-cycled every 12-18 months depending on the genotype. The availability of long-term storage of cassava germplasm in reduced space, free of genetic change, and at a low cost can be achieved by cryopreservation.

A research project on cassava cryopreservation began at CIAT in 1988; the project comprises three phases: (i) the first phase was carried out in cooperation with IBPGR (1988-90) and resulted in the recovery of plants from frozen shoot tips in liquid nitrogen. With the cv. MCol 22, recovery rates ranged from 20 to 40%. However, several cassava cvs. tested only showed a low response or did not respond at all (Ann. Report, CIAT 1991). (ii) the second phase of the research (1990-present), was designed to improved the previous protocol in order to increase the recovery rate of plants, minimizing genotypic differences. Lower temperature and higher illumination of donor cultures increased recovery from liquid nitrogen with consistent rates 50- 60%. Use of high concentration of sucrose in lieu of sorbitol and DMSO in the pre-culture stage, allowed high rates of plant recovery from frozen shoot tips. Work continues to adjust the cryoprotection, freezing, and post-thawing culture phases. Ultra rapid freezing, i.e. direct immersion of shoot tips into liquid nitrogen, resulted in similar or higher recovery rates than slow freezing. On the other hand, recovery rate of otherwise unresponsive genotypes has significantly increased with the improved technique. This work has paved the way to the development of a long-term, base gene bank of cassava clones using liquid nitrogen. (iii) the third phase of the research will focus on developing further the technique, especially with regard to genotype response and evaluation of genotypi stability; finally critical logistical aspects of cassava cryopreservation will be tackled.

Cassava (Manihot esculenta Crantz), an annual root crop, is extensively grown as a source of human food in the tropical regions of the world.

Because of modern agricultural practices, the genetic variability of crops is gradually diminishing, thereby, the potential danger of genetic erosion of both cultivated and wild cassava germplasm resources may be attributed to the replacement of primitive cultivars by new varieties or hybrids and the incorporation of land to agriculture in the areas of genetic diversity. Such danger, and the requirement of genetic variability for use in the improvement of the crop, justifies cassava germplasm conservation efforts.

Vegetatively propagated crops require that high levels of heterozygosity be preserved (which otherwise can be lost trough seeds or pollen), thus, vegetative organs are preferred to maintain valuable gene combination. However, in most clonal crops, the perennation through vegetative organs requires continuous field cultivation; this in turn demands intensive labor and is expensive. Furthermore, the risk of loss due to pests and diseases is high (Roca, 1984).

Currently, *ex-situ* conservation of cassava at CIAT is carried out both in the field and in the laboratory as shoot tips cultures. Two types of *in vitro* gene-bank conservation have been proposed (Withers and Williams, 1985): a. the *in vitro* active gene-bank (IVAG) where cultures are maintained under slow growth and b. the *in vitro* base gene-bank (IVBG) where cultures are cryopreserved. The IVAG is to a large extent, being developed for cassava, potatoes, sweet potatoes, banana and sugar cane, and constitutes a working collection. Its counterparts would be represented by the field collection and the sexual seed collection under short-term storage.

The IVBG constitutes a base collection; cryopreservation is still not fully developed for a given crop. This approach enable full maintenance of germplasm genotypic stability. Its counterpart would be represented by the sexual seed collection under long-term storage.

Ultra-freezing is ideal for the conservation of germplasm, as it stops all cell functions allowing preservation an indefinite time. The *in vitro* gene-bank of cassava at CIAT presently comprises 4300 clones in a laboratory of 50 m^2 and is probably the largest and most complete *in vitro* collection in the world. This is an active collection (IVAG) in the sense that clones have to be transferred every 12-18 months to fresh culture medium.

Conservation of cassava clones for long-term in small space, free of genetic change and at low cost, can be achieved by Cryopreservation. It's a good alternative to conserve.

In addition to stopping the cell metabolism and growth, mutations can be avoided which might be due to storage or external factors when other methods of germplasm conservation are applied (Kartha, 1984).

In the past, cryobiology was centered on the preservation of fruits, vegetables and other plant parts. These procedures allowed the material to be preserved and to increase its storage time. It is now possible to extend these cryobiologic methods to the conservation of plant germplasm. Suspended animation, prevention or delay of processes of cell deterioration and indefinitive preservation of plant genomes are now the main objectives of this science.

METHODOLOGY AND RESULTS

CIAT and IBPGR have joined in an effort to develop a protocol for the conservation in liquid nitrogen with cassava as model, it will be a more efficient, safer and economic method.

A research project on cassava cryopreservation began at CIAT at 1988, the project comprises three phases: 1. the first phase was carried out in cooperation with IBPGR (1988-1990) and resulted in the recovery of plants from frozen shoot tips in

liquid nitrogen; 2. the second phase of the research (1990-present), was designed to improve the previous protocol in order to increase the recovery rate of plants; 3.- the third phase on the research will focus on developing the technique, further especially with regard to genotype response and evaluation of genotypic stability, and finally critical logistical aspects of cassava cryopreservation will be tackled.

CIAT's Methodology

1. Extraction of shoot tips between 2-4 mm
2. Preculture in C4 medium (1M sorbitol and 0.1M DMSO) for 3 days
3. Cryoprotector (1M sorbitol, 10% DMSO and 0.1M sucrose) addition for 2 hours on ice
4. Drying for 1 hour
5. Control freezing rate:
   Wait to 5°C
   0.5°C to -15°C
   -15°C to -20°C
   -20°C to -17°C
   1°C to -40°C
   End
6. Transfer to liquid nitrogen (-196°C)
7. Thawing, swirl in 37°C bath
8. Reculture medium:
   a. Equilibrium media (R1 and R2) for 2 days each
   b. Normal proliferation medium 4E
9. Evaluation of viability and shoot formation, 1 month.

Using this methodology it was possible to obtain consistently for first time plants from frozen shoot tips. The parameters evaluated were viability (tissue that survives and show capacity to grow) and shoot formation (explants that can form shoots capable of growing in plants). With the cv. MCol 22, recovery rates ranged from 20% to 40% . However, several cassava cvs. tested showed only a low response or did not respond at all (Annual Report, CIAT, 1991). (Table 1)

With respect to the first protocol, in the second phase at CIAT, we are working to improve this methodology and find that lower temperatures and higher illumination of donor cultures increased recovery from liquid nitrogen with consistent rates of 50-60%. Use of high concentration of sucrose (0,75M) in lieu of sorbitol and DMSO in the pre-culture stage, allowed high rates of plant recovery from frozen shoot tips. In this phase we tested three media in the preculture step and found that when it compared with the C4 medium (first protocol) the amount of recovery plants were different and showed significant differences (Table 2).

The work continues to adjust the cryoprotection, freezing, and post-thawing culture phases.

Ultra-rapid freezing , i.e. direct immersion of shoot tips into liquid nitrogen, resulted in similar or higher recovery rates than slow freezing. On the other hand, recovery rate of otherwise unresponsive genotypes has significantly increased with the improved technique (Table 3).

At the moment with the improved methodology we can estimate the cost of this technology in contrast to the other form of conservation of cassava that is used at CIAT (Table 4).

CONCLUSIONS

A cryopreservation technique for cassava will allow to greatly reduce germplasm storage cost. The high costs of maintaining a germplasm collection are due largely to the labor required in the field as well as in the active *in vitro* bank. Cryopreservation should decrease these costs, and it should reduce the space needed to preserve a collection as large as the one maintained at CIAT. The conservation of cassava germplasm in the form of shoot tips in liquid nitrogen will play a basic role in handling and studying the genetic variability of this important crop using new and already known methods of genetic manipulation. In addition cryopreservation of pollen, seeds (or zygotic embryos), somatic embryos and other cells and tissue will be fundamental for achieving the same goal, which is basic to the cassava improvement programs at CIAT and elsewhere.

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Table 1. Effect of the cassava genotype on viability and shoot formation after cryopreservation of shoot tips.

Cultivar	Average value	
	Viability*	Shoot**
MCOL 22	92.72 (A)***	52.22(A)
CM 922-2	81.39(AB)	48.65(A)
MCOL 1468	73.73(B)	10.00(BC)
MARG 2	67.72(B)	37.45(A)
MPAR 193	51.66(C)	13.97(BC)
MCUB 27	45.31(DC)	11.42(BC)
MECU 48	32.94(DE	21.03(B)
MPAN 125	24.83(EF)	12.92(BC)
MGUA 14	21.22(EFG)	2.00(C)
MPER 303	15.26(FGH)	0.00(C)
MCR 113	14.44(FGH)	2.77(C)
MBRA 12	11.00(FGH)	0.00(C)
MDOM 2	5.25(GH)	3.12(C)
MVEN 232	5.02(GH)	0.00(C)
MMEX 71	3.90(H)	0.00(C)

* viability: tissue that survives and shows capacity to grow.
** shoot formation: explants that can form shoots capable of growing in plants.
*** averages with the same letter do not show significant differences at 0.05 level.

Table 2. Effect of pre-freezing cultures medium on viability and shoot development from shoot tips (cv. MCol 22) after freezing in liquid nitrogen.

Preculture medium	Viability	Shoot
0.75M sucrose, 0.01% CA*	88(A)	56 (A)
1M Sorbitol, 0.1M DMSO**	90(A)	32(B)
0.35M Sucrose, 1g m-Inositol***	56(B)	26(B)
Micropropagation medium	0	0

* Without basal salt or hormones. CA: Charcoal Activated.
** Basal medium 4E.
*** ½ basal medium, without hormones.

Table 3. Effect of pre-freezing culture medium on tissue viability and shoot development of recalcitrat cvs after freezing in L.N.

Medium*	MCol 22**		MMex 71		MVen 232	
	Viabil	Shoot	Viabil	Shoot	Viabil	Shoot
1	88	72	40	8	32	18
2	84	56	32	4	40	20
3	92	52	4	0	5	0

* 1: Basal 4E, 0.5M sorbitol, 0.01M DMSO, 0.1M sucrose.
2: Basal 4E, 0.5M sorbitol, 0.001M DMSO, 0,25M Sucrose.
3: Control medium: basal (4E), 1M sorbitol, 0.1M DMSO.
** Control cv.

Table 4. Maintenance costs of cassava germplasm under three conservation methodologies (CIAT, 1992).

Method of conservation	Collection size N°access	Area utilized m²	Estimated cost/year U.S. $
Field gene bank	5300	50000	30000
in vitro gene bank (IVAG)	4850	50	25000
in vitro base gene bank Cryopreservation	6000	1	5000

MUTATION INDUCTION BASED ON IN VIVO AND IN VITRO TECHNIQUES AS TOOLS FOR CASSAVA BREEDING

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Mutation induction with radiation and chemical mutagens in Cassava (*Manihot esculenta*) is aimed at optimizing genetic variation in the frame of a Coordinated Research Program (CRP) sponsored by the Italian government in cooperation with the FAO/IAEA Joint Division and a number of African institutes mutagenic treatments were applied to *in vivo* and *in vitro* cuttings.

In vivo techniques were already established and applied to a breeding program in Ghana aimed to select cassava clones with increased starch quality and mealiness. In the IEAEA Laboratories (Seibersdorf) *in vitro* technologies are currently investigated and applied to cassava clones. The paper reports on methodological investigations of *in vitro* mutagenesis with physical and chemical mutagen in nodal cuttings of cassava and the evaluation of early assessable M_1V_1 effects which are indicative for a mutagenic response. Most significant differences in mutagenic efficiency were obtained when the induced effect to acute and fractionate gamma doses treatments were compared.

The potential of *in vitro* tissue culture of cassava, for mutation breeding will be also discussed.

INTRODUCTION

Cassava is a multi-purpose plant and its role as a staple food and as livestock feed is well established. Traditional cross breeding is somewhat constrained by its sporadic flowering and the relatively low number of seeds produced. Moreover, hybrid breeding using inbred lines is not possible due to severe inbreeding depression.
However, its vegetative propagation allows desirable traits originating during the lengthy breeding process to be fixed.

Mutation induction aims to optimize genetic variation with minimal plant injury. In this paper we will describe two complementary approaches to mutation induction in cassava, in vivo and in vitro. The two methods rely basically on the same philosophy i.e. vegetative explants are treated with mutagens and the population is multiplied to dissolve the chimeric situation and establish homohistont plants which are finally screened for useful traits.

Results of this work originate from a Coordinated Research Program of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture in Vienna, which was supported by the Italian Government.

MATERIALS and METHODS

1) Improvement of cooking quality by in vivo mutation breeding

Cuttings of four cassava varieties (segregants of improved IITA varieties) were irradiated with gamma rays (^{60}Co source). The M_1V_1 was planted in the field in Kumasi, Ghana during the 1988-1989 season. Cuttings taken from these plants were used to establish the M_1V_2 generation during the 1988-1989 and 1989-1990 planting seasons. In July 1990 the plants were harvested and screened for cooking quality. Plants were harvested individually and the tubers cooked in boiling water for 20 min after which they were rated for mealiness by an independent panel. A scoring system of 1 to 4 for non-mealy to very mealy was used. Only plants with a mealiness score of 3 and above were selected in M_1V_2 and screening for cooking quality and for genetic confirmation of this trait was continued in M_1V_3.

2) Establishment of an in vitro mutation induction system

The specific objective was to study early assessable parameters of primary injury in tissue culture which are indicative of a mutagenic response and permit estimates of mutation frequency due to correlations between parameters of primary injury and the induced genetic variation in homohistont plant regenerants. Another objective was to compare effects of acute vs. fractionated gamma dose applications and to investigate whether mutagenic efficiency i.e. the ratio between mutation rate and primary damage could be increased by split dose application.

Four clones of cassava i.e. M. Mal-2, M. Mal-3, M. Thai-1 and M. Col-1390 were mass propagated in a modified MS medium supplemented with inositol 100 mg/l, thiamin 1 mg/l, BAP 0.1 μM, NAA 0.01 μM, GA_3 0.1 μM, 30 g/l sucrose, pH 5.8 and B5 vitamins. The top two nodes were aseptically excised and representative populations of nodal cuttings treated in an hormone free medium with acute vs. fractionated ^{60}Co gamma radiation at a dose rate of 4,99 Gy min^{-1}. Dose fractionation implied splitting of a total dose in three fractions where the first dose fraction was the lowest to condition effectively an enhanced level of radioresistance (dose fractionation: 20 Gy = 5Gy + 5Gy + 10Gy, 30Gy = 10Gy + 10Gy + 10Gy, 40Gy = 10Gy + 15Gy + 15Gy). Moreover, a recovery period of 4 hours was applied between split dose application since preliminary experiments have shown that shorter intervals yielded lower radiation recovery factors. The in vitro irradiated nodal cuttings were transferred immediately after treatment into the liquid culture medium. Radiosensitivity was based on the number of proliferating explants, fresh weight and shoot height.

RESULTS AND DISCUSSION

i) In vivo mutation breeding

Mutation induction enhanced the limited genetic variation for mealiness and cooking quality in the agronomically superior disease resistant and high yielding IITA parent varieties to a varying, genotype specific degree (Table 1). The mean score for mealiness in control plants was 2 and variation was limiting. Promising M_1V_2 mutants have been identified that were mealy and also had relatively large starch granules. There were no differences between mealy and non-mealy tubers in the arrangements of cells i.e. in cell condition. However, when these were cooked, the cells in the mealy tubers could be easily disorganized while those in the non-mealy tubers continued to remain intact. Parameters involved in cooking quality e.g. starch content, starch
granule size and dry matter were correlated with mealiness, smoothness and elasticity of pounded paste. Data from these studies are being used to develop a simple screening technology for cooking quality in cassava.

ii) Effects of acute gamma radiation

Radiation damage increased with dose and followed a sigmoidal pattern of primary injury for all radiosensitivity parameters and cassava clones tested. Useful doses for mutation induction appear to be in a dose range between 20 and 35 Gy while doses beyond 40 Gy inhibited plant regeneration strongly and induced severe primary injury. A culture period of 40 days is recommended for the assessment of M_1V_1 parameters of radiation effects and their association with mutation frequency in M_1V_2 or M_1V_3 plants regenerated from nodal cuttings.

iii) Application of fractionated gamma ray doses

Among the factors influencing and/or modifying the effect of ionizing radiation are the total dose and dose rate. The mode of application i.e. acute vs. chronic or single vs. split dose application may have implications on radiation injury and mutation frequency (Chadwick and Leenhouts 1981). Dose fractionation of low LET radiations has long been known to reduce radiation damage by repair of physiological and genetical effects (Wolff 1966, Evans 1967). But Broertjes (1972) concluded that dose fractionation did not yield any significant increase of mutation rate in leaves of Saintpaulia. Our objective, however, was to improve parameters influencing positively mutagenic efficiency i.e. which maximize mutation rate and minimize physiological damage (Brunner 1991). Both, the number of dose fractions and the length of the interval between dose fraction were tested in preliminary experiments and found in agreement with the methodology described by Walther and Sauer (1990) for in vitro derived explants of Gerbera jamesonii. Splitting an acute dose into three fractions and separated by at least four hours intervals was found to produce consistently superior recovery compared with two acute dose fractions and shorter recovery intervals between split doses. The magnitude of

recovery is obviously influenced by the level of the first or primer dose which activates repair phenomena. The comparative development of fresh weight and node number after 40 days culture between fractionated and acute gamma doses is depicted in Fig. 1. Recovery effects were most pronounced at dose levels which induce severe radiation damage or lethality after acute irradiation. On the basis of the definition of recovery factors (f_{rec}), any identical dose effect of acute and split dose application manifests itself for a given biological endpoint by the value of 1 indicating also the level of the respective acute dose. Recovery factors as shown in Fig. 2 increase with dose and are most enhanced at very drastic doses which are applicable only by means of multiple dose fractionation. Both assessed parameters of radiation damage followed the same tendency as proven by a chi-square heterogeneity test for constancy of the set ratio of recovery factors. The radiobiological effects of split dose irradiations and their potential positive impact on an improved mutation induction methodology are only the first step within the scope of efficient in vitro mutation induction for breeding objectives. Large populations of homohistont M_1V_2 or M_1V_3 plants with desirable traits must be raised for a meaningful study of correlations between parameters of primary injury and the induced genetic variation.

Further systematic investigations on mutation induction in different tissue culture systems are required to define correct estimates of mutation frequency based on primary injury and an economically relevant contribution of in vitro induced genetic variation for crop improvement. Tissue culture commonly results in a time reduction of clonal reproduction and its value for mutation breeding relies mainly in the propagation rates that are much higher than in vivo. This technology was transferred to African countries during the FAO/IAEA/ITALY project "Improvement of Basic Food Crops in Africa Through Plant Breeding, Including the Use of Induced Mutation" where in vitro mutation breeding of cassava genotypes with resistance to viruses was attempted. The breeding work is still in progress and final results are not yet available. On the other hand, mutation induction was successfully used in breeding complex traits such as mealiness of cassava tubers and it is expected that in vitro technologies will increasingly complement the classical mutation breeding approach in an economically feasible way.

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Table 1. Frequency distribution of cooking quality scores of M_1V_2 populations 12 month after planting.

	Clone			
	ISU-W	1425LB	1425-W	518DB
Score*				
1.0-1.4	64	187	36	14
1.5-1.9	70	79	4	6
2.0-2.4	64	30	5	3
2.5-2.9	68	23	2	1
3.0-3.4	94	11	7	1
3.5-3.9	59	6	3	-
4.0-4.4	71	11	5	-
4.5-4.9	-	-	-	-
5.0-5.4	1	-	-	-
TOTAL	491	347	62	25

* 1 = not mealy, 5 = very mealy

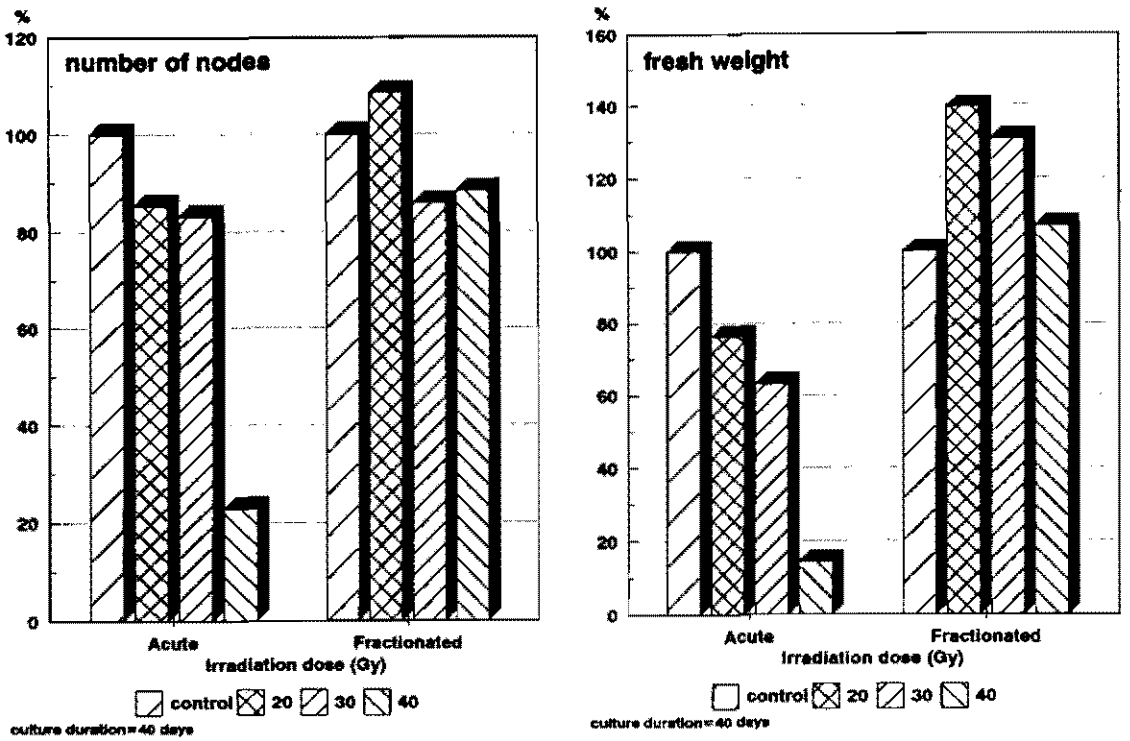

Fig. 1 Comparative development of fresh weight and node number in cassava M. Mal-2 after irradiation with acute and fractionated gamma rays grown in liquid medium for 40 days

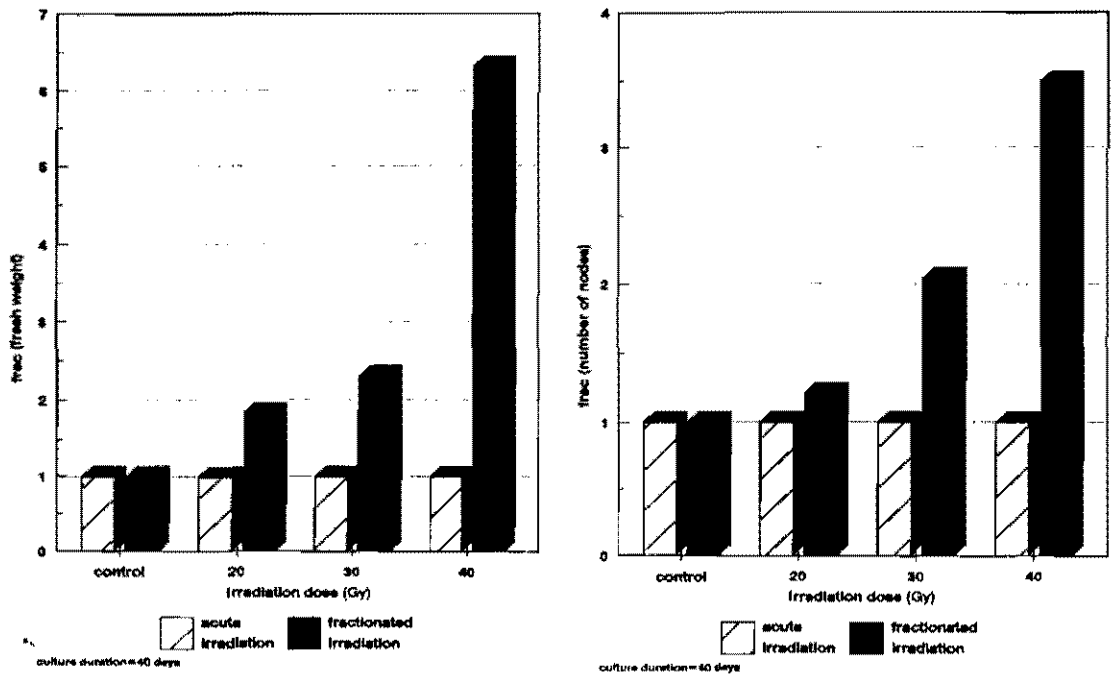

Fig. 2 Factor of recovery (f_{rec}, i.e. mean of radiation injury parameter/fractionated dose divided by the mean of radiation injury parameter/acute dose) between fractionated and acute gamma irradiation in cassava M. Mal-2

THE INDUCTION OF SOMATIC EMBRYOGENESIS OF RECALCITRANT CASSAVA CULTIVARS USING PICLORAM AND DICAMBA

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In addition to CMC 76, leaf lobes of two recalcitrant cassava cultivars (CMC 40 and MCol 113) were able to undergo somatic embryogenesis on semi-solid MS media supplemented with either picloram or dicamba. The leaf lobes were incubated on this medium for 20 days prior to transfer to hormone-free MS medium. The recalcitrant cultivars required higher concentrations of both picloram and dicamba than those used with CMC 76 (6.0 mgl^{-1} vs 3.0 mgl^{-1} and 33.0-66.0 mgl^{-1} vs 3.3 mgl^{-1}, respectively) to produce somatic embryos. In the case of picloram, a longer period of incubation resulted in a higher total somatic embryo production.

INTRODUCTION

Somatic embryogenesis has been successfully induced from young leaf lobes of cassava (Stamp and Henshaw, 1982; Sudarmonowati, 1990) on medium containing 2,4-D. The capability of undergoing embryogenesis, however, greatly depended upon genotypes; all parts of certain cultivars failed to undergo embryogenesis on medium supplemented either with different levels of 2,4-D, or with NAA alone or in combination with cytokinin (Sudarmonowati, 1990).

At present, the embryogenic system is the only *in vitro* regeneration system in cassava that has potential for use in genetic transformation procedures, it is therefore, important to be able to regenerate a wider range of genotypes. In this report, the effects of picloram and dicamba on somatic embryogenesis of recalcitrant cassava cultivars were investigated.

MATERIALS AND METHODS

Two ranges of size (1-3 and 3-5 mm) of immature leaf lobes of cassava cultivars CMC 76, CMC 40 and MCol 113 from plants maintained *in vitro* were cultured on different media containing seven different levels of picloram (0.06, 0.6, 1.0, 3.0, 6.0, 9.0 and 12.0 mgl^{-1}) or five levels of dicamba (1.0, 3.3, 10.0, 33.0 and 66.0 mgl^{-1}). Unless otherwise stated, the explants were incubated for 20 days on these media prior to transfer to hormone-free MS medium for a further 21 days before the total number of embryos were counted for the 10 replicates. Longer periods of incubation on medium supplemented with picloram were tried to investigate their effect on the production of somatic embryos of CMC 76 and of CMC 40.

RESULTS

1. The effect of picloram on somatic embryogenesis with cassava cultivars CMC 40, MCol 113 and CMC 76.

Both CMC 40 and MCol 113 were able to undergo embryogenesis in response to picloram, but the concentrations of picloram required were higher than those required by CMC 76. Both CMC 40 and MCol 113 required 6.0 mgl^{-1} to induce embryogenesis from 1-3 mm leaf lobes and higher than 6.0 mgl^{-1} to induce embryogenesis from 3-5 mm leaf lobes, while those of CMC 76 only required 1.0 mgl^{-1} picloram. The optimum concentration for both CMC 40 and MCol 113 was 12.0 mgl^{-1}, while that for CMC 76 was 3.0 mgl^{-1} and 9.0 mgl^{-1} depending on the size of leaf lobes used (Figs. 1 and 2).

Cassava cultivar CMC 40 seemed to be the least responsive to picloram, but if the leaf lobes were incubated for longer than 20 days, somatic embryogenesis was enhanced (Fig. 3). A longer period of incubation on medium supplemented with picloram also resulted in a higher total production of embryos with CMC 76 (Fig. 4).

2. Effect of dicamba on the induction of somatic embryogenesis with cassava cultivars CMC 40, MCol 113 and CMC 76.

Of the cultivars tested, CMC 40 required the highest concentration of dicamba (66.0 mgl^{-1}) to be able to undergo embryogenesis, followed by MCol 113 which required 33.0 mgl^{-1}. In contrast, CMC 76 only required 3.3 mgl^{-1} of dicamba (Fig. 5).

CMC 76 proved to be superior to both cultivars CMC 40 and MCol 113 in terms of total production of somatic embryos.

Dicamba at a concentration of 33.0 mgl^{-1} seemed to be optimal for the induction of embryogenesis of not only MCol 113 but also CMC 76.

DISCUSSION

The results showed that somatic embryogenesis in cassava is strongly influenced by genotype and that each genotype responded differently to picloram or dicamba. Cultivars which failed to produce somatic embryos when they were cultured on media supplemented with 2,4-D, were able to respond to either picloram or dicamba but the required concentrations of these growth regulators were considerably higher than those used for inducing somatic embryogenesis with other plant species such as *Pisum sativum* (Jacobsen and Kysely, 1984), *Gasteria* and *Haworthia* (Beyl and Sharma, 1983), *Vigna mungo* (Sinha et al., 1984), and *Zea mays* (Conger et al., 1987).

The results also showed that the effect of the size of leaf lobes used as explants was important in terms of the production of somatic embryos. The effect

of the physiological state of explants on somatic embryogenesis has been reported by several investigators. In the Gramineae, the developmental stage of the explant was found to be the most critical factor in obtaining the optimum response and in establishment of vigorously growing embryogenic tissues (Vasil, 1982).

The ability of picloram and dicamba to induce somatic embryogenesis in recalcitrant cultivars will benefit the cassava genetic transformation programme which needs a reliable regeneration system for many cultivars.

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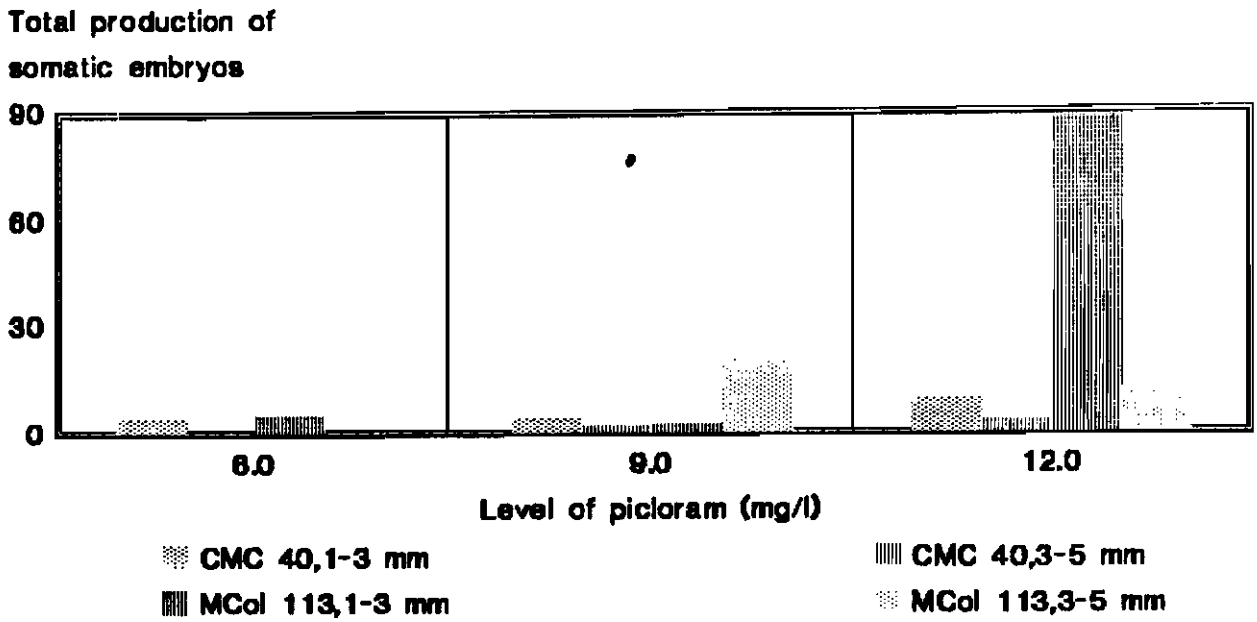

Fig. 1. The effect of picloram and the size of leaf lobes on somatic embryogenesis of recalcitrant cassava cultivars.

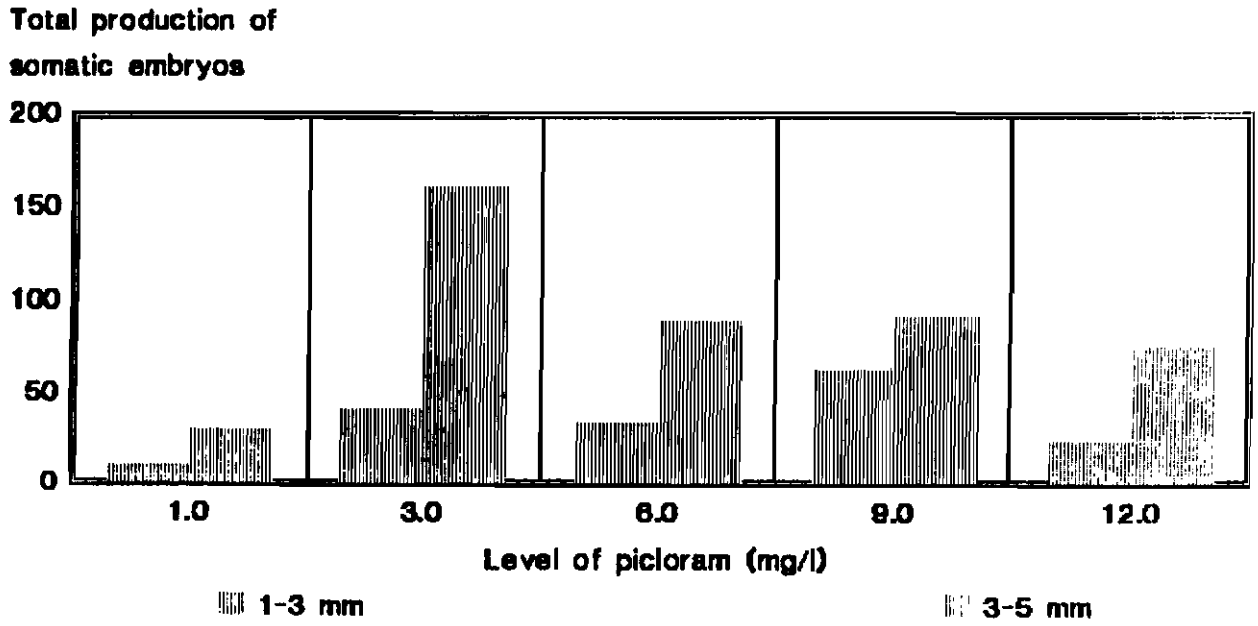

Fig. 2. The effect of picloram and size of leaf lobes on somatic embryogenesis of cassava cultivar CMC 76.

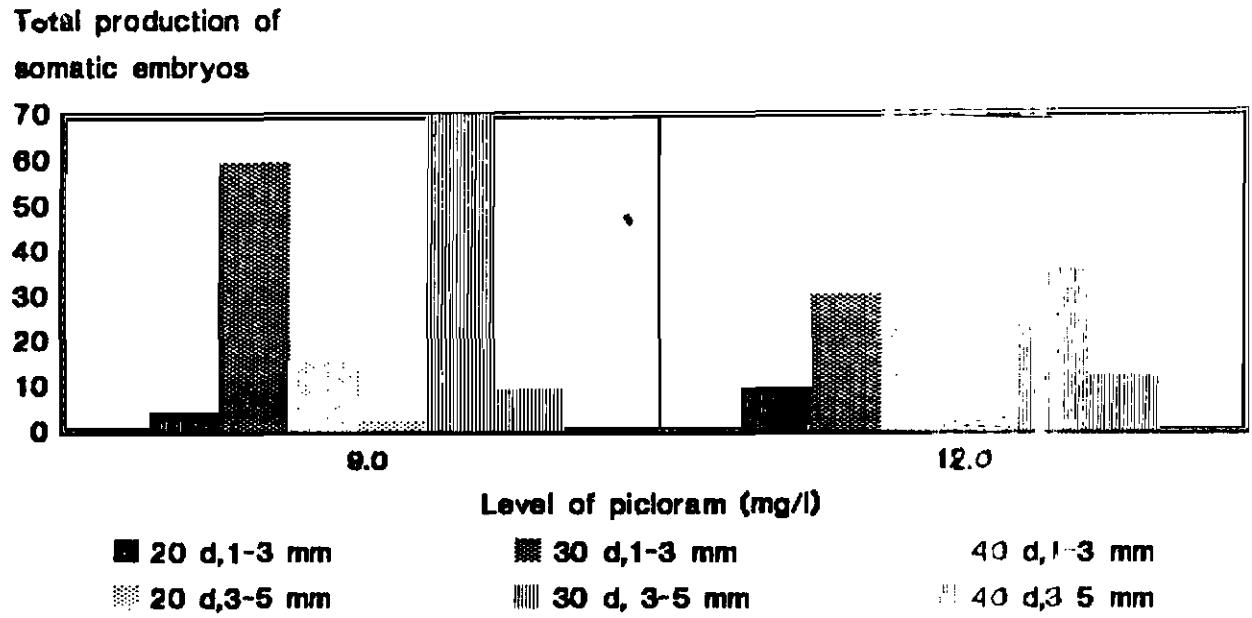

Fig. 3. The effect of picloram and period of incubation on somatic embryogenesis with cassava cultivar CMC 40.

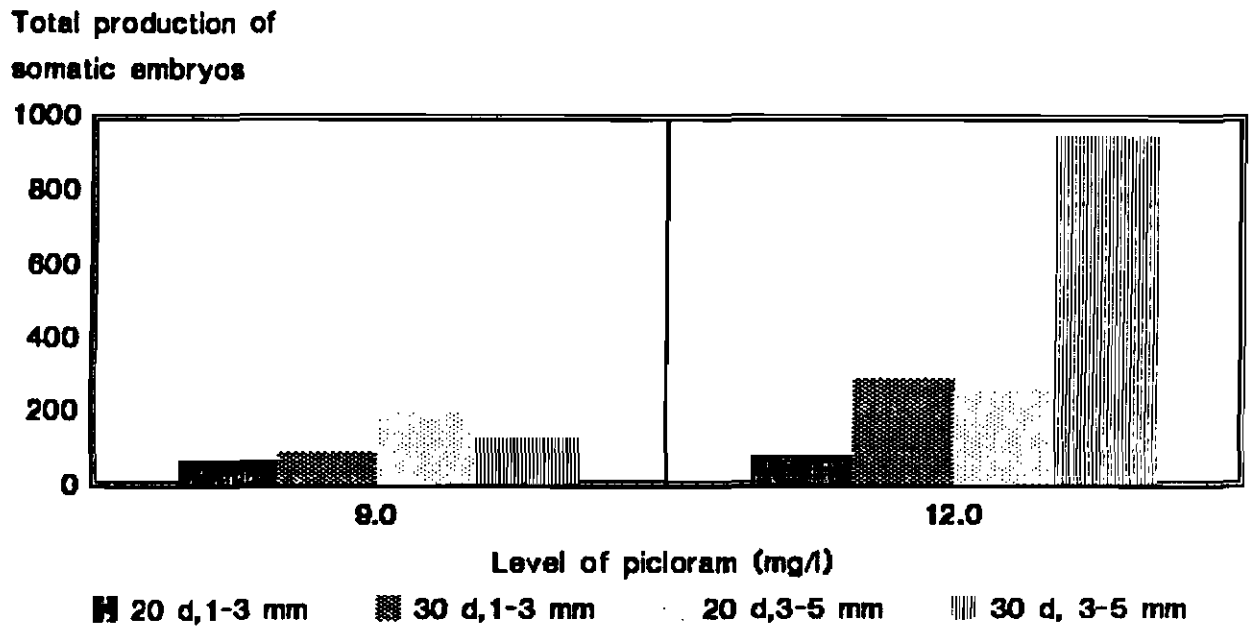

Fig. 4. The effect of picloram and period of incubation on somatic embryogenesis with cassava cultivar CMC 76.

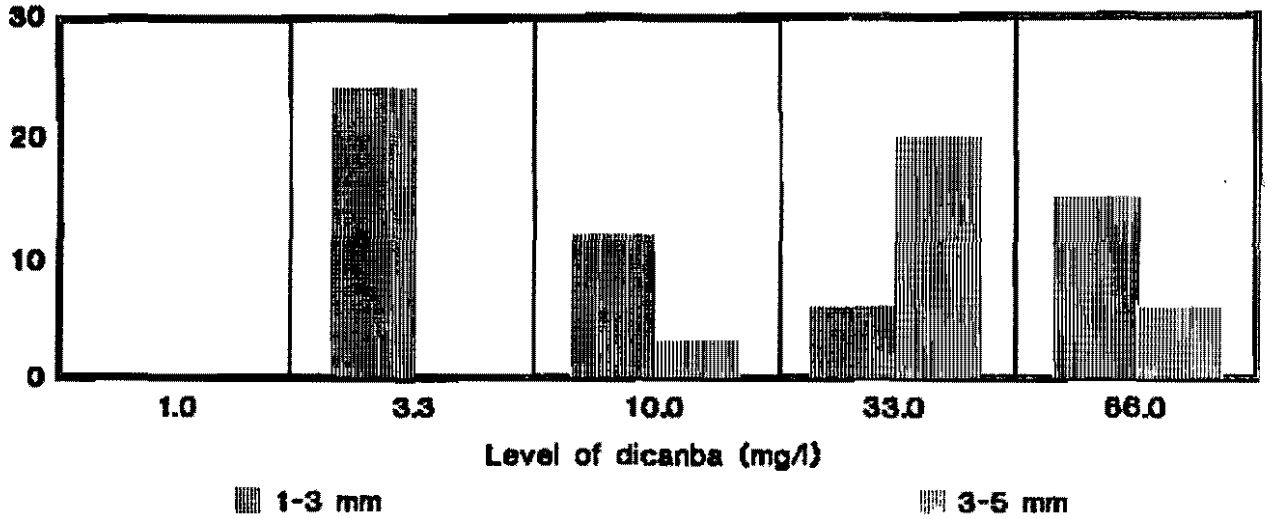

Fig. 5. The effect if dicamba on the induction of somatic embryogenesis with cassava cultivar CMC 76.

THE INDUCTION OF SOMATIC EMBRYOGENESIS IN FIFTEEN AFRICAN AND ONE SOUTH AMERICAN CASSAVA CULTIVARS

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Picloram was shown to be more effective than 2,4-D at inducing somatic embryogenesis from leaf lobes of fifteen African genotypes of cassava. This response was genotype--dependent and could be separated into high, medium and low. A system for the recovery of plantlets from somatic embryos of the genotype CMC 76 has been developed.

INTRODUCTION

Although organogenesis has been reported in cassava (Manihot esculenta Crantz) (Tilquin, 1979; Shahin and Shepard, 1980) somatic embryogenesis is the more common and reproducible morphogenic response in this important crop species.

The formation of somatic embryos and their subsequent recovery to whole plants was first achieved from seedling tissue by Stamp and Henshaw (1982). They used an induction medium supplemented with 2-8mgl^{-1} 2,4-D and in following reports this response was extended to the production of primary and secondary embryogenesis from young clonal leaf material in the genotype CMC 76 (Stamp and Henshaw, 1986; Stamp and Henshaw, 1987).

Szabados et al. (1987), employing the same culture system in order to screen a number of South American varieties, obtained embryogenesis from all of those tested, although the degree of response varied considerably between the genotypes allowing them to be classified into groups with high, medium or low embryogenic potential. Whole plants were recovered from the genotypes M Col 1505 and M Col 22 by subculture of the embryos onto a medium supplemented with a reduced auxin concentration, BAP and GA_3.

To date no such information has been available concerning the embryogenic capacity of African varieties of cassava. These are considered to be of importance because of their potential for genetic improvement and thus it is desirable to establish an understanding of their In vitro qualities. Data is presented concerning the embryogenic potential of fifteen African genotypes in comparison with the model genotype CMC 76. In addition the effectiveness of the auxin picloram, first investigated in cassava by Sudarmonowati (1990), is assessed against that of 2,4-D, while a system for the recovery of embryos to plantlets in CMC 76 is also reported.

MATERIALS AND METHODS

Media and culture conditions:

Plant material was cultured on Murashige and Skoog basal medium (Murshige and Skoog, 1962) supplemented with 2% w/v sucrose (MS2) and various growth regulators. Media were adjusted to pH 5.8 and 0.75% w/v Oxoid No. 3 agar added prior to autoclaving at 1.87 bar for 15 minutes. Media were dispensed in 25ml aliquots into 9cm petri dishes and sealed with Parafilm M. Cultures were incubated at 28^{+}1°C with a 16/8 photoperiod and 30uMm^{-2}s^{-1} irradiance.

Experimental material:

All experimental material was obtained from in vitro grown plants. One South American, CMC 76 (from the Centro Internacional de Agricultura Tropical, Colombia) and fifteen African genotypes (from the International Institute of Tropical Agriculture, Nigeria) were propagated by nodal cuttings and grown in glass jars containing 30ml MS2. Leaf lobes I-9mm in length were excised from these plants and placed, vein down-wards, on MS2 supplemented with various concentrations of 2,4-D or picloram in the range 10^{-6} - $5x10^{-4}$ M. Lobes from three plants were placed on each dish with four plates established from each genotype. The tissue was scored for the formation of somatic embryos 28 days after explanting.

The recovery of somatic embryos to plantlets in the genotype CMC 76 was achieved by culturing small groups of secondary embryos approximately 0.5cm across onto MS2 medium devoid of growth regulators for 2-3 weeks. This was followed by subculture of individual embryos to a range of media containing BAP, ABA and GA_3. These were then scored at weekly intervals for the development of leaf and root formation and the onset of stem elongation. Recovered plantlets were transferred to MS2 medium in jars.

RESULTS

Embryogenic response

An embryogenic response was achieved with leaf lobes 1-5mm in length from all of the genotypes investigated. Lobes greater than 5mm in length had very low embryogenic potential. CMC 76 reacting at around 80%, was superior to all of the African genotypes and responded equally to 2,4-D and picloram (Table 1). Although all fifteen African genotypes produced some embryogenesis this was a genotype-dependant response which could be classified into the relative groupings of high, medium and low embryogenic potential.

In both the high and medium groups picloram was superior to 2,4-D at inducing embryogenesis in all of the genotypes, causing at least a two-fold greater response and in the case of TMS 40160 up to a seven-fold increase. In the low

response group, this pattern did not exist and for the majority 2,4-D was equally, if not more effective than picloram. All of the genotypes, except TMS 90853 (see Table 1) produced a maximum response when exposed to the auxin at concentrations between

Leaf lobes ranging between 1 and 9mm in length were placed on MS2 medium supplemented with 2,4-D or picloram in the concentration range 10^{-6} - 5 x 10^{-4}M for 28 days. Lobes from twelve plants were used for each genotype and the data shown are means from three such experiments. The values listed represent the maximum response achieved by each genotype, this being achieved with lobes between 1 and 5mm long and, in all but one case, from auxin concentrations in the range 10^{-5} - 10^{-4}M inclusive, although the optimum concentration within this range differed between the different genotypes.

The amount of embryogenic tissue produced from each responding leaf lobe and the quality of the resulting embryos also varied with the genotype. These parameters, however, were generally correlated with the frequency of response; the greater the percentage embryogenesis the more, and better the quality of the embryos produced.

Embryo recovery

A system has been developed for the reliable recovery of somatic embryos to plantlets in the genotype CMC 76. Groups of secondary embryos (produced by serial subculture of embryogenic tissue at four weekly intervals on 2.5 x 10^{-5}M 2,4-D or picloram) 0.5cm across were subcultured onto MS2 for 2-3 weeks. After this time embryos in which the cotyledons had developed to the pale green foliose stage were separated and transferred as individuals onto one of the media shown in Table 2. All the media were capable of encouraging leaf formation after 28 days, but apart from occasional root formation, many embryos had failed to develop further. Only the media containing GA_3 were effective at inducing more than 50% of the embryos to undergo stem elongation and development to the plantlet stage.

DISCUSSION

In this study picloram proved to be a considerably more effective auxin than 2,4-D for the induction of somatic embryogenesis in a range of African genotypes. This, together with that of Sudarmonowati (1990) is one of the few reports to cite the successful use of picloram in a morphogenic culture system, and indeed it would appear that this auxin must be used if high-frequency embryogenesis is to be achieved in cassava.

As reported by Szabados et al. (1987) the relative degree of response could be clearly separated into high, medium and low categories. This was reproducible for this set of genotypes, indicating that there is some underlying genetic control of embryogenesis in cassava.

A system has been developed for the recovery of plantlets from somatic embryos of the genotype CMC 76. As with Szabados et al. (1987), it was found that the inclusion of GA_3 in the medium was required if a high frequency of embryos were to be converted to plantlets. GA_3 would appear to be essential for the extension of the stem and the formation of mature leaves.

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Table 1. Maximum Percentage Embryogenic Response after 28 days from leaf lobes of various genotypes of Cassava exposed to 2.4-D and Picloram.

Genotypes	Type of auxin in the Range 10^{-5} - 10^{-4} M		Relative Response
	2, 4-D	Picloram	
CMC 76	81.5	84.0	
TMS 83350	14.5	42.5	
TMS 90257	20.2	43.6	High
TMS 60142	14.2	50.4	
Kataoli	10.0	52.5	
TMS 40160	3.0	21.2	
TMS 90853	12.6	24.8*	
TMS 90059	11.4	24.6	Medium
TMS 60444	14.2	35.7	
TMS 30555	--	21.1	
TMS 60506	8.0	6.0	
TMS 91934	5.0	10.5	
TMS 30040	2.2	3.8	Low
TMS 63397	0.0	1.6	
TMS 30786	9.0	3.8	
TMS 30572	6.1	3.6	

* Maximum response in this genotype took place at 5 x 10 6M picloram.

Table 2. Development after 28 Days of Somatic Embryos from Secondary Embryogenic Tissue of the Genotype CMC 76.

Medium	Percentage Response		
	Root formation	Leaf formation	Stem elongation
MS2	2.0	74.3	0.0
10^{-6}M BAP	5.5	75.0	8.3
10^{-7}M ABA	0.0	50.0	0.0
10^{-6}M GA_3	22.5	70.0	23.5
10^{-6}M BAP/10^{-7}M ABA	0.0	54.1	0.0
10^{-6}M BAP/10^{-6}M GA_3	8.0	64.7	52.9

Number of somatic embryos per treatment was 36.

CASSAVA TISSUE CULTURE AND TRANSFORMATION: IMPROVEMENT OF CULTURE MEDIA AND THE EFFECT OF DIFFERENT ANTIBIOTICS ON CASSAVA

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The culture medium most often used for *in vitro* culture of cassava is based on that developed by Murashige and Skoog. In the published papers on cassava tissue culture this medium is usually modified by optimizing the concentration of its organic component (e.g., growth regulators, sucrose), but not of its mineral elements.

In our experiments with shoot tip cultures, we found that raising the concentration of cupric sulfate in the MS-medium from 0.1 uM (original concentration) to 2.0 uM resulted in an increase in shoot length and fresh weight of about 50%. Using young leaf lobes for the induction of embryogenesis, 2.0 uM cupric sulfate increased the number of lobes producing embryos in three independent experiments from about 5-25% to about 20-70%.

As selective marker for transformation experiments we had chosen the nptII gene, which confers resistance to aminoglycoside antibiotics. Using kanamycin for the selection of *Agrobacterium*-treated somatic embryos (see abstract by Chavarriaga *et al*,) so far we have obtained chimeric embryos. The regeneration of plantlets or the induction of secondary embryogenesis starting from transformed tissues on media with kanamycin was not possible. For that reason we tested alternative antibiotics (glyphosate, phosphinotricin, and hygromycin) for their effects on non-transformed cassava embryo clumps. The results show that both glyphosate and hygromycin suppress embryogenesis at comparatively low concentrations, while permitting callus formation at higher concentrations. In contrast to that, phosphinotricin inhibits embryogenesis and formation of callus to a similar extent and therefore seems to be better suited for the selection of transformed Tissues.

INTRODUCTION

The final goal of our research is to transform cassava tissues with viral coat protein genes and to regenerate plants that are resistant to the corresponding viruses (see paper by Fauquet *et al.;* Schöpke *et al.,* in press). In cassava, the only reproducible way to regenerate plants from tissues is through somatic embryogenesis. Cotyledons, young leaves, and shoot tips can be used as a source for somatic embryos. To establish an efficient transformation system there are many parameters to investigate and to optimize. They can be divided into two major classes, i.e., parameters affecting plant regeneration, and parameters related to the transformation itself. In this paper we describe investigations dealing with both classes, i.e., with the optimization of tissue culture media and with the effect of antibiotics on cassava tissues.

IMPROVEMENT OF CULTURE MEDIA

During our studies we tried to improve the regeneration efficiency of cassava. Although the MS-medium (Murashige and Skoog 1962) in general seems to be good for cassava tissues, it might not be the best one. Meyer and Van Staden (1986) found that the mineral salt composition of MS-medium is sub optimal for the growth of nodal explants taken from greenhouse-grown cassava plants. They devised a modified mineral medium that improved shoot growth of nodal explants of four cassava cultivars by more than two times, root growth by two to five times. Part of this effect was due to an increase of the micronutrients molybdenum and zinc. In our experiments we investigated the effect of copper on the development of shoots from nodal explants.

Materials and Methods:

Nodal explants derived from *in vitro* shoot cultures of cassava cultivar CMC 76 were used for the experiments. The shoots were cultured in GA7 vessels (Magenta Corp., Chicago, USA), with 100 ml medium and nine plantlets per vessel. The basal culture medium (BM) was composed of mineral salts and vitamins according to Murashige and Skoog (1962), 20 g/l sucrose, and 2.0 g/l phytagel (Sigma, St. Louis, USA). The pH was adjusted to 5.7 before autoclaving. For the experiment shoot cultures were used that had been grown for six weeks on either BM (0.1 μM $CuS0_4$) or on BM with 2 μM $CuS0_4$. Nodal explants were cut, keeping approximately 8 mm below the node and 3 mm above. They were collected in sterile water and then randomly distributed to GA7 vessels with BM containing various concentrations of Cu^{2+} (see Fig.1A), 100 ml per vessel.

The explants were inserted with their basal part into the medium. For each treatment three vessels were established. Culture conditions: photoperiod 16 h (fluorescent tubes Sylvania cool white, 90 - 110 μM $m^{-2}s^{-1}$ PAR), 25°C, humidity not controlled. After six weeks, the plantlets that had developed from the nodal explants (one shoot per node) were evaluated for fresh weight, length, and for the number of nodes.

Results:

In general, plantlet development after 6 weeks was better compared to the control (MS-medium containing 0.1 μM Cu^{2+}) at all concentrations of Cu^{2+} tested. Fresh weight (Fig. IA), number of nodes per shoot, shoot length, and root growth (data not shown) all followed the same pattern, i.e., an increase in the Cu^{2+} concentration had a positive effect with an optimum around 2.0 - 4.0 μM. Even at 8.0 μM Cu^{2+}, which is a concentration 80 times higher than in the MS-medium, the average fresh weight was increased by up to 25%. The comparison between shoots coming from nodal explants that had been cultured either on medium with 0.1μM Cu^{2+} or with 2.0 μM Cu^{2+} indicates that the beneficial effect of increased Cu^{2+}

concentrations is more pronounced for the explants coming from medium with the lower concentration.

EFFECT OF DIFFERENT ANTIBIOTICS ON CASSAVA TISSUES

A problem still to be solved is the selection of transformed cells and the regeneration of these to plants. Initially, we used the NPT-II gene (conferring resistance to aminoglycoside antibiotics) as a selectable marker. From experiments with young leaf lobes we concluded that cassava is very sensitive to kanamycin and geneticin. However, in contrast to the results with primary explants, callus that had formed on young leaf lobes after one week on medium without kanamycin was able to survive and grow after transfer to medium with 100 mg/l kanamycin, although at a very slow rate. Similar results were obtained with embryo clumps: secondary embryogenesis was strongly suppressed, but the tissues were not killed and little callus growth was observed on medium with 400 mg/l kanamycin. Additionally, kanamycin and geneticin suppress somatic embryogenesis at concentrations lower than 20 - 30 mg/l. To avoid these problems, we investigated the effect of alternative antibiotics on non-transformed embryo clumps, namely glyphosate, phosphinotricin, and hygromycin.

Materials and Methods:

Experiment 1: embryo clumps which had originated from young leaf lobes of *in vitro* plants of cassava cultivar CMC 76 (Stamp and Henshaw 1986) were subcultured every four to five weeks for 17 months on medium for the induction of secondary embryogenesis. This medium was the same as for the leaf lobes, but 2,4-D (2,4-dichlorophenoxyacetic acid) reduced from 4.0 mg/l to 2.0 mg/l). Four weeks after the last transfer, explants of 2 - 3 mm diameter were transferred to medium for secondary embryogenesis, but with different concentrations of the antibiotics glyphosate, phosphinotricin, and hygromycin. Culture conditions: photoperiod 16 h (fluorescent tubes Sylvania cool white, 30 μM $m^{-2}s^{-1}$ PAR), 25°C, humidity not controlled, 1 petri dish with 10 explants per treatment. After 4 weeks, the fresh weight of the embryo clumps and the weight of that part of the clumps comprised of typical embryos (yellowish, smooth surface, no callus between embryos) were determined.

Experiment 2: using the same line of embryo clumps, but subcultured for 21 months, and the same methods as described under Experiment 1, a broader range of phosphinotricin concentrations was tested (see Fig. ID).

Results:

The three antibiotics tested had a similar effect on the development of embryo clumps (Fig. IB). The increase in fresh weight after 4 weeks is smaller at higher antibiotic concentrations. However, in the case of hygromycin, this effect is not very pronounced.

A different picture emerges if one compares the amount of embryo tissue developed after four weeks. In the control the clumps are composed of 26 % embryos on a fresh weight basis. With glyphosate at 42 and 84 mg/l, the average fresh weight of the whole clumps has increased to values comparable to the control, while the percentage of embryos is reduced to 3 % and zero, respectively. blygromycin has a similar effect. At concentrations which permit the clump to increase to a fresh weight similar to that of the control (0.5, 2.5, 5.0 mg/l), the part of the clumps composed of embryos is reduced to 18, 7, and 4 %, respectively. With both glyphosate and hygromycin the tissue making up the rest of the clumps is friable callus, i.e., the antibiotics selectively inhibit embryogenesis and favor callus formation at the concentrations tested. In contrast to this, at concentrations of phosphinotricin up to 10.0 mg/l the development of the embryo clumps is affected differently (Fig. 1C). The growth of the clumps as a whole is inhibited, but the relative amount of that part of the clumps composed of embryos remains fairly constant and on a level similar to the control. The tissue not composed of embryos in this case is not callus, but misshaped, partly brownish embryos.

The experiment where a broader range of phosphinotricin concentrations was applied (Fig. ID) showed that 20 mg/l is sufficient to stop the development of embryo clumps completely.

CONCLUSIONS

The results obtained with increased copper concentration in the basal culture medium for shoot development demonstrate the Cu^{2+} content of the MS-medium is suboptimal for that purpose.

Therefore we now routinely include 2.0 μM Cu^{2+} in all media for cassava tissue culture. Preliminary results with the induction of somatic embryos from young leaves demonstrated that also here increasing the Cu^{2+} concentration has a positive effect. Our observations are in accordance with Teasdale (1987) who comes to the conclusion that (for tissue culture of forest trees) none of the more frequently used culture media contain sufficient Cu^{2+}. He recommends a concentration of 5 μM.
The antibiotics tested with embryo clumps acted in different ways. Glyphosate and hygromycin inhibited embryogenesis at low concentrations while permitting callus growth at relatively high concentrations. On the other hand, phosphinotricin at low levels inhibited the development of embryos and of non-embryo tissue in the same way, i.e. callus development was not enhanced compared to embryo development. At 20 mg/l, the lowest concentration which suppressed completely the growth of embryo clumps (Fig. ID), some typical yellowish embryos were still observed. These were most probably ones remaining unchanged from the original explant.

In order to select for transformed cells or tissues, in the case of glyphosate and hygromycin one would have to use relatively high concentrations to inhibit or kill non-transformed cells. These concentrations then might interfere with the regenerative capacity of transformed cells. With phosphinotricin the level needed to

inhibit callus growth is the same as that needed to inhibit embryo growth and formation of secondary embryos. This may be an advantage, because the probability for a transformed cell to enter the pathway of embryogenesis might be higher. Within our current investigations we are trying to transform cassava tissues with the bar-gene (conferring resistance to phosphinotricin) to test this hypothesis.

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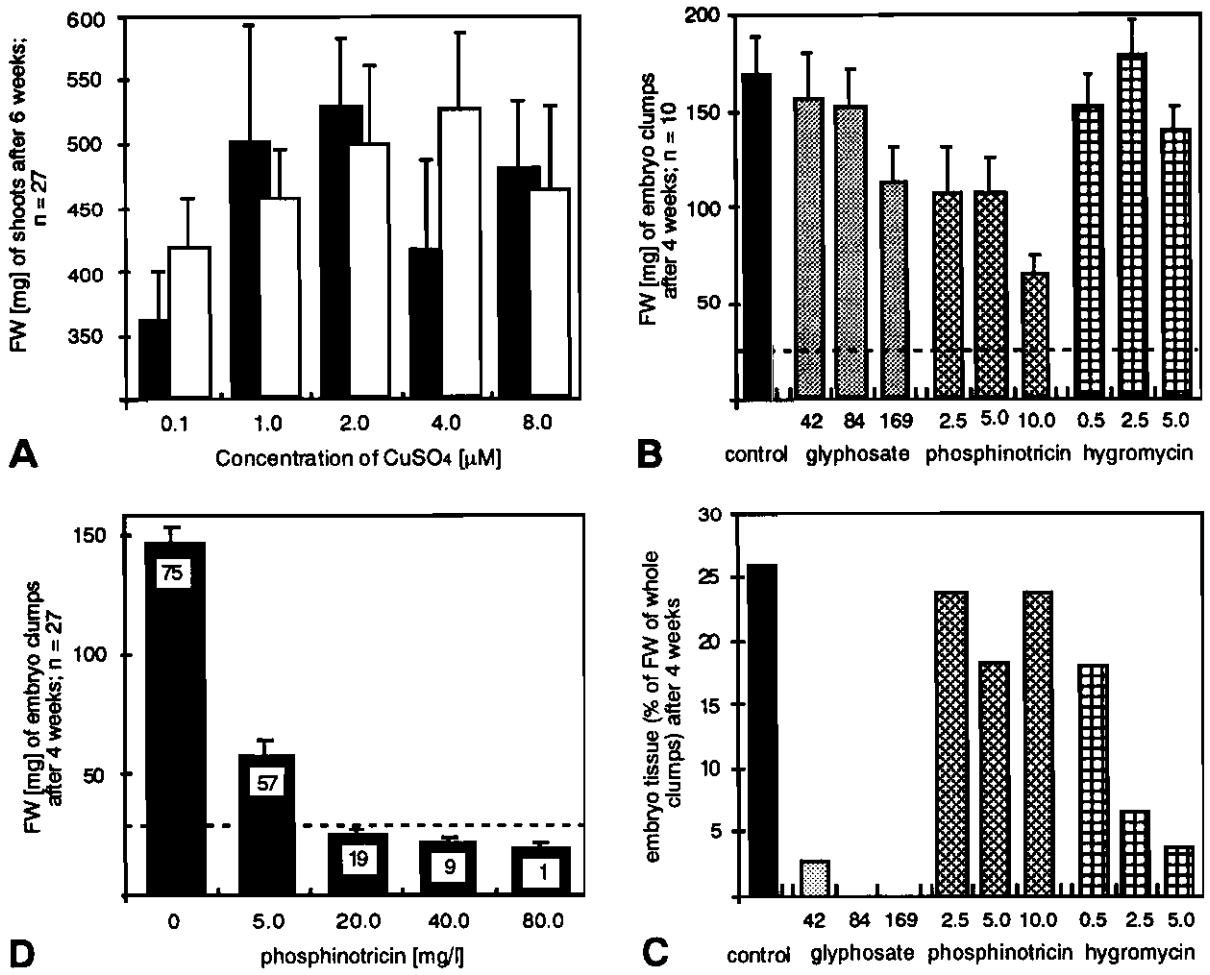

Fig. 1: A) Effect of $CuS0_4$ on plantlet development. Nodal explants coming from shoots that had been grown either on MS-medium (black columns) or on MS-medium with CuS04 increased to 2 μM (white columns) were transferred to media with a range of CuS04 concentrations. After 6 weeks, the fresh weight of the plantlets was determined.

B) - D): Effect of different antibiotics on the development of cassava embryo clumps after 4 weeks on media for secondary embryogenesis. B) Effect of glyphosate, phosphinotricin and hygromycin (conc. in mg/l) on the fresh weight of whole embryo clumps. The dotted line indicates the average weight of the original explants (embryo clumps with a diameter of 2-3 mm). C) Same experiment as B), but here the amount of embryo tissue expressed as percentage of the fresh weight of the whole embryo clump is given. D) Effect of different concentrations of phosphinotricin. The bars represent the fresh weight of the whole clumps after 4 weeks. The numbers inside the bars give the amount of embryos per clump, expressed as percentage of the fresh weight of the whole clump. Dotted line:
see A. Error bars: Standard Error

CASSAVA SEED PRODUCTION PROGRAM BY MERISTEM CULTURE IN UNPRG - LAMBAYEQUE (PERU)

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The cassava seed production program by meristem culture at UNPRG, Lambayeque began in 1986 with CIAT and IBPGR/FAO collaboration. Many cassava cultivars collected in Peru were transferred to CIAT as in vitro cultures. These materials were processed through thermotherapy and meristem-tip cultured and indexed for known viruses. IBPGR/FAO provided support to implement a tissue culture laboratory. The peruvian germplasm, after cleaning and testing for freedom of viruses, were returned from CIAT and added to the germplasm collection maintained in vitro at UNPRG. The entire process for clean cassava "seed" production involved five steps; clonal selection in the field, treatment of stakes with thermotherapy (40°C day/35°C night) for three weeks, meristem-tip culture, node cutting in vitro propagation, potting and field propagation and distribution of clones to farmers. Between 1986 and 1990, farmers from 10 northern coast and northeastern sites in Lambayeque received 20 cassava cultivars each, comprising 50-100 stakes per cultivar. Fresh root yields of micropropagated clones in farmer's fields were 2.5 to 3.5 ton/ha higher than clones propagated conventionally, without changing the farmer's agronomic and cultural practices. Currently, the program received financial support from the CONCYTEC Project PCT/UNPRG, 002-91, CONCYTEC, Perú.

INTRODUCTION

The Cassava Seed Production Program using Meristem Cultures, in U.N.P.R.G., Lambayeque, Peru, started in 1986 with CIAT and IBPGR/FAO collaboration. Cassava cultivars collected in Peru and other countries with CIAT support and selected hybrids with high output of fresh roots were disinfected for systemic pathogens by the combined action of stakes thermotherapy and meristem cultures. In addition, cassava cultivars collected in Peru were propagated by meristem cultures at the U.N.P.R.G. Tissue Culture Laboratory. IBPGR/FAO supported the basic implementation of this Laboratory, thanks to a dual collaboration project between both institutions. This work presents the findings we obtained, thanks to the economic support which the Cassava Seed Production Program receives from the National Council of Science and Technology National Council.

MATERIALS AND METHODS

The methodology followed for the production of healthy cassava plants by meristem cultures has been widely reported by CIAT (1980). Culture mediums, both meristem cultures (4E), and nodal and rooting cultures (17N), were as described by CIAT (1980).

Stages are: a). Field parental plant selection b). Stake treatment with thermotherapy at 40/35°C for three weeks c). Isolation and meristem culture with 2-4

leaf primordia d). Nodal micropropagation e). Field clonal propagation f). Farmers' distribution.

RESULTS AND DISCUSSION

There are many viral diseases reported in cassava from Africa to Latin America (CIAT, 1985). However, thanks to the meristem culture techniques we can overcome this problem. When thermotherapy is used in addition to meristem culture, the eradication of pathogens is higher (Lizarraga **et al**. 1980; Delgado y Roca, 1985). There another decisive factor for success of meristem culture: the smaller the meristem, the more effective the method (Navarro **et al.**, 1976).

The wide use of this technique lets us clean many genotypes from well known viral diseases, increasing considerably the root outputs respective to plants propagated by infested stakes (Kartha and Gamborg, 1975; Kaiser and Teemba, 1979; CIAT, 1979; Adejare and Coutts, 1981; Delgado and Roca, 1985; CIAT, 1986; Roca, 1983).

Table 1 shows yields of some genotypes cleaned by meristem culture and the mean yield of local genotypes propagated with infested material. The agronomic management was the same for all.

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Table 1. Yield in fresh root (t/ha) of cassava genotypes disinfected by thermotherapy and meristem cultures. Pucala and Pacanga locations*

GENOTYPE	YIELD IN FRESH ROOTS (T/Ha)		
	PUCALA	PACANGA	X
MCol 22	14.0	13.0	13.5
MCol 33	15.0	19.0	17.0
MCol 2063	21.0	22.0	21.5
MCol 2215	23.0	17.0	20.0
MCol 2050	-	18.0	18.0
MCol 2032	-	14.0	14.0
MPer 441	-	10.0	10.0
MPer 443	-	13.0	13.0
MCub 49	9.0	-	9.0
X local genotypes	6.0	5.0	5.5

*Harvest at 9 months

EFFECT OF MERISTEM CULTURE MICROPROPAGATION ON THE VIGOR AND YIELD OF THE CASSAVA CLONE 'SEÑORITA'

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The cassava clone 'Señorita' is a local ecotype widely cultivated in the cassava-growing regions of Cuba, occupying large areas of different edapho-climatic conditions. Yield losses due to physiological or genetic problems have increased due to exposure of the crop to various stress factors in the yield and subsequent vegetative propagation. The clone has been propagated for more than 30 years without being rejuvenated.

Using meristem micropropagation techniques, it has been possible to create a program to recover the yield stability of 'Señorita'. A comparative study was carried out using plants obtained from meristem culture. An increase in yield of commercial tuber roots up to 15% was obtained using meristem culture. The results suggest that there is a need to use meristem culture to maintain the yields of this vegetatively propagated crop.

INTRODUCTION

During its growth cycle, cassava (*Manihot esculenta* Crantz) plants are exposed to different stress factors which affect the yield stability considerably. Yields further deteriorate when vegetative propagation is carried out for many years without appropriate selection and conservation of the planting material. These two factors contribute to a prolongation of physiological age. Aging of the planting material results in a decrease in the potential productivity of cassava clones. The recommended practices for reducing the effect of stress factors on the yield of commercial tuber roots are the use of pesticides and careful selection of planting material.

Meristem culture micropropagation allows the elimination of pathogens and rejuvenates the planting material, resulting in higher yields and reduced pesticide use. Once the methodology to obtain healthy plants through tissue culture techniques has been well established, farmers may be periodically provided with healthy rejuvenated stakes.

The main objective of this project is to determine the effect of meristem tissue culture on yield and stake multiplication.

MATERIALS AND METHODS

This project was carried out at the Instituto de Investigaciones de Viandas Tropicales (INIVIT), Santo Domingo, Villa Clara, Cuba. Clone 'Señorita' was selected for this project because it is a local ecotype, distributed throughout Cuba by INIVIT and it has been vegetatively propagated for more than 30 years under different edapho-climatic conditions. The selected plants were grown under dry conditions (without irrigation). The 20 cm long stakes were derived from different parts of the plant: primary stem, secondary and tertiary branches. In addition there was a treatment with mixed

stakes (derived from all three parts of the plant) and a treatment with stakes derived from primary stems of plants which were obtained through meristem culture according to Roca (1980).

A randomized complete block design with 4 repetitions was used. The plots were 9 m long with 4 rows containing 10 plants each, in order to evaluate the 2 central rows. The methodology used was recommended by the Instructivo Técnico para el Cultivo (MINAG, 1988). During the 12 months of cultivation the following observations were taken:

1. Sprouting; this was directly determined by counting the number of plants present in the two central rows of each plot.

2. The rate of closing of the crop; the crop is considered closed when only 25% of the sunlight gets through the canopy.

3. Yield of commercial tubers; this was determined by weighing all roots with a length of more than 20 cm and a diameter of more than 5 cm from the two central rows.

RESULTS AND DISCUSSION

1. Sprouted stakes per plot

Table 1 shows the result of the sprouting analysis. It shows that the sprouting in plots planted with stakes derived from primary stems is significantly ($p<0.05$) higher than sprouting in plots with mixed stakes. Similar results were obtained by Fairlei, 1970; Huertas, 1940; Guanzón, 1927; García and Rodríguez-Morales, 1983; and Morejón and Rodríguez-Morales, 1983. These results imply the importance of the source of the stakes used in the plantation.

2. The rate of closing of the crop

Table 2 shows that the treatments where stakes derived from primary stems were used, needed significantly ($0<0.05$) less days for closing than stakes derived from other parts of the plant. The rate of closing of the crop depends on physiological factors as well as on the genotype of the plant material. However, a higher vigor was found in the treatments with stakes derived from primary stems.

3. Yield of commercial tubers

Table 3 shows that yields resulting from primary stems of tissue cultured plants was significantly ($0<0.05$) higher than the remaining treatments. The second highest yields were obtained from primary stems derived from normal plantation plants. The lowest yields were obtained with stakes from tertiary branches.

Lozano (1983) reported similar increases in yield using meristem culture.

Roca (1985) suggests that the isolation and ulture of meristems allows the propagation of plants with a high probability of being free of viruses and other diseases and would be a good source of juvenile material.

Lozano et al. (1978) consider that as well as the climatic and edaphic factors that affect the cassava plant, the quality of the stakes is a very important factor, which depends basically on the sanitary conditions and agronomic characteristics of the donor plant.

The rejuvenation resulting from meristem culture and the high rates of healthy plants obtained can explain the superiority of stakes from primary stems of meristem culture over primary stakes from normal plants which may be carriers of pathogens.

CONCLUSIONS

1. The source of planting material is a determining factor in achieving high populations and higher root yield of cassava.

2. The yield and vigor of cassava clones can be restored through meristem culture.

3. Economic feasibility studies are needed to define a continuous 'seed' production plan.

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Table 1. Number of sprouted stakes per plot.

Treatment	X
1. stakes derived from primary stems	19.7 a
2. stakes derived from secondary branches	13.0 b
3. stakes derived from tertiary branches	11.2 b
4. mixed stakes	14.2 b
5. stakes derived from primary stems of plants obtained by meristem culture	20.0 a

ES ± 0.01
CV = 6%
Means followed by different letters are significant at $p < 0.05$ according to Duncan's test

Table 2. Days to field cover.

Treatment	X
1. stakes derived from primary stems	113.7 a
2. stakes derived from secondary branches	129.5 c
3. stakes derived from tertiary branches	126.0 c
4. mixed stakes	119.5 b
5. stakes derived from primary stems of plants obtained by meristem culture	112.0 a

ES ± 0.002
CV = 0.4%
Means followed by different letters are significant at $p < 0.05$ according to Duncan's test

Table 3. Yield of commercial tuber roots (t/ha).

Treatment	X
1. stakes derived from primary stems	36.4 b
2. stakes derived from secondary branches	23.4 d
3. stakes derived from tertiary branches	20.3 e
4. mixed stakes	26.5 c
5. stakes derived from primary stems of plants obtained by meristem culture	43.2 a

ES ± 0.007
CV = 2.1%
Means followed by different letters are significant atp < 0.05 according to Duncan's test

CULTURAL FACTORS AFFECTING CLONAL PROPAGATION AND MICROTUBERIZATION OF Dioscorea alata L. FOOD YAM.

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This study investigated the main effects and interactions of plant growth regulators and cultural parameters on yam shoot multiplication and microtuber induction of D. alata food yam. Agar-gel strength of 0.2 and 0.4% supported higher shoot multiplication levels (15 shoots/culture) than a Control concentration of O.8%. Proliferation of uniformized yam shoots was accomplished by a BAP pulse treatment approach.

Supplementing the T medium with 1.0 μM KIN substantially improved microtuber frequencies. Interaction of tested levels of KIN with a set level of 1.0 μM ABA, reduced microtuber frequency (by 14°%). However, microtuber induction tended to be higher if shoot cultures had a 21-day initial exposure to 16 h day light prior to incubation under 8 h. Leaves borne on cultured shoots had to he green and had to have an area of c. 2 cm^2 in order that induction responses would exceed 50%.

INTRODUCTION

Yam is very important for the provision of staple energy source in the form of carbohydrate for millions of people in many tropical countries (Onwuene, 1978). In West Africa, the per capita daily consumption is Q.5-1.0 kg (Coursey, 1967). The most reliable field propagule for the crop is the tuber which reduces the net yield by c 20-30% and constitutes a major constraint in its production (Okoli et al., 1982; Ng, 1988).

Alternative propagation systems including microtuber and clonal propagation techniques have been devised to facilitate international exchange of clean clones . Also, physiological parameters that affect tuberisation such as photoperiod and source/sink relationships which are difficult to investigate in vivo, can be more conveniently probed using microtuber systems. Media and cultural factors continue to be tested to obtain higher induction frequencies (previously c. 60%) and larger microtubers (>100 mg) capable of direct field planting.

The objective of this study was to improve levels of microtuberisation by modifying cultural parameters such as plant growth regulators, photoperiod duration and explant types in Dioscorea alata cv. Crop Lisbon.

MATERIALS AND METHODS

For shoot multiplication, four agar gel strengths (0.2, 0.4, 0.6 and 0.8%) were used as media supports in MS medium with benzylaminopurine (BAP) supplementation.

Kinetin (KIN) at 1.0, 2.5 and 5.0 μM and abscisic acid (ABA) at 1.0 μM and their combinations were used as supplements in the T medium for microtuber induction.

Eight treatments were derived by in vitro exposure of cohorts of shoot explants to short (16 h) and long (8 h) daylengths for 0, 7, 14 and 21 days and thereafter incubated under short and long daylengths, respectively to complete growth cycle.

Single-node shoots were excised from Stage II mother cultures and classed into 5 explant classes on a visually assessed state of maturity and size of leaves as follows: 1), >2cm^2), <2 cm^2 3), Expanding leaf (pinkish) 4), Unexpanded leaf and 5) Leafless nodes.

Sample size for microtuberisation treatments was 8 explants replicated three times and held under standard growth conditions for microtuber induction as previously defined by Mantell and Hugo (1988). Quantitative data were collected on microtuber weights and frequencies 10-12 weeks after incubation.

RESULTS

Supplementation of MS media witH 1.1 μM EAP increased shoot proliferation levels by C. 15 times over the control (plain MS medium). When single-node shoots were excised from cultures induced by this level of BAP, multiple shoot formation increased with BAP concentration (Cycle II, Fig. 1).

Agar concentration levels usually applied for microtuber induction (0.4%) enhanced multiple shoot formation. Combined application of 1.1 μM BAP and soft agar (0.4%) sustained higher numbers of multiple shoots than this level of agar in the absence or in the presence of pulse applications of BAP (Fig. 2).

KIN supplementation in the T medium did not significantly influence microtuber frequency levels of cultures (c. 60%). However, unlike results obtained by Mantell and Hugo (1989), there was a marked decrease in microtuber induction at the 2.5 μM KIN level (Table 1). Addition of 1.0 μM ABA depressed microtuber induction levels by as much as 15% (viz plain T medium plus ABA treatment, Table 1). There was a strong KIN effect on microtuberisation since it increased when KIN combined with the set level vf 1.0 μM ABA. This also resulted in smaller microtubers on shoots (<40 mg).

Incubation of cultures under 16 h photoperiods for 7-21 days prior to incubation under 8 h photoperiods enhanced microtuber induction frequencies dramatically (Table 2). The reverse order of photoperiod treatments had the opposite effect. Incubation of explants under continuous 8 h or 15 h photoperiods significantly depressed ($P < 0.05$) microtuber induction frequencies.

Results of the Explant type treatments suggested that the leaf sizes borne on single-node explants at incubation, influenced the levels of their responses to microtuber induction and bulking. For good results (>70%), it was preferable for explants to have fully mature green leaves (Table 3).

DISCUSSION

The results suggested that in the plain MS medium, a low agar-gel strength was appropriate for induction of multiple shoots in D. alata yams. This observation is consistent with results obtained by Peiris (1991, Pers. comm.) where liquid rather than semi-solid agar-gelled media induced higher shoot numbers in anthurium (Anthurium spp.). It must be emphasized however, that the high shoot production levels attained by continuous BAP supplementation were accompanied by formation of some fasciated and disfigured shoots which were morphologically incompetent to produce microtubers and this introduced uncertainty with regard to the genetic fidelity of any shoots subsequently produced. These were unlike shoots induced in either plain MS medium or BAP pulse treatments.

It is likely that the soft semi-solid media supplemented with 1.1 M BAP produced multiple shoots through enhanced axillary bud fecundity present in D. alata, and this increased the propensity for meristematic development as observed by Wickham et al. (1982). There were sufficient indications that the 1.1 μM BAP pulsing treatment induced sufficient numbers of normal shoots for uninterrupted large-scale microtuber production.

The results suggested that supplementation with 1-2.5 μM KIN in the T medium should enhance bulking of microtubers large enough (>100 mg) for field planting. The plain T medium would appear to produce similar effects. There would he great advantages in producing D. alata microtubers in the absence of plant growth regulators as has been the case with potato (Garner and Blake, 1989), since genetic fidelity of vegetatively produced germplasm is less likely to be impaired by factors such as somaclonal variation.

The results suggested that the 8 h photoperiod regime was essential for optimal microtuber induction levels and this condition ought to be applied after 7-21 days under 16 h photoperiods or at PNC formation which normally occurs at 28-48 days after subculturing. The beneficial effects of exposing plants to the long photoperiod regime initially were amply demonstrated. High microtuber frequencies were obtained when long days (16 h) preceded short day (8 h) treatments. This is a novel finding and has not been reported previously in the literature. Microtuber weight data suggested that microtuber weights increased as shoot cultures stayed longer on tuber induction media under the 16 h photoperiod regime. A corollary to this observation was that, even though continuous exposure of explants to 8 h photoperiods did not enhance induction frequencies, this photoperiod regime was advantageous to microtuber bulking and confirmed the need to apply both photoperiods for optimum microtuber frequencies and bulking.

There was a strong positive association between microtuber induction frequencies and shoot leaf sizes at incubation. One most desirable outcome of this experiment was that if cohorts of explants bore leaves of uniform size (of 2.0 cm^2), then 100% induction frequencies could be achieved. This suggested that variations in microtuber frequencies in previously published works were perhaps due to disparities in explant shoot leaf sizes and their relative states of maturity at the time of subculture. The results also added more credibility to the assumption that it is yam leaves of an appropriate physiological age which possibly contain the tuber-inducing factor(s).

There was a 50% reduction in the mean microtuber weights as shoot leaf size reduced from >2 cm^2 to <2 cm^2. One interpretation of this trend could be that explants with expanded mature leaves at incubation received the signal(s) for tuber induction and bulking earlier, whereas the ones that either bore smaller leaves or were leafless produced only vegetative shoots and subsequently these did not show any appreciable microtuber formation since there was a substantial delay in the attainment of physiologically mature leaves able to respond to the 8 h induction treatment.

Evidence that tuberisation is a multistage physiological process made up of several distinct phases like the ones that have been proposed for flowering by Wareing and Phillips (1986) could be indicated by the data obtained in the current study. There are likely to be three (or more) distinctive phases involved in yam tuber induction: firstly, preconditioning, in which leaf tissues have to attain a certain stage of development before they are capable of responding to a series of independent or interacting signals, secondly, elicitation, the process whereby environmental and physiological signals of tuberisation are perceived and translated into active tuberisation responses and thirdly, tuber bulking when following tuber induction (PNC induction in yam), there is a need for a continuing source/sink interactions whereby photosynthates or sucrose (provided by an artificial culture medium) are accumulated in axillary meristems to produce sizeable microtubers. These three stages were likely to be influenced independently or interactively, by explant leaf sizes (Stage 1), photoperiods (Stage 2) and sugar levels/plant growth regulators (Stage 3).

In conclusion, this study has provided evidence that D. alata shoot multiplication can be increased by pulse treatments of 1.1 μM BAP applied in alternating 4-6 week subculture cycles. Explants for microtuber induction should bear mature green leaves (2 cm^2) and given a dual photoperiod exposure tc achieve both increased microtuber frequencies and weights.

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Table 1. Effect of KIN ± ABA in T medium on microtuber frequency and weight.

Treatment (μM KIN)	MTF T-ABA	T+ABA	MTW T-ABA	T+ABA
0.0	63.9	32.3	309	48.1
1.0	67.5	46.4	215	58.4
2.5	35.7	50.0	190	34.3
5.0	60.4	45.0	164	31.1
LSD @ 5%	16.6	24.5	135.9	6.3
CV (%)	25.2	42.0	6.2	10.8

MTF = Microtuber frequency (%)
MTW = Microtuber weight (mg/culture batch).

Table 2. Effect of photoperiod duration on microtuber frequency and weight.

Treatment	Microtuber Frequency(%)	Weight (mg)
1 (8h, 7d, 16h)	0.0	0.0
2 (8h, 14d, 16h)	2.7	42.0
3 (8h, 21d, 16h)	4.3	42.0
4 (8h continous)	21.0	79.0
5 (16h, 7d, 8h)	67.0	71.0
6 (16h, 14d, 8h)	65.7	112.0
7 (16h, 21d, 8h)	62.7	84.0
8 (16 h continous)	4.3	115.0
LSD @ 5% (*)	22.7	-
CV (%)	49.6	-

(*) Analysis based on arc-sine transformed original data.

Table 3. Influence of shoot leaf size on microtuber frequency and weight.

Explant Class	Microtuber Frequency (%)	Microtuber Weight (mg)
1	87.7	109
2	67.0	52
3	12.7	89
4	21.0	34
5	12.7	7
LSD @ %% (*)	30.2	-
CV (%)	46.0	-

(*) Analysis based on arc-sine transformed original data.

FIG.1: Effect of BAP on shoot proliferation.

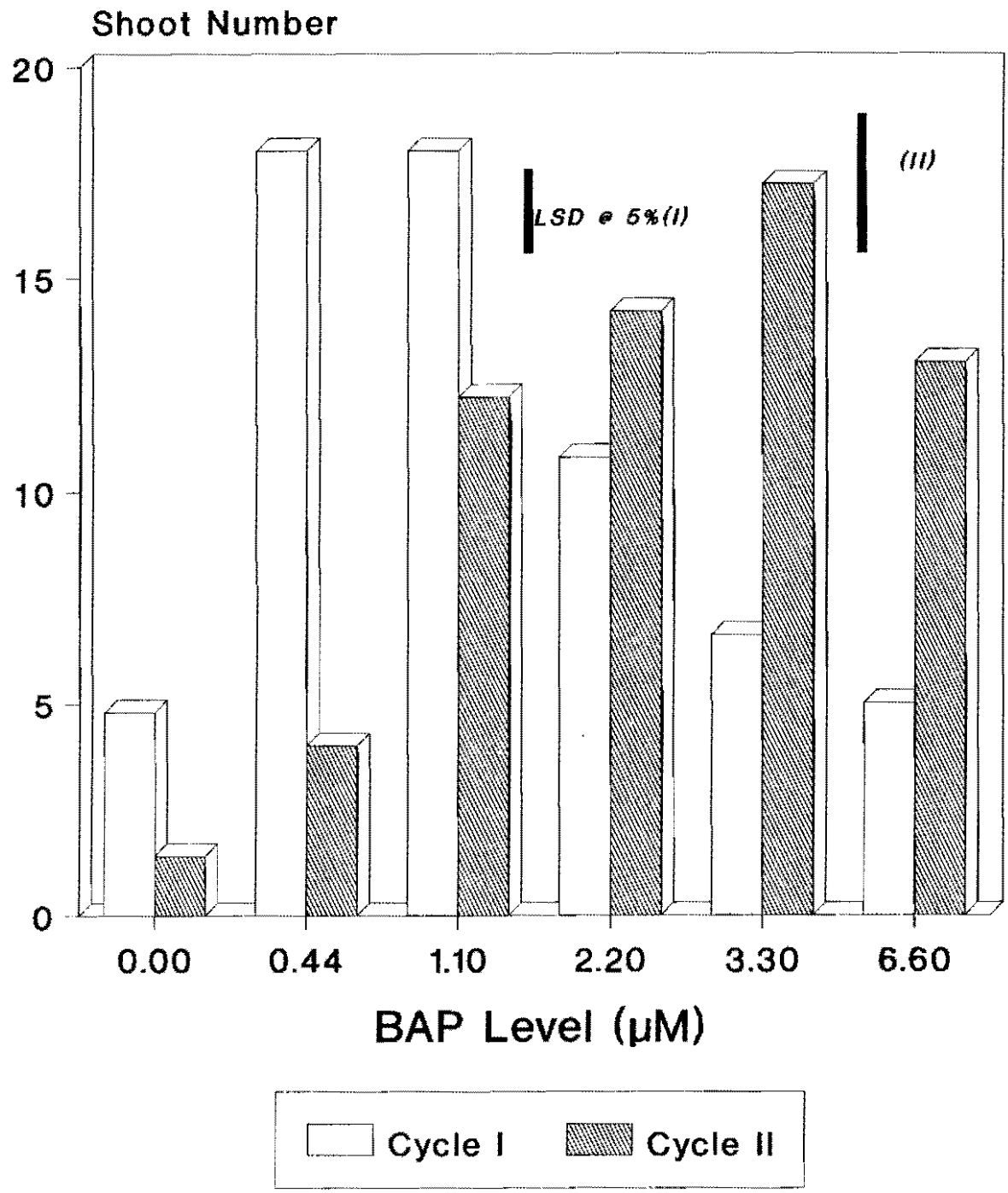
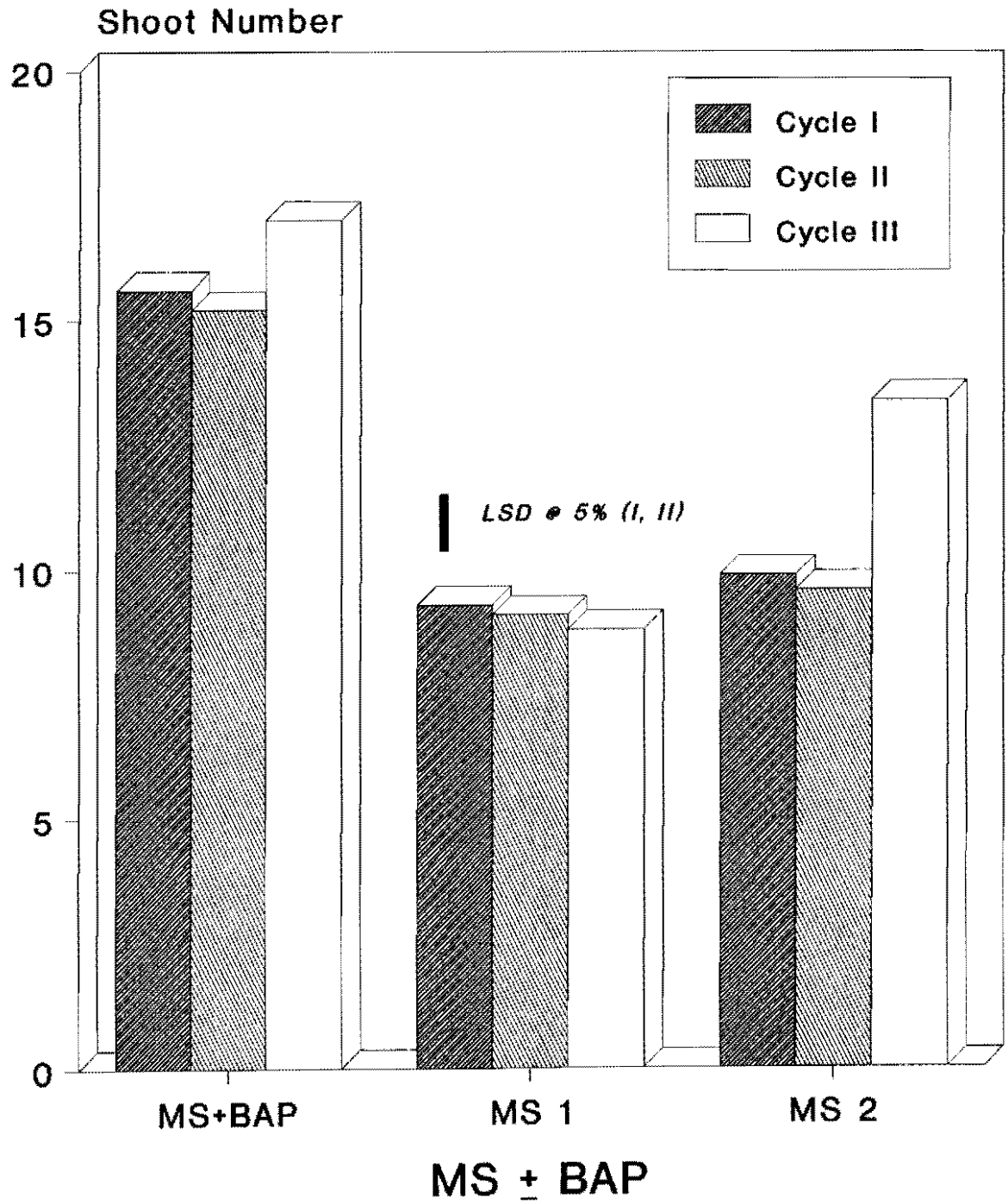


FIG. 2: Combined effect of Soft Agar and BAP treatments on number of shoots.



MAINTENANCE OF A CASSAVA GERMPLASM COLLECTION IN THE SOUTH PACIFIC

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Three Fijian cultivars of cassava, (*Manihot esculenta* Crantz) were examined for their responses to the presence of the osmoticums, mannitol and sorbitol, in combination and in isolation, in the culture medium. The highest concentration of mannitol and sorbitol, (1% mannitol + 1% sorbitol) utilised in the experiment resulted in the greatest reduction in growth; plant height was reduced by 61% compared to plants cultured on osmoticum-free medium. Low levels of sorbitol, (0.5%), improved the growth of the plants; plants had shorter internodes, thicker stems and a lower rate of senescence. Recover from the lag phase resulting from long-term culture, (12 months), of cassava on mannitol - containing medium was optimised when the medium contained 4% sucrose.

INTRODUCTION

The cassava germplasm collection for the South Pacific Island countries is held at the IRETA Tissue Culture Unit, University of the South Pacific, Western Samoa. The collection consists of 23 cassava accessions, comprising of Fijian varieties and material imported from CIAT for its resistance to cassava bacterial blight. The IRETA Tissue Culture Unit also holds taro, sweet potato, yam, banana and vanilla, some 300 accessions in all, the majority of which are taro and sweet potato. The subculturing requirement for maintenance of this collection places a demand on labour input, consequently techniques with which to reduce this labour input are under investigation for all crops.

Slow growth of plant tissue cultures can be induced by several methods, such as reduced temperatures or the presence of growth regulators in the culture medium (Withers, 1988). Several of these methods have already been examined with cassava. At CIAT, reduction in storage temperature from 28°-30° to 22°C decreased the rate of shoot elongation by approximately 20%. A 5-25mm (1% to 5%) concentration of mannitol in the medium effectively arrested growth, however, at the lowest storage temperature, tissue viability is affected. Tissue culture viability was significantly increased by the addition of 0.088m and 0.18m sucrose at both low and high storage temperatures (Roca, WM et al., 1989). At the IRETA Tissue Culture Unit initial work on storage of cassava has concentrated on the use of osmoticums in the culture medium.

MATERIAL AND METHODS

Plant Material

All experimental material was derived from tissue culture plantlets of three Fijian varieties, Merelesita, Vulatolu and Yabia Damu, maintained on Murashige and Skoog

Plant Salt Medium, (Murashige, T., *et al*, 1962) supplemented with 0.088m sucrose, (3%), 100 mg/l inositol and 0.4 mg/l thiamine hydrochloride.

Slow Growth Treatments

The basic medium was Murashige and Skoog (1962) Plant Salt Medium supplemented with 3% sucrose to which three different concentrations (0.05%, 1.0%) of both sorbitol and mannitol were added to provide nine treatment media (see Table 1). Nodal explants (up to and including the third node from the apical bud), were excised from the source plant material and cultured on treatment medium in test tubes approximately 55cm in size and containing 10cm of medium. Plant growth was assessed at monthly intervals by the measurement of plant height and number of nodes. A final assessment was made after six months of growth in culture.

Lag Phase Recovery

The basic culture medium used was as described for the slow growth treatments to which different concentrations of sucrose were added, 2%, 3% and 4%. Nodal explants for this experiment were obtained from the same material as used for the slow growth treatment. Culture tubes and method of assessment was as described for the slow growth experiment.

Experimental Conditions

With all media, pH was adjusted to 5.6, and Gelrite was used as the gelling agent. All cultures were incubated at 25°C +/-1°C under a 16h daylength with a light intensity of 46μmol $m^{-2}s^{-1}$ PAR. The number of replicates per treatment for all treatments was eight.

RESULTS

Slow Growth Treatments

As the percentage of mannitol and sorbitol in the medium increases, plant growth with respect to height decreases (Fig. 1). Explants cultured on medium containing the highest concentration of sorbitol and mannitol (1.0%M + 1.0%S) had the lowest mean plant height of all treatments; those explants cultured on a medium lacking in osmoticums had the highest mean plant height. Where node number was the parameter assessed, very little difference was observed between the treatments (Fig. 2). However, it was noted that mannitol induced a more distinct response than sorbitol. Analysis of variance showed the treatments to be significantly different at the 1% level of probability.

No abnormality in growth was observed after six months of culture. Leaf senescence occurred with all plants. Treatments containing sorbitol in the medium resulted in sturdier plants. Plants cultured on medium containing 0.5% sorbitol, and on

medium containing no osmoticums, showed the least senescence, with plants cultured on medium containing 0.5% sorbitol having thicker stems, and the greater number of green leaves on the oldest growth.

Lag Phase Recovery

The results from this experiment indicated that cassava plants overcome the lag phase often observed after long-term culture on mannitol-containing medium, if subcultured onto Murashige and Skoog Plant Salt Medium containing 4% sucrose. As Figs. 3 and 4 show, culture medium containing 4% sucrose induces a faster growth rate in terms of plant height and number of nodes, than culture on media containing 2% or 3% sucrose.

CONCLUSION

Slow Growth Treatments

As the results show, the response of cassava to culture on sorbitol-and/or mannitol containing media was one of a reduction in growth rate. The use of these osmoticums allowed the culture of cassava for periods of six to nine months. After this time, with all treatments, the plants had attained the full height of the culture containers. It is likely that a greater reduction in growth rate could have been achieved if higher concentrations of the osmoticums had been used. Work done by Henshaw et al, (1980), showed that the period of storage can be extended to approximately 57 months when abscisic acid (10mg1), sucrose (up to 8%) and/or mannitol (3 to 6%) is incorporated into the medium. In this experiment higher levels of the osmoticums were not used as past experience in the IRETA Tissue Culture Unit has shown that the use of 2% mannitol in the medium results in the loss of viability of cassava plants, particularly with repeated subculturing.

Lag Phase Recovery

The results did not reveal much variation between the 2% and 3% sucrose treatments, but it would seem that the presence of 4% sucrose in the medium optimises recovery of cassava plants from culture on mannitol-containing media. This response was observed by Taylor (pers. comm.) with Solarium tuberosum spp tuberosum. The incorporation of 4% sucrose in medium containing 0.01 mg1 6-benzylaminopurine increased the viability of cassava cultures stored at 20° to 22°C to 90% from 52%, when the medium contained 2% sucrose (Roca, 1982). The effect on the final osmotic potential of the medium must be similar to that achieved by including 0.5% sorbitol in the medium, as the improvement in growth obtained with both these media was of the same order.

ACKNOWLEDGMENTS

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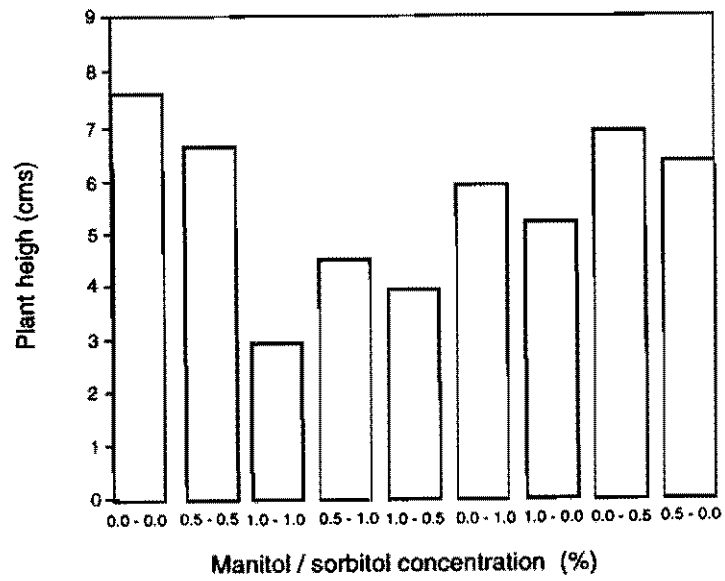


Fig. 1. The effect of mannitol and sorbitol in the culture medium on the growth of cassava in vitro.
a: plant height.

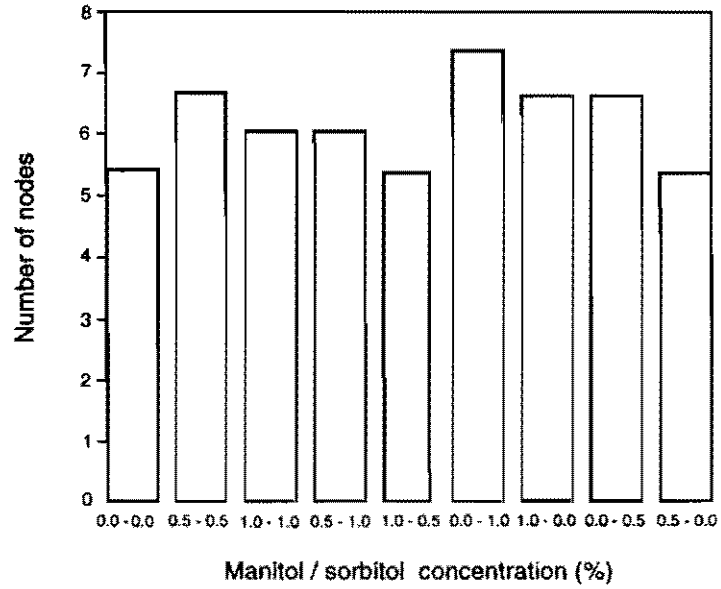


Fig. 2. the effect of mannitol and sorbitol in the culture medium on the growth of cassava in vitro.
b: number of nodes.

Fig. 3 Plant height

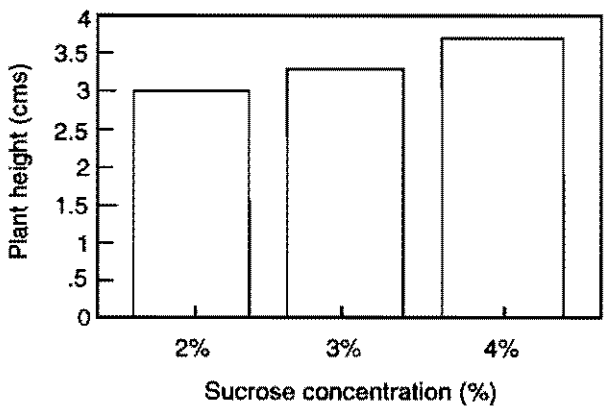
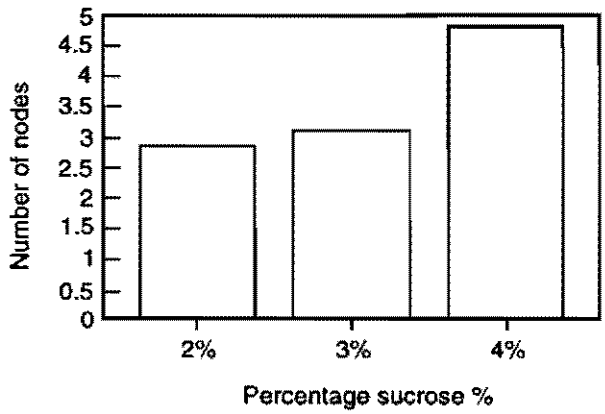


Fig. 4 Number of nodes



IN VITRO MANAGEMENT OF CASSAVA GERMPLASM AT CIAT

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The cassava germplasm collection held at CIAT, which is the world largest, is conserved both in the field and as an in vitro collection. The field collection now has 5035 clones, more than 95% of which is conserved in the in vitro collection. CIAT has established a tissue culture laboratory (TCL) in 1979 for in vitro cassava germplasm management, particularly in three species: (1) germplasm conservation, (2) production of disease free clones in collaboration with CIAT's Virology Research Unit (VRU) and (3) germplasm exchange. Cassava clones in the in vitro gene bank are conserved under slow growth conditions. The average period between subcultures was 12.8 months, with a range of 12.1 to 19.5 months. Some differences in subculture period according to country of origin were found. Since the initiation of the TCL, pathogen (especially virus) elimination through meristem culture, combined with thermotherapy, has been an important activity. A total of 958 and 870 clones has been cleaned and indexed by using diagnostic techniques (ELISA) for CCMV and CsXV, respectively, and 145 clones have been indexed for frog skin disease (FSD) by grafting on to an indicator clone ("Secundina"), while another 167 clones are being indexed for this disease. During the period 1979-1991, 3070 clones have been shipped in vitro to 43 countries in answer to 205 germplasm requests. The active germplasm distribution in throughout 13 years demonstrates the scale and efficiency of the in vitro germplasm exchange system which has been developed in collaboration with national and international agencies. The wild relatives of cassava are receiving increasing attention for their potential as sources of useful traits for cassava improvement. A total of 397 genotypes of 29 wild Manihot species are being maintained in vitro.

A study was initiated in 1990 to develop appropriate methods for improved management of micropropagation and conservation of wild Manihot species. Out of 29 species tested, 24 were found to respond better in culture media different from the present one (CEC). Using improved media, 178 genotypes of 29 wild species were successfully transferred to soil in a screenhouse for use in field gene bank and isozyme analysis. In vitro methods have played a pivotal role of CIAT's efforts for cassava germplasm conservation and exchange.

INTRODUCTION

Sustained progress in plant breeding requires a broad genetic base. A rich and diverse germplasm collection is the backbone of every successful crop improvement program. Important traits should be incorporated into new cultivars to solve production and use constraints so that new cultivars, which are the final products of breeding efforts, can successfully penetrate specific targeted markets. Genetic diversity becomes more important as cropping intensity and monoculture continue to increase in all major crop-producing regions of the world.

Within the Consultive Group of International Agriculture Research (CGIAR) system, CIAT has accepted global responsibility for conserving cassava germplasm. This implies an obligation to provide a high level of security for conservation,

complete characterization and evaluation, thorough documentation, and a system for effective pathogen-free exchange of germplasm.

In most crop species, true seeds are used as a means of germplasm conservation. Cassava, however, is a highly heterozygous, clonally propagated crop and thus, true seeds cannot be used for conservation of cassava clonal accessions.

CIAT maintains the cassava collection in both in-vitro and field gene banks. The field gene bank offers easy availability of plant material for evaluation and crossing work. As a valuable, complementary method of backing up field cassava germplasm conservation, in-vitro conservation provides a means of maintaining a large collection in a small space, free of pests and diseases, and without risk of loss from climatic or soil stresses. Cassava pathogen-tested clones are conserved in vitro to allow the propagation of disease-free plants at any time.

IN VITRO GERMPLASM CONVERVATION

The in vitro gene bank now conserves 4788 clones from 23 countries (Table 1). This is equivalent of 95% of the total number of accessions maintained in the field. The transfer of field-grown plants into the in vitro system is almost completed. Further growth in the number of accessions maintained in vitro will result from introduction of new accessions from other countries. (CIAT, 1991).

Cassava clones in the in vitro gene bank are conserved under the following slow-growth conditions: (1) constant temperature of 23-25 °C maintained day (12 h) and night (12 h); (2) 1000-1500 lux illumination; (3) a slightly modified Murashige-Skoog (MS) culture medium; (4) test tubes, 20 x 150 mm, capped with aluminum foil; and (5) 3 to 5 test tubes per clone. (Roca, et al 1982).

Under these conditions, in vitro plantlets need to be subcultured every 12 to 18 months, depending on genotype. In collaboration with CIAT's Data Services Unit, the GRU has analyzed frequency of subculture for 3536 clones since initiating the in vitro conservation work. The average period between subcultures was 12.8 months, with a wide range of 12.1 to 19.5 months. Some differences in subculture period according to country of origin were found (Table 1). It was interesting to note that cassava clones from Nigeria, Fiji, and Thailand, which represent secondary centers of cassava genetic diversity, occupied the four longest periods between subcultures. This may have been because these material arrived at CIAT relatively recently when the conservation methodology had already was improved significantly.

The present status of the wild *Manihot* species collection is given in Table 2. The table present also recommended culture medium for each species based on a student thesis study at CIAT. The collection is small, considering more than 100 wild *Manihot* species reported. Nevertheless, it represents the starting point of a long-term strategy for exploiting these potentially valuable genetic resources. Crossability studies, preliminary evaluation, and biochemical characterization have begun.

Further acquisition of wild germplasm through collaboration with national programs is a high priority (Baca, A.E., 1991).

PATHOGEN ELIMINATION

To ensure disease-free in vitro plantlets, a disease elimination technique was developed by the Biotechnology Research Unit of CIAT for cleaning pathogens from clones. This consists of cultiring small (0.2 - 0.3 mm) meristem tips from apical buds of newly sprouted shoots at 40°C day and 35°C night temperatures during three to four weeks. In cases where no stakes are available, i.e., germplasm was introduced to CIAT as in vitro plantlets, then in vitro thermotherapy is applied. Rates of virus elimination depends, to a large extent, on the size of the explant used for culture and on whether thermoterapy was applied or not, as well as on the virus strain concerned (CIAT, 1982).

The disease-free status of the plants is validated through appropiate indexing. viruses and virus-like pathogens of major concern for elimination at CIAT in the past year have been the cassava common mosaic virus (CCMV), cassava Colombian Symptomless Virus (CCSV), cassava virus X (CsXv) and Frog Skin disease (FSD).

A total of 958 and 870 clones have been cleaned and indexed by using diagnostic techniques (Elisa) for CCMV and CSXV, respectively, and 312 clones have been indexed for frog skin disease (FSD) by grafting on to an indicator clone ("secundina").

The purpose of this "clean up" is provide healthy material for (1) distribution of elite clones (highly selected breeding lines) and specific bank germplasm to national programs, (2) rescue of field-grown clones damaged by severe disease or pest attack, (3) provision of clean seed stock for Colombial regional variety trials, and (4) return of clean germplasm to its country of origin.

INTERNATIONAL GERMPLASM EXCHANGE

The movement of plants from one country or region to another plays an important role in the transfer of technology carried out by national and international agricultural organizations (CIAT, 1982). (Table 3)

The overal transfer process may comprise the following steps:

1. Production and multiplication of disease-free clones.

2. Preparation of cultures for distribution.

3. Evaluation, packing and shipment of cultures.

4. Handling of cultures at the receiving end.

5. Release of materials.

During the period 1979 - 1991, 3070 clones have been sent to 43 countries in answer to 205 requests. The active germplasm distribution in throughout 13 years demostrates the scale and efficiency of the in vitro germplasm exchange system which has been developed in collaboration with national and international agencies.

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Table 1. Number of cassava clones by source country maintained in vitro banks as of September, 1991 and range of subculture periods during storage.

Country of Source	In vitro	Average Period (month)
Argentina	16	12.8
Bolivia	3	13.1
Brazil	1087	12.9
Colombia	1895	12.1
China	2	-
Costa Rica	148	13.0
Cuba	74	14.1
Dominican Republic	5	14.0
Ecuador	116	13.8
Fiji	6	15.1
Guatemala	91	12.7
Indonesia	51	13.5
Malaysia	67	12.9
Mexico	100	13.4
Nigeria	19	19.5
Panama	41	12.1
Paraguay	193	14.3
Peru	405	12.8
Philippines	6	14.9
Puerto Rico	7	12.7
Thailand	21	15.9
USA	10	13.5
Venezuela	111	12.4
Ciat hybrids	314	13.5
Wild Species	29	-
Total	4817	

Table 2. Recommended culture medium based on response of wild *Manihot* to in vitro culture in five culture media.

Species	Culture medium recommended[a]
M. *aesculifolia*	CEC
M. *alutacea*	CC
M. *anomala*	CC
M. *caerulescens*	CEC
M. *carthaginensis*	4EM
M. *cecropiaefolia*	CEC
M. *chlorosticta*	GA
M. *epruinosa*	GA
M. *filamentosa*	CEC
M. *flabellifolia*	CC
M. *fruticulosa*	GA
M. *glaziovii*	CEC
M. *guaranítica*	CC
M. *hastatiloba*	MEC
M. *irwinii*	CC
M. *jacobinensis*	CC
M. *longipetiolata*	CEC
M. *orbicularis*	CEC
M. *peltata*	CC
M. *pentaphylla*	CEC
M. *pilosa*	CC
M. *pseudoglaziovii*	CC
M. *purpureo-costata*	MEC
M. *rubricaulis*	CC
M. *sparsifolia*	GA
M. *triphylla*	MEC
M. *tristis*	MEC
M. *violacea*	4EM
167.1-71323	CC
595.1-75698	MEC
604.7-75663	MEC
666.10-7400	MEC

a. CC = ½ MS + 4% S + 0.25 mg/l IBA + 0.2% CA
CEC= ½ MS + 4% S + 0.25 mg/l IBA
4EM= ½ MS + 2% S + 0.03 mg/l BAP + O.05 mg/l GA3 + 0.017 mg/l ANA + 1.0 mg/l NIC + 1.0 mg/l PRI.
GA = ½ MS + 4% S + 0.03 mg/l BAP + 0.05 mg/l GA3 + 0.20 mg/l IBA + 1.0 mg/l NIC + 1.0 mg/l PRI + 0.2% CA
MEC = ½ MS + 3% S + 0.03 mg/l BAP + 0.05 mg/l GA3 + 0.20 mg/l IBA + 1.0 mg/l NIC + 1.0 mg/l PRI.

Table 3. Total cassava germplasm distributed from, or introduced to CIAT using in vitro techniques.

REGION	DISTRIBUTED FROM CIAT		INTRODUCED TO CIAT	
	No. Countries	No. Clones	No. Countries	No. Clones
America				
South	7	1308	5	1681
Central	7	349	4	282
North	2	147	1	10
Caribbean	5	632	-	-
Asia	9	411	4	142
Africa	3	83	1	19
Europa	6	106	-	-
Oceania	3	34	1	6
Total	42	3070	16	2140

SOMATIC EMBRYOGENESIS OF ARGENTINE CASSAVA VARIETIES

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INTRODUCTION

Since the first reports in tissue culture of carrot (Steward, 1985; Reinert, 1958, 1959), plant regeneration through somatic embryogenesis has been accomplished in various plant species (Williams and Maheshwari, 1986).

In cassava, somatic embryos and plant regeneration have been induced by in vitro culture of cotyledons, embryonic axes of seeds, as will as by culture of leaf lobes of in vitro cloned plants (STAMP and HENSHAW, 1982, 1987; SZABADOS et al, 1987).

In this investigation, the effects of two media and three physical conditions have been tested on leaflet cultures of 13 varieties of cassava.

MATERIALS AND METHODS

Plants of cassava (Manihot esculenta Crantz) were used in this investigation. Twelve Argentine varieties (Palomita, Carapé, 76, Cati-Guá, Cambí, Misionera, Blanca, Amarilla, Azul Misionera, 256, Caroba, Pombero), as will as a Colombian variety (Col. 1505), kindly supplied by CIAT, were employed as source of explants. All plant materials were grown either in the field or in the greenhouse of the Instituto de Botánica del Nordeste (Corrientes, Argentina). The experiments were done during both spring and summer seasons of the years 1990 and 1991.

Immature leaflets (4 mm in length) isolated from either aseptically grown plants or from greenhouse-grown plants were incubated. Aseptic plants were obtained by in vitro shoot apical culture on standard propagation medium, which is a modification of the medium devised by KARTHA et al (1974): MURASHIGE and SKOOG (MS) basal medium (1962), supplemented (BAP), 0.02 mg/l of Naphthaleneacetic acid (NAA) and 1 mg/l of Gibberellic acid (AG_3).

The medium was solidified with 0.5 % Sigma Agar. Cultures were grown under a 14 hr. photoperiod (3 W/m^2) at 27 ± 2°C. The plants were propagated by nodal cuttings every month.

The procedure employed for the induction of somatic embryogenesis was described by STAMP and HENSHAW (1982, 1987) and by SZABADOS et al (1987). Immature leaflets were surface sterilized in 70 % ethanol for 1 min followed by immersion in 0.16 % sodium hypochlorite for 5 min, and subsequently rinsed three times with sterile distilled water.

The nutrient medium consisted of either 1) major and minor salts, as well as vitamins according to MS medium or 2) salts of MS medium and vitamins as in B5 medium (GAMBORG et al, 1968). Both media were supplemented with 3 % sucrose and 8 mg/l of 2,4- dichlorophenoxyacetic acid (2,4-D). The pH of the medium was adjusted to 5.8 with KOH or HCl prior to adding the agar (0.4 %). Test tubes (containing 5 ml of medium) were autoclaved at 1 atm during 20 min.

The environmental conditions for leaflet cultures were a constant temperature of 27 ± 2 °C, 14 hr photoperiod (3 W/m^2 or 10 W/m^2) as well as darkness.

RESULTS

After 30 days of culture, callus formation occurred in all of the varieties tested but the percentage of the leaflets which produced calli was a function of the composition of the medium and physical conditions of the incubation, the variety, as well as the growth conditions of the plants (Figs. 1 and 2). In general, the highest light intensity promoted callus formation in explants isolated from in vitro plants. However, darkness was more effective in producing callus from leaflets of greenhouse-grown plants. (Figs. 1 and 2). Although somatic embryogenesis was found to occur in 4 out of 13 varieties tested, in only one (**Col. 1505**) have the embryos germinated and whole plant regeneration been achieved (Table 1).

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TABLE 1: PRODUCTION OF SOMATIC EMBRYOS AND PLANTS IN CALLI OF THIRTEEN VARIETIES OF CASSAVA

VARIETIES	SOMATIC EMBRYOS	PLANTS	VARIETIES	SOMATIC EMBRYOS	PLANTS
1505	+ +	+	Cambí	—	—
Palomita	+	—	Azul misio-	—	—
76	+	—	nera		
Carapé	—	—	Caroba	—	—
Cati-Guá	—	—	Blanca	—	—
Pombero	—	—	Amarilla	—	—
Misionera	—	—	256	—	—

References: + + : Production of embryos in more than 80 % of the calli.
+ : Production of either embryos or plants in 50 - 80 % of the calli.

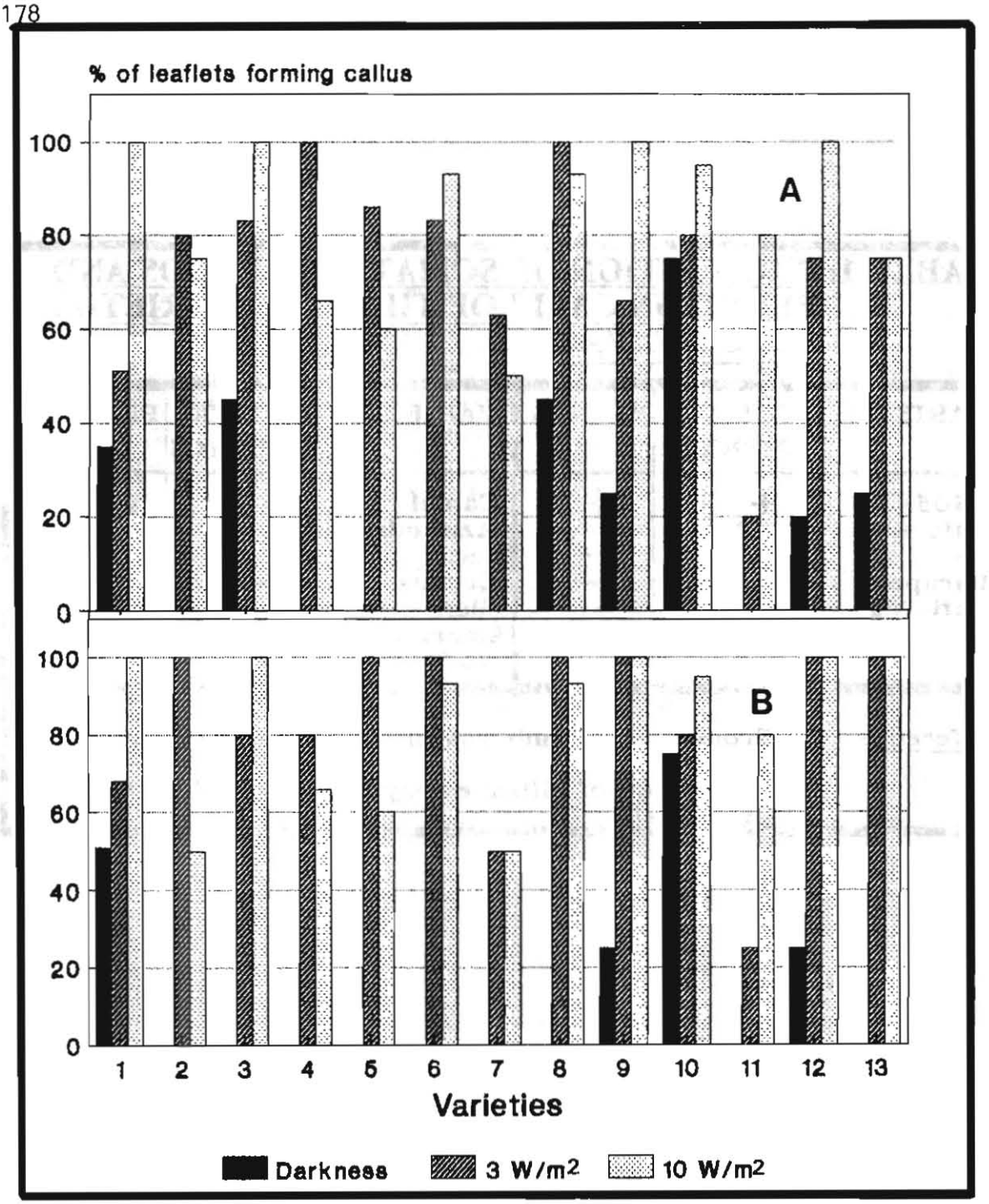

Fig. 1: EFFECTS OF THREE ENVIRONMENTAL CONDITIONS ON THE CALLI INDUCTION OF THIRTEEN CASSAVA VARIETIES BY *IN VITRO* CULTURE OF LEAFLETS ISOLATED FROM *IN VITRO*-GROWN PLANTS:
A: MS + 8 mg/l 2,4-D
B: MS (B5 Vit.) + 8 mg/l 2,4-D

REFERENCES:
1- 1505
2- Cati-guá
3- Palomita
4- Pombero
5- Misionera
6- 76
7- Caroba
8- Cambí
9- Azul Misionera
10- Carapé
11- Blanca
12- Amarilla
13- 256

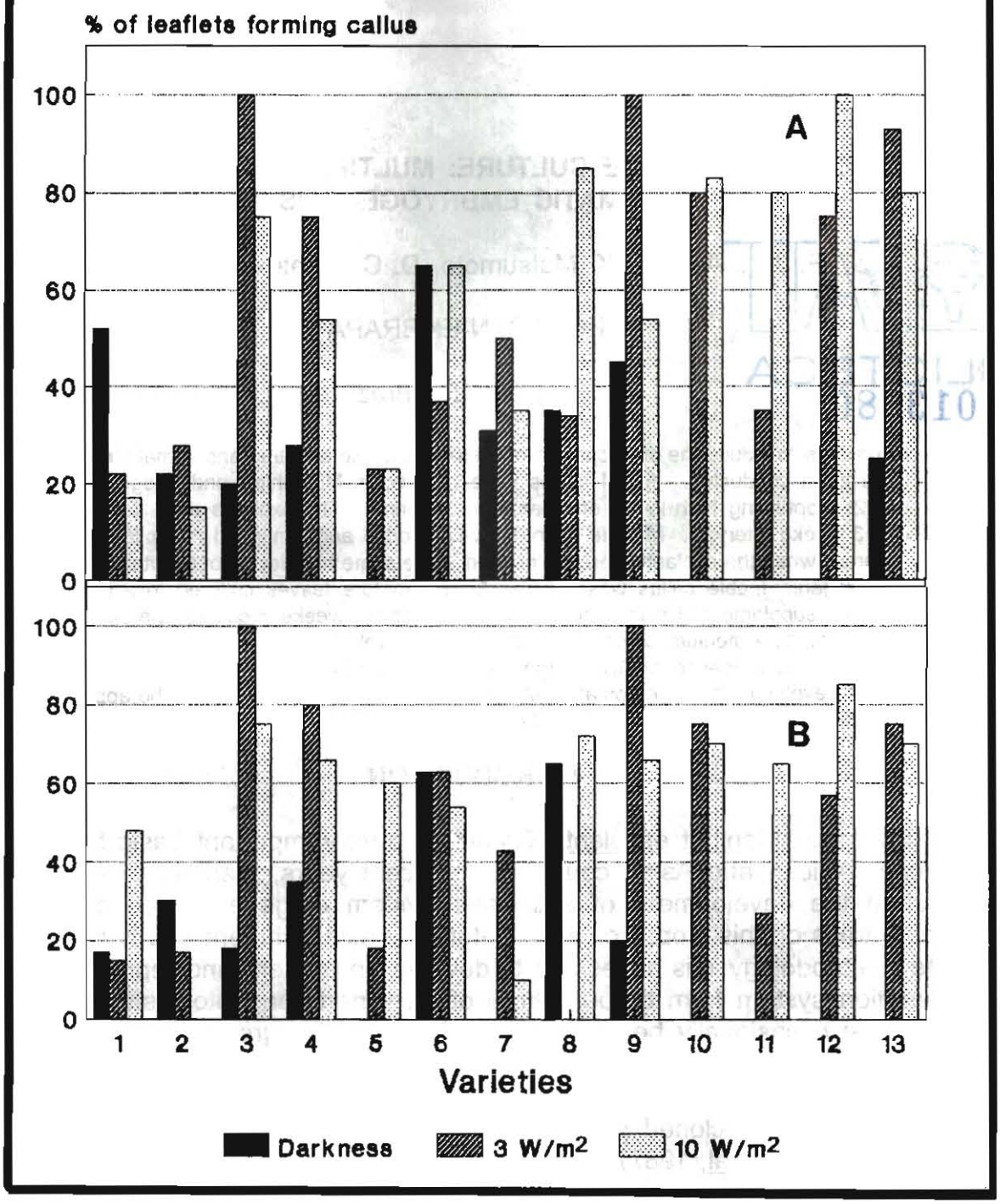

Fig. 2: EFFECTS OF THREE ENVIRONMENTAL CONDITIONS ON THE CALLI INDUCTION OF THIRTEEN CASSAVA VARIETIES BY *IN VITRO* CULTURE OF LEAFLETS ISOLATED FROM GREENHOUSE-GROWN PLANTS:
A: MS + 8 mg/l 2,4-D
B: MS (B5 Vit.) + 8 mg/l 2,4-D

REFERENCES:

1- 1505	5- Misionera	9- Azul Misionera
2- Cati-guá	6- 76	10- Carapé
3- Palomita	7- Caroba	11- Blanca
4- Pombero	8- Cambí	12- Amarilla
		13- 256

CASSAVA TISSUE CULTURE: MULTIPLE SHOOTS AND SOMATIC EMBRYOGENESIS

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Conditions to induce the development of cassava multiple shooting and somatic embryos have been conducted. Apical nodes were cultured in Murashige and Skogg medium (1962), containing high levels of 6-benzylaminopurine. The explants were subcultured at 2-3 weekd intervals. Multiple shooting was induced and continued multiplication has occurred when the explants were maintained in the same high levels of growth regulator. Embryogenic friable callus was induced from immature leaves cultured in a modified medium, supplemented with 10 mg.L-1 2,4-D. After two weeks in culture, the callus was transferred to a medium containing a lower auxin level, 0.1 mg.L-1 2,4-D and 1 mg.L-1 of GA3. The presence of GA3 in the culture medium was an important factor for the further development of the somatic embryos. The results obtained should be applicable in studies of cassava genetic manipulation.

INTRODUCTION

Cassava (Manihot esculenta Crantz) is a very important basic food in Latin American, African and Asian countries. In recent years, there has been growing interest in the development of a transfer system of genes which could confer desirable traits on this crop. However, before the establishment of any plant genetic transfer methodology it is necessary to develop an efficient and reproducible plant regeneration system from tissue culture of plant parts or protoplasts. Leaves and shoots have occasionally been regenerated from callus grown from stem sections (Tilquin, 1979), or from calli developed from leaf mesophyll protoplasts (Shahin & Shepard, 1980). Somatic embryos have been induced on cotyledons and on young leaf lobes of in vitro cloned plants (Stamp, 1987; Stamp & Henshaw, 1987a and 1987b; Szabados et al, 1987).

The aim of this work is the study of the morphogenetic behaviour of nodal stem segments and the improvement of a plant regeneration system through somatic embryogenesis from immature cassava leaves.

MATERIALS AND METHODS

Nodal segments.

Cassava plantlets (var MCOL 22) cultivated on a modified MS medium (Murashige and Skoog, 1962) containing 3% sucrose, were used as an explant source. Nodal stem segments were excised and inoculated on MS medium containing different combinations of NAA (0-0.01-0.03-0.1-0.3 ml/l) and BAP (0-0.1-0.3-1.0-3.0 mg/l). The explants were grown at a constant temperature of 28°C under

a 14 hours photoperiod (2000 lux). Multiple shoots were then transferred to MS medium containing 2% sucrose and 0.2% active charcoal to inducing rooting.

Immature leaves.

Cassava immature leaves (3-5 mm) were excised from in vitro plantlets and inoculated on MS medium containing different concentrations of 2,4 D (0-1.0-10.0-15.0-20.0 mg/l) to induce somatic embryogenesis. After 15 days of cultivation, embryogenic calli were transferred to MS medium supplemented with different combinations of 2,4 D (0-0.01-0.1 mg/l), BAP (0-0.1-1.0 mg/l) and GA_3 (0-1.0-5.0 mg/l). In these two cultivation stages, the explants were incubated at 28 ± 2°C in a 12 hours photoperiod (3000 lux).

RESULTS AND DISCUSSION

Nodal segments

Nodal segments were cultured on MS medium under different hormonal conditions. Plantlets with a tuberized root were observed in segments cultured on medium supplemented with 0,1 mg/l of BAP and NAA (Fig. 1A). Multiple shoots were observed from a single node when the nodal segments were cultured in presence of 3 mg/l of BAP (Fig. 1A and 1B). When these shoots were isolated and subcultured in the same medium, formation of multiple buds was observed in the basal leaves and the calli formed in the nodal segments (Fig. 1C). The buds were transferred to medium without growth regulators, where they formed new shoots (Fig. 1B and 1D). These shoots eventually formed roots and turned into normal plants.

Immature leaves

Immature leaves were cultivated on medium containing different concentrations of 2,4D. In all of them, intumescence of the tissues and formation of embryogenic nodular tissues (Szabados et al, 1987), and calli were observed after 2 weeks of culture (First culture stage). Embryogenic calli were transferred to medium containing different combinations of GA_3, BAP and 2,4D (Second culture stage). The highest somatic embryo formation was observed when immature leaves were cultured on medium containing 10 mg/l 2,4D in the first culture stage and in the presence of 1 mg/l GA_3 plus 0,1 mg/l BAP in the second culture stage (Fig. 2A).

While light did not affect the embryogenic induction process, GA_3 was very important for elongation of the embryos. Somatic embryos were excised from the calli and kept on medium containing the best hormonal conditions of the second culture stage, where secondary embryogenesis was obtained (Fig. 2B). (Stamp & Henshaw, 1987). Under these conditions, the embryos regenerated plants that were transferred to medium without growth regulators for root inducing. Our results indicate that plant regeneration of cassava is possible, using the previously described

procedure of somatic embryogenesis (Szabados et al, 1987) with some modifications.

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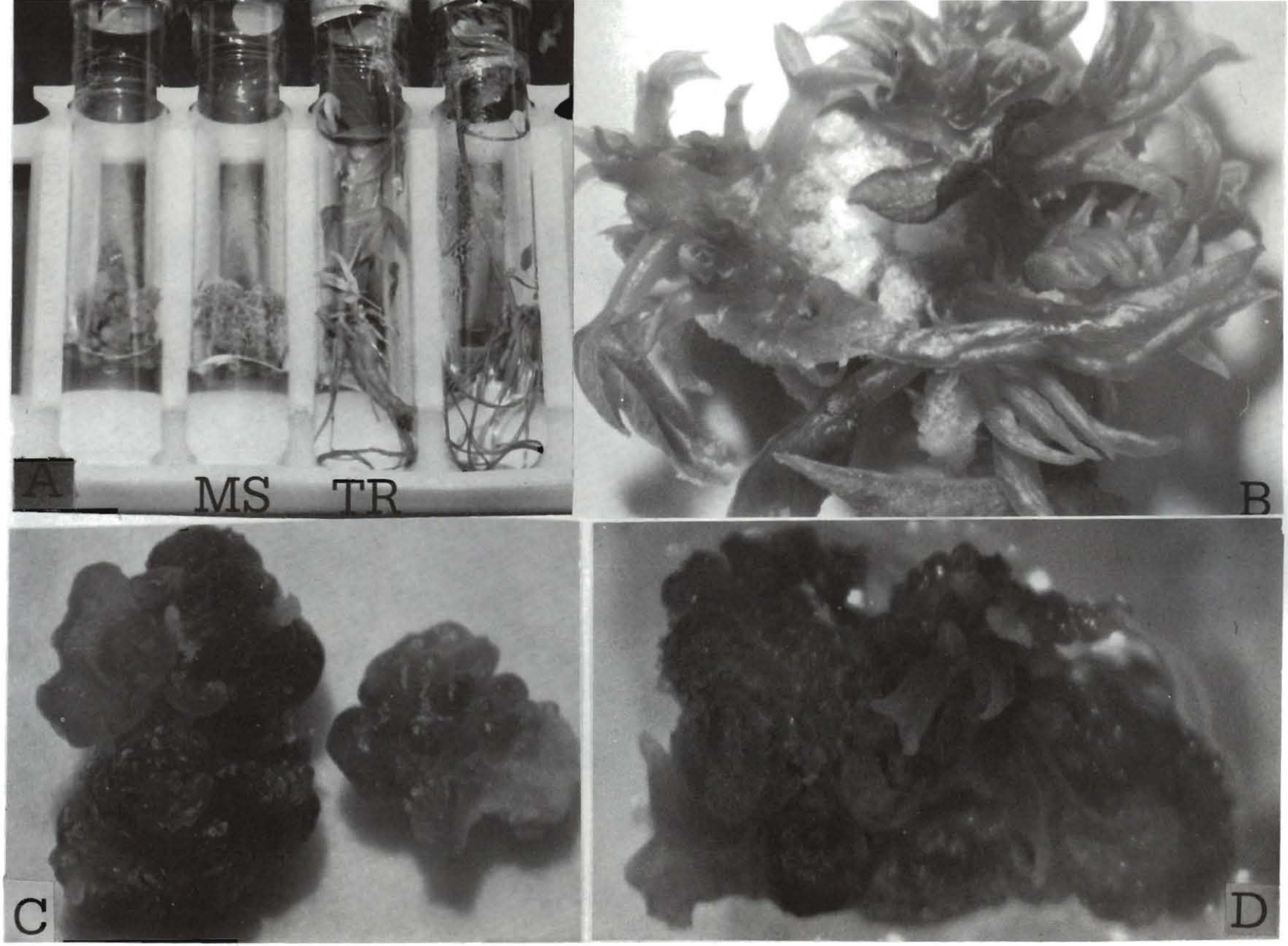

Figure. 1: A) Multiple shoots induced of nodal segment C) Multiple shoots (detail) (10x)
B) Plantlet with a tuberized root D) Multiple buds developed in basal leaves from shoots (15x).

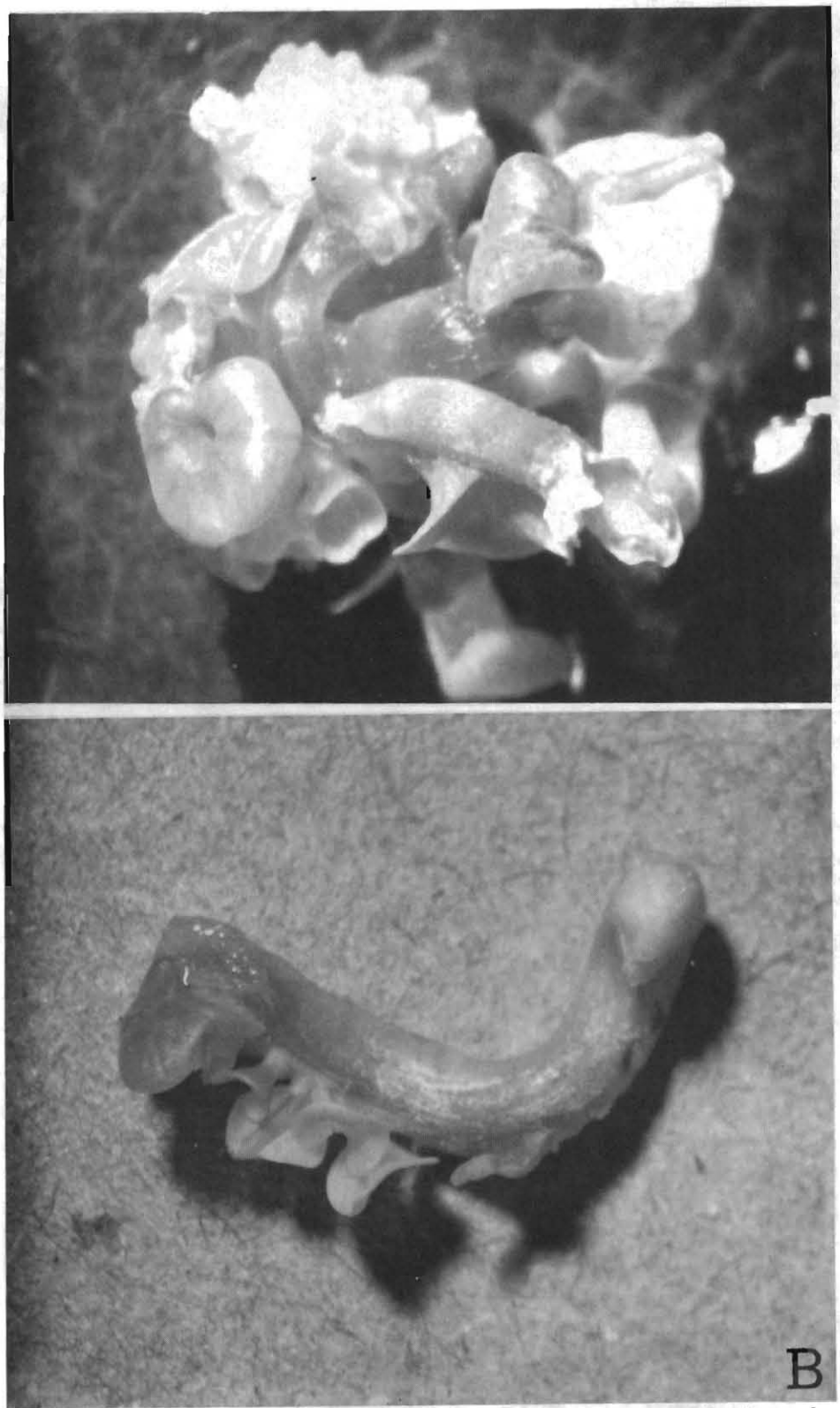

Figure. 2: A) Somatic embryos developing on immature leaf explant (10x).
B) Proliferating secondary somatic embryos (10X).

DEVELOPMENT OF METHODOLOGIES FOR THE ISOLATION AND CULTURE OF CASSAVA IMMATURE POLLEN AND ZYGOTIC EMBRYOS

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Highly efficient systems for the isolation and in vitro culture of cassava pollen and zygotic embryos have been developed. These methodologies have allowed the manipulation of large quantities of isolated pollen from immature and mature anthers for use in studies towards haploid induction and pollen cryopreservation. Conditions were also developed for the isolation, in vitro culture and germination of cassava immature zygotic embryos. The technique was used to recover interspecific Manihot hybrid populations for use in the construction of a cassava molecular linkage map. Culture media and conditions for isolation and culture of pollen and zygotic embryos, and results of work carried out towards the induction of haploidy, will be presented.

INTRODUCTION

Efficient systems for the isolation and in vitro culture of cassava pollen would be a way to avoid the possible detrimental effect of anther wall tissue on microspore development observed in previous cassava anther culture research. In vitro germination of cassava zygotic embryos will be useful in designing protocols for germinating and growing interspecific hybrid embryos as well as for the former to contribute to the construction of a cassava molecular map.

We have developed a very efficient system for the rapid isolation and in vitro culture of large quantities of mature and immature cassava pollen in a very short time, starting with male flower buds.

METHODOLOGY AND RESULTS

- **Isolation and in vitro culture of pollen**. Mature and immature male inflorescences from four cassava var. (HMCI, CM91-3, CM523-7 and CM507-37) were used. Flower buds 0.8 to 2.5 mm. in length, corresponding to tetrad to late uninucleate microspore stages were gently macerated in 5% sucrose solution sterilized by filtration. According to pollen size (40-100 μm), the slurry was passed through 2 filters of 750 and 150 μm to eliminate somatic tissue. The slurry suspension was collected in a centrifuge tube. The filtrate was allowed to sediment and the supernatant discarded, followed by 3 washes (re-suspensions) with sucrose solution and finally with culture medium. The contents of 50 flowers in a vol of 5 ml sucrose solution resulted in a density of 10,000 microspores ml^{-1} (as determined by hemocytometer count), cultured in 15-mm petri dishes, in a hanging drop system, at 26°C, dark and high humidity conditions (Fig. 1).

- Isolation and in vitro culture of immature cassava pollen has allowed us to obtain cell proliferation from microspores cultured at the tetrad stage. Induced microcallus was obtained by direct pretreatment of the isolated microspores with high osmoticum (Fig. 2).

1. **Zygotic embryo isolation, culture and growth.** Immature seeds obtained from 3 var. (M Col 122, M Cub 18 and M Cub 62) were collected in the field at different stages of fruit development. Under aseptic conditions the seeds were split along the raphe, with the aid of forceps and scalpels to remove embryogenic axes. Immature embryo axes (with their cotyledons separated) were cultured between 25 to 45 days after pollination (torpedo to cotyledonary stage).

2. We were able to promote in vitro development of zygotic embryo axes isolated from 35 day-old seeds of the 3 varieties used. Germination started 5 days after isolation and culture in the dark at 28°C. Among the treatments, the best was 1 mg/l GA, 2% sucrose, 1/2 strength MS, 1 mg/l thiamine, 100 mg/l inositol, pH 5.6, and medium solidified with 0.7% agar. Average embryo fresh weight increased from 0.2 to 26 mg in two weeks. Fig. 3 shows in situ (A-D) and in vitro (E-H) development of cassava zygotic embryos (MCub 18).

3. The system for the isolation and in vitro culture of immature zygotic embryos has been successfully applied to recover interespecific Manihot hybrid plants (M. esculenta x M. aesculifolia) for use in the contruction of a cassava molecular map.

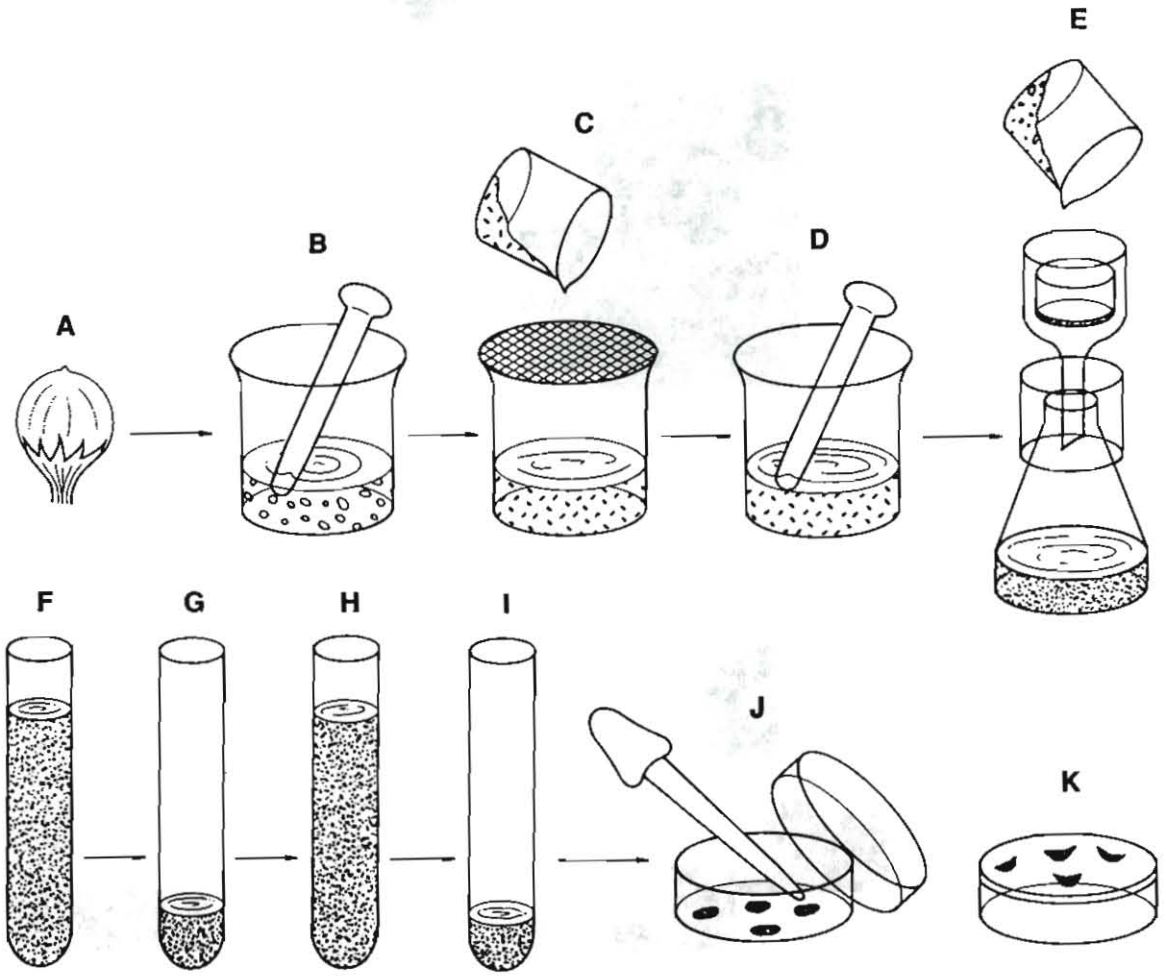

Figure 1. Steps A-K followed to isolate large numbers of cassava microspores at the tetrad stage.

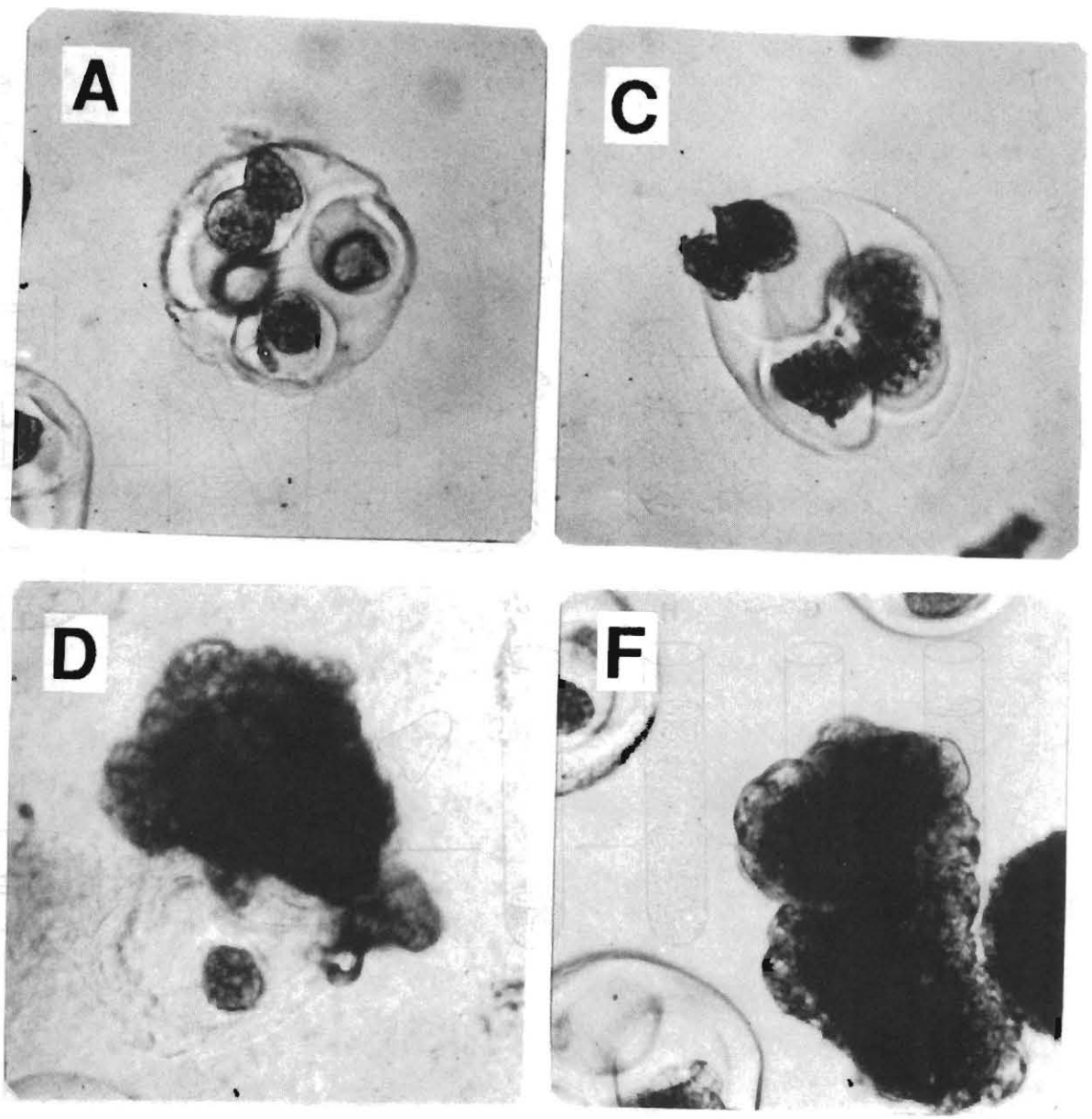

Figure 2. Induction of mitosis and cell proliferation from isolated cassava microspores at the tetrad stage: A. isolated tetrad-stage microspore; C. first mitosis of tetrad cells; D. and F. micro-calli grown from microspores.

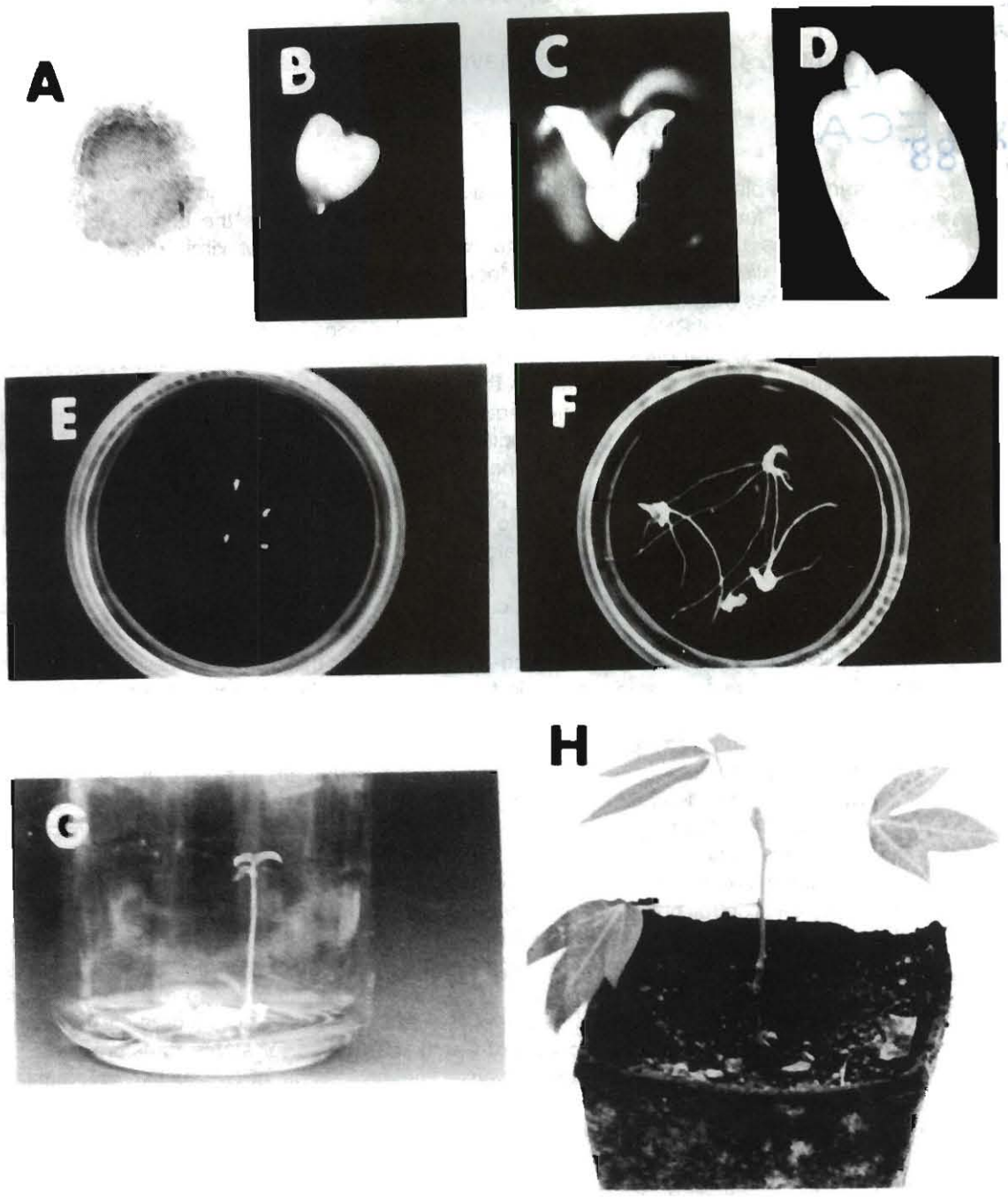

Figure 3. Isolation, culture and growth of cassava immature zygotic embryos: (A & B) heart-shaped embryos isolated from fertilized ovaries; (C & D) torpedo and cotyledonary-shaped embryos; (F-G) growth of embryos in vitro; (H) seedling transplanted into soil from a zygotic embryo.

GENETIC ENGINEERING TECHNOLOGIES TO CONTROL VIRUSES AND THEIR APPLICATION TO CASSAVA VIRUSES

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Resistance to plant viruses through genetic engineering has been investigated with the application of four different approaches. A recent approach is the use of ribozymes, which involves using catalytic RNAs to cause the cleavage of viral RNA molecules. Results of *in vitro* experiments have documented cleavage of viral RNAs but *in vivo* experiments has thus far not been reported. The second technique is to attempt to block the translation of viral RNAs by expressing genes that encode sequences complementary (i.e. antisense) to viral genes. Only a limited number of cases have been reported, but little success has been documented. A third approach is to block virus replication, using competitor sequences, subgenomic sequences and satellite RNAs. The last technique is the expression of viral genes which will interfere with virus replication and/or, diffusion in the plants; replicase gene, movement proteins and capsid proteins. The most promising technique is that of the coat protein (CP), which consists of the integration of a gene encoding viral coat protein into the plant genome. An increasing number of examples using the coat protein mediated resistance (CP_MR) strategy are available, and resistance specificity and efficacy have been evaluated for viruses belonging to thirteen groups; furthermore, several successful field experiments have been conducted.

The application of the CP-MR has been investigated for the prevalent cassava viruses; the African cassava mosaic geminivirus (ACMV) and the cassava common mosaic potexvirus (CCMV). The CP gene of both viruses have been integrated in *Nicotiana benthamiana*, a tobacco species susceptible to both viruses. The ACMV CP gene is expressed at a very low level, the corresponding mRNAs are in a very limited amount and the CP is only detectable in few transgenic lines, at a low concentration (max. 0.01%). Therefore the protection to ACMV is also very limited. Homozygous transgenic plants, challenged with a low virus inoculum, escape infection to a maximum of 30%, the infected plants show the same severity of symptoms and the same amount of virus content as the control plants. In the case of CCMV, the amount of mRNA and CP are extremely high (more than 2% of protein in some cases) and as a consequence the protection is very high. Thirty five days after inoculation, 100% of transgenic plants escape infection for virus inoculum up to 100μ/ml. The plants are also resistant to virus RNA infection but susceptible to PVX, a related potexvirus, and are moderately resistant to other CCMV strains. The same CP constructs were used for stable transformation of cassava calli by *Agrobacterium tumefaciens*. The expression of CCMV CP was similar as for tobaccos but the expression of ACMV CP was about 10 times higher and was detected in 20% of the transgenic cassava calli. Stable transformation of cassava primary embryos has been achieved by *Agrobacterium* as well as by particle bombardment, but only chimeric embryos have been obtained. Efforts are now concentrating on the improvement of the level of expression of ACMV CP and efforts to select fully transformed cassava plants are intensified.

INTRODUCTION

This paper aims at presenting a summary of current knowledge for controlling viruses by genetic engineering and the application of this technique to cassava viruses. Biotechnology is changing very rapidly and besides the well established coat protein mediated resistance strategy (CP-MR), there are new methods for

blocking the replication of plant viruses. We will review the results obtained with the most important techniques. The application of biotechnology has been attempted on two viruses infecting cassava, the African cassava mosaic virus (ACMV) and the cassava common mosaic virus (CCMV). The method used is the CP strategy and the results obtained on tobacco and cassava calli will be summarized. Transfer of these results to cassava is made difficult by the fact that regeneration of transgenic cassava plants has not been yet achieved and the status of knowledge at that respect will be given in two other papers into this proceedings.

MULTIPLE STRATEGIES FOR CONTROLLING VIRUSES

With the increase of the knowledge of the virus replication and their genome organization, scientists are testing different approaches for controlling virus infection. Different viral genes or sequences, inserted into the plant genome, interfere with the virus replication in a beneficial way for the infected plant.

A. Ribozyme strategy.

A novel approach to achieve virus resistance is the use of autocatalytic RNA cleaving molecules, also called "ribozymes". Cleavage is effective both on the positive and negative strand of the RNA and is highly specific and associated with conserved sequence domains. Several studies have been conducted to determine the optimal in vitro conditions of cleavage (Gerlach 1989; Haseloff and Gerlach 1988). Replicase genes of tobacco mosaic virus (TMV) and barley yellow dwarf virus (BYDV) encoding sequences bearing specific virus cleavage sites have been integrated in transgenic plants and should generate sequence specific endonuclease activities (Gerlach 1989), but no in vivo results have been published yet.

B. Translation strategy.

By translation strategy we refer to the strategies involving integration in the plant genome of sequences generating complementary sequences to viral RNA that interfere with the translation of viral genes by hybridization of the coding sequence. It has been reported that synthesis of complementary RNA (antisense RNA) can decrease the accumulation of gene products in both procaryotes and eukaryotes. It is likely that antisense RNAs anneal with sense RNAs to form a double strand complex which is rapidly degraded or which inhibits translation of the RNA. Several viral CP antisense constructs including TMV, cucumber mosaic virus (CMV) and potato virus X (PVX) have been integrated into plants and tested for resistance to infection (Cuozzo et al., 1988; Hemenway et al., 1988; Powell et al., 1989). In all these cases resistance has been reported against infection by the homologous virus but only at low virus inoculum concentrations. Recently a similar study done with the potato leafroll virus (PLRV) (Kawchuk et al., 1991) proved to induce resistance to the virus inoculation of the same level as the CP-MR.

C. Replication strategy.

One of the first steps of the virus cycle is replication of the viral genome and it seems logical that any approach to block this phase should be an efficient way to protect plants. Two approaches have been considered to block a virus infection, the antisense and the sense approaches.

1. Antisense approach of the replication strategy. The antisense approach of the replication strategy attempt to block the replication of virus by hybridization of complementary sequences to the replicase viral gene or to sequences recognized by the replicase during replication. The complete antisense sequence of the replicase gene of the tomato golden mosaic virus (TGMV) was integrated into tobacco genome and several lines were reported to exhibit a high level of resistance when challenged with different concentrations of TGMV (Day et al., 1991).

2. Sense approach of the replication strategy.

2.1. Competitor RNA. A study using sense sequences comprising the tRNA-like structure of TYMV in order to compete with the similar viral sequences and thereby decrease virus replication activity has been conducted.

In vitro studies have shown such competition (Morch et al., 1987) and in vivo experiments show some level of resistance (Cellier et al., 1991). In contrast a similar approach used with the TMV seemed not to induce any resistance (Powell et al., 1990).

2.2 Subgenomic DNA. Some viruses produce subgenomic molecules during virus infection; for example several geminiviruses produce subgenomic DNA molecules of the B component. Insertion of one copy of such DNA of the african cassava mosaic virus (ACMV) into the tobacco genome reduced disease symptoms when the plants were challenged with ACMV (Stanley et al., 1990). Symptom amelioration is associated with a reduction in the level of viral DNA, including B DNA which is responsible for the symptomatology, and the resistance is specific to ACMV.

2.3. Satellite RNA. Another approach taken to confer protection against viruses is to cause the expression of virus satellite (Sat) RNAs. SAT RNAs are associated with several viruses and are dependent upon a helper virus for their replication and spread in the infected plant. It has been reported that the presence of Sat RNAs in cucumber mosaic virus (CMV) infected tobacco reduces disease symptoms (Mossop and Francki 1979). Similarly tobacco plants infected with a mixture of tobacco ring spot virus (TobRSV) and ToBRV Sat caused amelioration of symptoms (Gerlach et al., 1986). Transgenic plants that express these satellite sequences were shown to decrease symptoms and virus replication (Gerlach et al., 1987; Harrison et al., 1987; Jacquemond et al., 1988). Recently this strategy has been applied to tomato (Tien et al., 1990; Tousch et al., 1990), and proven to be efficient for the reduction of symptoms both in greenhouse and field experiments.

Not all satellite sequences provide symptom attenuation but sometimes they can also cause necrosis; the sequences responsible for severe symptoms are reduced to a few nucleotides. There is a risk that amplifying a satellite in transgenic plants may result in some of the molecule reverting to a necrotic form, causing dramatic symptoms when naturally infected by the helper virus. This possibility will greatly limit the utilization of the Sat RNA strategy unless further studies can demonstrate a great stability of the system.

2.4. TMV replicase strategy. Lately, a new source of genetic engineered resistance has been identified involving the transformation of plants with non structural viral genes. Tobacco plants transformed with the TMV 54 kDa gene, which is derived from a portion of the replicase complex, are immune to extremely high concentrations of TMV virions or RNA (up to 500 g/ml) of the strain U1 (Golemboski et al., 1990). This immunity is highly specific to the strain U1, or a mutant YS1/1 of TMV and susceptible to the other strains, including the related U2 strain of TMV. This approach is undertaken with several viruses and results with the expression of the entire replicase gene of the potato virus X are producing a high level of resistance into transgenic tobacco plants (Braun and Hemenway 1992) and a portion of the replicase gene of CMV as well (Anderson, pers. com.).

STATUS OF THE COAT PROTEIN-MEDIATED RESISTANCE

Among the different strategies for controlling viruses by genetic engineering, the CP-MR is currently the most promising. CP-MR refers to the resistance to virus infection caused by expression of a CP gene in transgenic plants. Many examples have been published and the efficiency in terms of protection, stability and specificity has been evaluated for several viruses. The type of resistance and the mechanisms of action of the CP-MR have been investigated and a large amount of information is available. Finally both laboratory experiments and field tests with different crops have been conducted and the first commercial use of this type of resistance is scheduled for 1995.

A. Efficacy of coat protein-mediated resistance.

The efficacy of CP-MR is demonstrated by the number of examples where resistance has been achieved, by the spectrum of specificity of protection, and also by the type of resistance achieved.

1. Specificity of coat protein-mediated resistance. Since 1986, the date of the first publication describing the CP strategy (Powell et al., 1986), there have been a number of reports of CP-MR involving a variety of different viruses and host plants (Beachy et al., 1990, Fauquet, 1992 #232). There are viruses belonging to thirteen different virus groups and hosts that belong to dicotyledons and monocotyledons. Most of the examples are from non-enveloped ssRNA(+) viruses, but it seems that the morphology of the virus, the fact that the viral genome is divided or not, and the type of viral genome organization does not matter for the obtention of resistance.

There are positive examples of CP-MR for two groups of enveloped ssRNA(-) viruses :Tenuivirus and Tospovirus. As for the DNA viruses, we only have information about geminiviruses were the CP-MR has been achieved, the tomato yellow leaf curl virus from Thaland (TYLCV-Th) and the African cassava mosaic virus (ACMV) but in these cases the CP levels were extremely low and the protection limited (Rochester, pers. com.).

2. Spectrum of coat protein-mediated resistance. The best TMV CP(+) tobacco lines were inoculated with members of different virus groups including CMV, AlMV, PVX, and PVY, but there was no noticeable resistance against infection by any of the tested viruses (Anderson et al., 1989). Though there is no protection for viruses belonging to other virus groups, there is a growing evidence that the CP-MR has a large spectrum of specificity within the group and that a particular CP can provide resistance to more than the homologous virus.

A complete study on the spectrum of resistance of transgenic plants expressing a CP gene was carried out on tobamoviruses on tobacco (Nejidat and Beachy 1990), and tomato (Sturtevant et al., 1991). On the basis of these studies it was concluded that viruses that are related to the CP of TMV by greater than 60% are less able to infect the resistant lines than are less related tobamoviruses.

Results of experiments of CP-MR in the Potyvirus group, particularly important since many economically important plant viruses belong to this virus group, demonstrated that expression of a particular CP gene can induce protection for several other potyviruses (Ling et al., 1991; Stark and Beachy 1989). The CP gene sequences of these potyviruses are homologous to the level of 65%.

In the case of tobraviruses the heterologous protection is also effective for viruses having about 60% homology (van Dun and Bol 1988). For cucumoviruses it has been proven for several cases that the CP-MR is extended to several strains among and across the two subgroups of the Cucumovirus group (Quemada et al., 1991).

3. Multiple manifestations of coat protein-mediated resistance. In each of the examples of CP-MR described to date, resistance is manifested by several features: reduction of the number of infection sites, reduction of the spread within the plant, reduction of the symptoms intensity and reduction of the virus replication.

3.1. Resistance to inoculation. First, there is a reduction in the numbers of sites where infection occurs on inoculated leaves. Fewer starch lesions were produced after inoculation with PVX on CP(+) tobacco plants than on CP(-) plants (Hemenway et al. 1988), and there are fewer chlorotic lesions caused by TMV infection on tobacco plants that express the TMV CP gene than on those that did not (Powell et al. 1986). Likewise, the numbers of necrotic local lesions caused by TMV infection on CP(+) Xanthi nc tobacco local lesion were 95 to 98% lower than on CP(-) plants (Nelson et al., 1987).

3.2. Resistance to virus spread within the plant. The second manifestation of resistance of CP engineered plants is a reduced rate of systemic disease development throughout the CP(+) plants. Thus if inoculation results in infection on the inoculated leaves, the likelihood that the infection will become systemic is considerably lower. Grafting studies in which a stem segment of a transgenic TMV(CP+) tobacco plant was inserted between the rootstock and apex of a wildtype tobacco, demonstrated that the (CP+) segment prevented the virus from moving to the upper part of the grafted plant. This experiment shows that the CP may play a role in the long distance movement of the virus and consequently resistance has a component that affects systemic spread of the infection, at least in the TMV - tobacco system (Wisniewski et al., 1990).

3.3. Resistance to symptom expression. A third manifestation of resistance is a reduced rate of disease development on systemic hosts that are CP(+). In most of the examples of CP-MR, CP(+) plant lines were less likely to develop systemic disease symptoms than those that were CP(-). Several plant lines that expressed the PVX CP gene did not become severely infected when inoculated with high levels of virus (Hemenway et al. 1988). Similar results were reported for CP-MR against CMV (Cuozzo et al. 1988), TMV (Powell et al. 1986), PVY and TEV (Stark and Beachy 1989) and other viruses. On the contrary in other cases the transgenic plants which are becoming infected showed similar symptoms to the control plants as for example the CMV in transgenic tobacco plants (Quemada et al. 1991) and the cassava common mosaic virus (CCMV) in transgenic Nicotiana benthamiana (Fauquet et al., 1991).

3.4. Resistance to virus multiplication. Another manifestation of resistance is lower accumulation of virus in CP(+) compared with CP(-) plant lines. ELISA and semi-quantitative western blots have been used to quantify virus accumulation in inoculated leaves and other plant parts in most examples of CP-MR (Cuozzo et al. 1988; Hemenway et al. 1988; Lawson et al., 1990; Nelson et al. 1987; Powell et al. 1986). In certain examples of resistance, plants accumulate no virus after inoculation (Hemenway et al. 1988; Lawson et al. 1990), and can be, therefore, considered to be immune to infection under the conditions of the tests. In other cases, the virus accumulation in the infected transgenic plants is normal and comparable to control plants (Fauquet et al. 1991; Quemada et al. 1991).

All resistance manifestations of CP-MR can usually, but not always, be overcome by inoculating with relatively high concentrations of virus. A virus concentration of 10 g/ml of 10 μg/ml of TMV nearly breaks the CP-MR to TMV in a system where 0,01 μg/ml causes disease in CP(-) plants (Powell et al. 1986). Fifty μg/ml are needed to overcome CP resistance to AlMV, PVX, PVY and TEV (Hemenway et al. 1988; Lawson et al. 1990; Stark and Beachy 1989; Tumer et al., 1987) and 100 μg/ml of CCMV are required to break the CP-MR in tobacco plants (Fauquet et al. 1991). Resistance is largely overcome by inoculation with RNA rather than virions in many cases but the PVX CP(+) lines of tobacco and potato

(Hemenway et al. 1988; Lawson et al. 1990) and the CCMV lines of tobacco (Fauquet et al. 1991), this might be a specific property for potexviruses.

The majority of evaluation of CP-MR has been assessed by mechanical inoculation of the virus and of course the most important criteria for virus resistance is its evaluation in natural modes of contamination, i.e. in vegetative propagation and with the natural vectors. Information related to these points are limited but significant. In the case of the dually engineered resistance against PVX and PVY in potatoes (Lawson et al. 1990), it has not been possible to recover in the potato tubers any of these two viruses. At least one line of potato showed a good level of resistance through aphid inoculation of PVY. PLRV, a member of the luteovirus group, is non-mechanically transmissible and the CP-engineered potato plants have all been challenged by using aphid inoculation (Kawchuk et al., 1990; Tumer et al., 1991), and demonstrated some degree of resistance. CMV CP(+) transgenic tobacco plants have been challenged with viruliferous aphids and proved to be resistant as for mechanical inoculations (Quemada et al. 1991).

B. Field experiments with coat protein-mediated resistant plants.

There have been several field tests of virus engineered resistant plants.

Tobacco plants that expressed the CP gene of AlMV were field tested in Wisconsin in 1988 [Krahn, pers. com.]. At 85 days after inoculation only 9% of the CP(+) plants had developed a systemic infection, while 93% of the CP(-) plants had systemic infections.

The first field test with TMV resistant tomato plants, from cultivar VF36, was conducted in 1987 (Nelson et al., 1988). No more than 5% of the CP(+) plants developed disease symptoms by fruit harvest, while 99% of the VF36 plants developed symptoms. Lack of visual symptoms was associated with a lack of virus accumulation. Fruit yields of the infected VF36 plants decreased 26-35% compared to healthy plants, whereas yields from CP(+) line were equal to those of uninoculated VF36 plants.

To determine if the TMV CP gene conferred protection against infection by field isolates of ToMV, tests were conducted in 1988 in Florida and Illinois. The TMV CP gene conferred resistance against ToMV-Naples C infection under Florida field conditions and against two strains of TMV under Illinois field conditions. Only weak protection was conferred against infection by the ToMV strains under Illinois field conditions (Sanders et al., 1990). Tomato lines expressing ToMV CP gene were highly resistant to infection by ToMV-Naples C in both sites. And plants that expressed both TMV and ToMV CP genes were equally well protected against TMV and ToMV.

Field tests were conducted with Russet Burbank potato plants expressing the CP genes of PVX and PVY (Lawson et al. 1990). PVY causes significant yield

depression in potato and, in combination with PVX, PVY produces a severe disease called "rugose mosaic". To determine if expression of PVX and PVY CP genes would protect potato plants from the synergistic effects of PVX and PVY infection in the field, plants propagated from CP(-) Russet Burbank and from plant lines expressing both CP genes were inoculated with both PVX and PVY and transplanted into the field (Kaniewski et al., 1990). Plants from four CP(+) lines were significantly protected from infection by PVX. However, three of the lines were not protected from infection by PVY when simultaneously inoculated by both viruses. Plants of one line, however, were highly resistant to PVX and PVY. Tuber yields at maturity in uninoculated plots were the same for all the lines. In contrast, tuber yields of all inoculated lines were markedly reduced, except the line resistant to both viruses, which was unaffected by virus inoculation.

For the last 3 years, field trials of cucurbits transformed with the CMV CP gene have been conducted with success (Gonzalves et al., 1991). In 1991, four transgenic lines of the cv. Poinsett 76 were compared with resistant cv. Marketmore 76, infection was allowed to occur naturally by aphids using a low percentage of infected plants as initial virus sources. After four month of growth, the transgenic plants performed much better than the control plants, 8% of transgenic plants showed symptoms versus 98% for the control and 8% for the resistant line.

APPLICATION OF THE CP-MR TO CASSAVA VIRUSES

The objective is to produce by genetic engineering cassava plants resistant to two cassava viruses; the African cassava mosaic virus (ACMV) and cassava common mosaic virus (CCMV). These viral diseases were chosen because of their economic importance respectively in Africa and South America (Fauquet and Beachy 1989). The technique used here is the CP-MR. Among the different examples of resistance produced by genetic engineering its seems that in most cases an important criteria for obtaining a stable and substantial resistance is a stable and high level of expression of the inserted gene. The level and pattern of gene expression is greatly dependent upon the chimeric gene construct used, on the site of insertion of the foreign gene in the plant genome and on the number of inserted genes. These two last factors are not under the control of the investigator. On the other hand, it is possible to study the influence of each part of the chimeric gene on the expression of the gene; the transcriptional promoter, the leader sequence of the mRNA, the coding region, the untranslated region and the termination sequence.

In such project the most significant difficulty is cassava regeneration and transformation. We consequently need to use indirect methods to optimize the gene constructs, and to study their expression. In our case this has been done with Nicotiana benthamiana because it is a host for both cassava viruses and can be readily transformed and regenerated. Furthermore we carried transient assays with the particle gun and finally cassava calli transformation. The N.b. model can tell us if the chosen strategy is efficient for controlling these viruses, the transient assays

and calli transformation can tell us which gene construct is functional and which promoter has the highest level of expression in cassava cells.

A. Constructs with ACMV and CCMV coat proteins.

1. Construction of chimeric genes with the coat protein of ACMV. The ACMV is a geminivirus with a genome composed of two molecules of single stranded DNA of approximately 2.7 kb (Bock and Harrison 1985). The sequence of the genome was completed in 1983 (Stanley and Gay 1983) and the CP located on the positive strand of DNA A segment was isolated. Several constructs have been made with the CP coding region, with: 1) different promoters (p35S, pE35S, p35S+4xOCS...), 2) two viral leader length sequences, 3) two different termination sequences, 4) in a sense and antisense orientation. Finally a construct was also developed that included an improved translational consensus sequence.

2. Construction of chimeric genes with the coat protein of CCMV. CCMV is a potexvirus and its genome is composed of one molecule of single stranded RNA of approximatively 6.3 kb (Costa and Kitajima 1972). Total cDNA cloning and sequencing of the virus has been done (Lee et al., in preparation) and the CP coding region located near the viral 3' end has been sequenced. The CP of CCMV has a molecular weight of 25 kd and comparisons of its amino acid sequence with six other potexviruses showed 47 to 62% of homology amongst those viruses compared. In the case of CCMV we developed constructs with only the enhanced promoter of the cauliflower mosaic virus (Ep35S) (Kay et al., 1987), but we varied the viral leader sequences and the viral termination sequence in order to test their effect on the level of expression of the CP gene in transgenic tobacco plants.

B. Evaluation of CP mediated resistance in transgenic tobaccos plants.

The evaluation of the CP-MR in transgenic tobacco plants is realized with regenerated lines which are selected for expressing the CP gene, therefore showing a signal of mRNA corresponding to the coding sequence of the CP and having a certain amount of CP. The resistance evaluation of the plants is done by challenging them with different concentration of the homologous virus and related strains and/or viruses.

1. Transgenic tobacco plants with ACMV.

1.1. Expression of ACMV CP in transgenic plants. We regenerated 67 different lines of Nicotiana benthamiana transformed with 7 different constructs. They were all resistant to kanamycin, and we have analyzed the lines having the sense and antisense CP gene of ACMV. Over the 49 tobacco lines transformed with the ACMV CP only 6 had a detectable amount of protein with the same molecular weight as the CP and reacting with the ACMV antibody. It has to be noticed that there is an enormous background of aspecific reactions with the proteins of control plants and that all the different ACMV antibodies used, would they be polyclonal or

monoclonal, have not been able to solve that problem. Therefore it is very difficult to ensure that these bands are typically CP bands, but if they are, the corresponding amount of CP is about 0.001-0.005% which is a very small amount of CP. In the same 49 transgenic lines we searched for an mRNA corresponding to the coding sequence of the ACMV CP and all the lines tested were negative except the 6 lines for which a CP band was detected. But it has been necessary to purify polyA+ mRNA in order to detect a signal, this demonstrating that the mRNA are apparently very unstable or have a short life period, so that they are rare in the plant and consequently the amounts of CP are also low.

1.2. Challenge of the ACMV transgenic tobacco lines. The lines having a detectable amount of CP and showing an mRNA signal have been challenged with the purified ACMV virus at different concentrations ranging from 20 ng to 1$\mu\mu$g/ml. Only for low concentrations of 20 to 100 ng/ml we have been able to detect a level of resistance to ACMV. This resistance is characterized by a delay of infection, but once the plants are infected the symptoms have the same intensity after a few days and they contain the same amount of ACMV as for a typical ACMV infection. This result is consistent over the several lines tested and the engineered CP-MR is easily overcome with low concentration of virus.

2. Transgenic tobacco plants to CCMV.

2.1. Expression of the CCMV CP in transgenic plants. We have regenerated 126 lines of Nicotiana benthamiana which have been transformed with the different CCMV constructs, they were all resistant to the kanamycin but only the lines with the CCMV CP gene have been analyzed for protein and mRNA content and some of them were challenged with the CCMV. Over the 109 lines tested, about 65% had a detectable amount of CP, the background was very low and in more than 20% of the cases the CP amount was extremely high. The total amount of CCMV CP was often around 0.2% but it reached 2% in several cases. These levels of CP expression are in the order of 100 times more than the ACMV CP expression levels. We have also to note that several protein bands of a lower molecular weight, but serologically related, appeared in the western blots. The presence of mRNA, specific to the CCMV CP sequence has been searched and found in lines expressing the CP. It seems that there is a correlation between the amount of CP and the amount of mRNA in the same transgenic line. In contrary to the ACMV, it has not been necessary to purify the polyA mRNA to get a signal, indicating a level of mRNA of about 100 times more.

2.2. Challenge of the CCMV CP transgenic tobacco lines. Some of the best CCMV CP transgenic lines have been challenged with the CCMV strain Brazil (homologous strain) with concentrations varying from 100ng/ml to 1mg/ml. All the tested lines having a high level of CP expression were showing a high level of resistance. The resistance is firstly expressed by a decrease of the number of local lesions on the inoculated leaves and by a lower number of plants showing local lesions. It is needed to use a virus concentration of 100 μg/ml to reach 100% of

plants showing local lesions, while only 1 μg/ml is needed in the case of wild type plants. And even at that virus concentration the infected plants only show 80% of the number of local lesions on the control plants. The resistance is secondly expressed by a lower percentage of infected plants, at a concentration of 10 μg/ml 35 days after inoculation only 40% of the plants have symptoms and 32 days are needed at 100 μg/ml in order to reach 100% of plants infected. At 10 μg/ml and above the resistance of the transgenic plants is very spectacular as the control plants in most of the cases are dying. In the case of the infected transgenic plants the symptoms are not very different from the control plants and show severe necrosis. Furthermore, the infected transgenic plants contain, estimated by ELISA, a virus concentration which is not very dissimilar from the control plants. The transgenic plants are also resistant, in the same conditions, to viral RNA up to a concentration of 50 μg/ml. But these lines are apparently not resistant to another potexvirus like the PVX, even if we use very small virus concentration like 50 ng/ml. If we consider the homology of the amino-acid composition of their CPs, PVX is one of the most homologous virus to CCMV. These transgenic lines are also showing a degree of resistance to other strains of CCMV from Colombia and Paraguay.

C. Transient expression of foreign genes in cassava leaves.

The transient expression experiment allows a rapid evaluation of a construct avoiding all the problems associated with the transformation and regeneration. These tests are extremely useful especially if the studied plant is a recalcitrant plant, like cassava. It is also convenient because of the rapidity of the test, only 24 to 48 h are needed to get a response. The plasmid introduced in the cells is not integrated in the plant genome but it is expressed immediately, if the chimeric gene is active. This plasmid is later hydrolysed and eliminated from the cells. In order to visualize and to measure the gene activity the CP genes are replaced by a reporter gene like the b-glucuronidase gene (GUS gene). Only the particle acceleration technique (particle gun) offers the possibility to use differentiated tissues, reason why we chose it for testing our constructs in cassava. Several promoters have been tested and the two promoters derived from the 35S promoter (e35S and 4O35S) are equally active.and about 3 times higher than the 35S. The ubiquitin promoter has an activity similar to the 35S (Franche et al., 1991).

D. Transformation of cassava leaf-discs by Agrobacterium tumefaciens.

The cassava being a dicotyledonous plant is sensitive to Agrobacterium. and it appeared interesting to develop a transformation experiment on cassava calli because it is important to compare the expression of the different constructs obtained in a transient assay with a stable transformation assay, and because it is possible to transform cassava cells with CP constructs, to check for their expression and later make experiments of protection with protoplasts obtained from transgenic calli. Cassava leaf-discs were transformed (Horsch et al., 1985) with Agrobacterium tumefaciens GV3111 containing plasmids with the GUS reporter gene as well as ACMV and CCMV CP genes. The GUS activity has been shown in 70% of the calli

and the presence of the GUS gene has been demonstrated by southern. Experiments done with several constructs containing a range of diverse promoters demonstrated that the nature of the termination sequences is not important and that the enhanced 35S is three time more active than the regular 35S. They also show that the promoter modified with the OCS is 5 times more active and that the ubiquitin promoter has the same level of expression as the 35S promoter. The results obtained in the transgenic cassava calli are in total agreement with the transient assays on cassava leaves. Constructs with cassava virus CP genes have been used for transformation of cassava calli by Agrobacterium tumefaciens . After selection on kanamycin, these calli were analyzed for their DNA, RNA and protein content.

The results show that the transgenic cassava calli have been transformed and that they express mRNA corresponding to the expected size and that a large proportion of the calli are expressing the CP of ACMV (20-25%) and CCMV (90%) at a very high level (Franche et al., 1992). It is therefore apparent that cassava is able to express the CP of ACMV and CCMV at a reasonable level suitable for protection.

E. Genetic transformation of cassava.

Regeneration of cassava is only possible through direct somatic embryogenesis, consequently only gene delivery by Agrobacterium and particle gun with the embryogenic tissue is possible, thus limiting the chances of success (Schöpke et al., 1990). A ß-glucuronidase (GUS) gene with the cauliflower mosaic virus (CaMV) 35S promoter and the soybean 7S polyadenylation signal was used as reporter gene for transient and stable expression. For detection of transient gene expression, the GUS gene was inserted into the pUC19 plasmid from E. coli. For stable transformation by Agrobacterium, we used a binary vector. It contained the GUS gene and the neomycin phosphotransferase II (NPT II) gene, cloned into the plasmid pMON 505, and confering resistance to a group of aminoglycosides, including kanamycin and geneticin (G418).

1. Transformation with a particle gun. Various tissues derived from in vitro plants were bombarded with a particle gun and were tested for their ability to express the GUS gene. Transient expression was found in young leaf lobes, in leaf explants during the early phase of embryogenesis, in small somatic embryos, and in stem tissues. When embryo clumps were used as target, GUS expression was observed after 2 weeks on selection medium.

In some cases chimeric embryos where the meristematic region expressed the GUS gene were observed. The occurrence of GUS-expression in multicellular structures indicates that the gene was stably integrated in the genome and then passed on to the daughter cells during mitosis. But it has not been possible to regenerate full transgenic plantlets.

2. Transformation with Agrogacterium tumefaciens. Before trying to transform cells capable of regeneration, we used tissues like stem discs of greenhouse plants, leaf discs of old leaves and young leaf lobes of in vitro plantlets to obtain a general idea of the use of Agrobacterium with cassava tissues. In all cases it was possible to obtain GUS-expressing calli after several weeks of selection. Subsequent transformation experiments were performed with small and large somatic embryos. Experiments with larger embryos resulted in embryos which showed many GUS-expressing cells after several weeks of selection. They were located mainly on the hypocotyl or in wounded areas. In some instances they were found in areas where usually secondary embryogenesis is initiated, i.e. on the upper surface of the cotyledon near the leaf margin. These results are promising and by optimizing the system it should be possible to increase the number of embryos showing this type of GUS-expression and to select for transformed embryos.

It appears that the major problem for the production of transgenic cassava plants might be the selection of transgenic among non transgenic cells. Each specific type of tissue that is going to be used for transformation, has a different response to the selectable markers and therefore specific selection conditions have to be found. We are now establishing these conditions for different antibiotics and we are using other selectable markers (see other papers on cassava transformation).

CONCLUSION

There are many ways to control plant viruses by genetic engineering but the most developed and successful method is the CP-MR for which there are many examples and field experiments. Furthermore, it is now obvious that alternative methods can be used if CP-MR was not effective for a particular type of virus. The International Cassava-Trans Project, though it has not yet produced cassava plants resistant to ACMV and CCMV, has nevertheless answered many questions. A very strong resistance to CCMV has been achieved in Nicotiana benthamiana in many different tobacco lines and N.b. lines moderately resistant to ACMV have been detected, showing that the CP-MR can be effective for cassava viruses, at least in tobacco. The weak resistance against ACMV can be related to the low mRNA and CP content in the transgenic plants and further studies of mRNA stability are underway to improve the level of expression. It is not certain that a result in a tobacco model can be extrapolated to cassava but we have no other alternative. Thus far, there is no example where a resistance achieved in a tobacco has not been confirmed or improved in the "natural" host of the considered virus. We also demonstrated that cassava is able to express each of the gene constructs that we have produced and that the promoters used are efficient in different types of cassava cells. This could be a very important fact because ACMV is naturally transmitted by whiteflies which inject the virus in phloem associated cells, prior to the invasion of other cell types after the first cycles of the viral replication. Cassava calli cells can be transformed and selected, and transformed cassava calli express the CP genes of ACMV and CCMV. Finally we have been able to regenerate cassava plants from somatic embryogenetic tissues in a limited number of cassava cultivars and we are

currently using this route of regeneration to transform cassava by Agrobacterium tumefaciens and the particle gun.

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CYCLIC SOMATIC EMBRYOGENESIS AND PLANT TRANSFORMATION IN CASSAVA

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With a four step procedure embryos and plants were obtained with a number of clones originating from Africa, South America and Indonesia. The culture procedure consisted of four steps. In step one embryos were induced on young leaf lobes (1-6 mm) on a Murashige and Skoog (1962) based medium supplemented with sucrose and 2,4-D. The optimal 2,4-D concentration varied for each clone. In step two embryos were germinated on an MS medium with BAP. Isolated embryos can be used to start a new cycle (step 1--> step 2) or cultured for shoot development in step three. In step four shoots were rooted on a hormone free medium. Somatic embryos were used as starting material for transformation experiments using *Agrobacterium tumefaciens* as the DNA mediating vector.

For primary embryogenesis it was found that the growing conditions of the plants of which the lobes were taken, especially the light intensity during growth, were crucial to the response of the leaf lobes and the subsequent formation of embryos. Once embryos were formed it was no problem to obtain more embryos by going through a number of embryogenic cycles. The response of somatic embryos was much better than that of leaf lobes. After one year of culture (equaling 10 successive cycles) the production of embryos and the conversion into shoots was comparable with that of 2nd cycle embryos. The use of liquid medium and fragmentating of the embryos enhanced the production of new embryos. Infection with *Agrobacterium tumefaciens* was integrated in the cyclic somatic embryogenesis system at the beginning of step 1 and at the beginning of step 3. With the first approach partly transformed embryos were obtained and with the second approach partly transformed shoots were obtained.

INTRODUCTION

Despite some reports on adventitious shoot formation [7,11], the only good documented regeneration method for cassava is somatic embryogenesis [3-6, 8-10]. Here we present an easy four step procedure to obtain large numbers of embryos and plants from a variety of different genotypes. The elegance of the system is the cyclic development of embryos from isolated somatic embryos [5,6]. (Fig. 1).

MATERIALS AND METHODS

Plant Material

Single bud cuttings of the Colombian clone M. Col 22 and the Nigerian clones TMS 30555, TMS 50395, TMS 60506, TMS 90059, TMS 30211, TMS 60444, TMS 30395, TMS 90853, TMS 4(2)1244, TMS 30001 and TMS 30572 were propagated on Murashige and Skoog [2] medium supplemented with 20 g/l saccharose and 0,7 g/l agar.

The standard condition in the culture room was: temperature of 30 °C, daylength of 12 hours and a light intensity of 40 $\mu Em^{-2}s^{-1}$. Deviations from the standard are mentioned in the results section.

RESULTS AND DISCUSSION

Primary Embryogenesis using Leaves as Explants

Different types of explants (petioles, roots, stems, leaf lobes), relatively young and old, originating of in vitro and in vivo grown plants and of different genotypes were cultured on media supplemented with 2,4-D, NAA, IBA and IAA with or without BAP. Morphogenesis was only seen when young leaf explants were cultured on 2,4-D supplemented media and only somatic embryos were formed. After 10 days step 1 they were visible as nodular embryos and after 15 days as torpedo shaped embryos. In the second step of the procedure, on MS medium + 0.1 mg/l BAP, torpedo shaped embryos developed into structures with a distinct hypocotyl and green cotyledons; so called germinated embryos (GE).

The percentage explants forming GE and the average number of GE per initial explant were recorded to discriminate between the different treatments. The genotype M. Col22 was used as a model clone to optimize the production of primary somatic embryos of leaf explants. The length of the initial leaf explant was one of the most important factors [4,8]. Generally speaking leaf lobes which had started to unfold, are not capable of initiating embryos. The response of explants of in vivo grown plants was depended on the time of the year. In January 80 percent of the leaf explants responded with the formation of GE and the production was 22.2 GE per initial leaf explant (GE/IE). Before and after January the response was lower (< 3 GE/IE).

As it was impossible to standardize the growth of in vivo grown plants, in vitro plants were used as source of leaf explants. On the same step 1 medium as used for in vivo lobes, the production of GE was more than a factor 20 lower than in the January experiment. Doubling of the 2,4-D concentration to 8 mg/l increased production (between 0.5 and 3.5 GE/IE). Higher 2,4-D concentrations did not further enhance the production. Besides 2,4-D, also Dicamba and Picloram formed GE, but not at a significantly higher level. The results with in vivo derived leaf explants suggested an influence of light on the embryogenic response, therefore in vitro plants were grown under different light regimes. Leaf explants isolated from donor plants grown in different daylengths gave no differential embryogenic response whereas a lowering of the light intensity during the growth of donor plants significantly enhanced the percentage of responding explants and the production (GE/IE). (Table 1)

However, the best production was obtained when donor plants were pre-treated with 2,4-D. For this, plants were grown in liquid medium on rockwool plugs. After 14 days of growth, 2,4-D was added to the plants (final concentration

8 mg/l). Two days after the 2,4-D pretreatment, leaf explants were isolated and cultured on step 1 medium. Seventy-one percent of the leaf explants, cultured on MS + 8 mg/l 2,4-D, responded with the formation of GE and the production was 9.4 GE/IE. The general application of 2,4-D pretreatment was further investigated on 11 Nigerian clones (Table 2). With the standard procedure 5 of the 11 Nigerian clones formed nodular embryos (visible after 10 days step 1) but of only 2 clones GE were obtained and with a very low production (< 0.1 GE/IE). After the pretreatment 10 clones had formed nodular embryos and 8 clones responded with the formation of GE (< 0.1 - 1.1 GE/IE).

Cyclic Embryogenesis using Germinated Embyos as Explants

GE, cultured again on step 1 and 2 medium formed secondary embryos. In this way cyclic cultures were obtained. In Fig. 2 A and B the percentage of responding explants and the number of GE per initial explant is given for five (step 1; 4 mg/l 2,4-D) or six (step 1; 8 mg/l 2,4-D) successive cycles of embryogenesis.

The response in the primary cycle was significantly lower than in all succeeding cycles. In the higher cycles between 56 and 85 % of the GE explants formed new embryos, independent of the used 2,4-D concentration (Fig. 2). The production of GE was between 6.8 and 9.9 GE/IE [5]. GE can stay in culture for at least 15 cycles without decrease of production (results not shown). One of the production determining factors is the developmental stage of the starting embryos. Two main categories were distinguished; torpedo shaped and germinated embryos. Torpedo shaped embryos have a distinct hypocotyl and are translucent. They are divided in Ia (without cotyledon primordia) and Ib (with cotyledon primordia). Stage II embryos are germinated embryos with distinct green cotyledons and are divided in IIA (young) and IIb (mature). About 50 % of the torpedo shaped embryos produced new embryos and the production was 2.9 GE/IE whereas 81 % of the germinated embryos produced new embryos (production 10.3 GE/IE).

Another important factor is the period between the transfer to step 2 medium and the start of a new cycle. Generally speaking GE with well developed green cotyledons should be transferred as fast as possible to a new step 1 medium. Further optimalizations were obtained by culturing in liquid instead of solid media and by culturing fragmented instead of intact GE. Fragmented GE in liquid medium produced 32.1 GE/IE [6].

Eight of the 11 tested Nigerian clones formed primary GE (Table 2). Forty-two percent of the primary GE produced secondary GE. Cyclic embryogenic cultures were obtained of 7 clones. The production of intact GE on solid medium in the third cycle varied, depending on the clone, between 5.3 and 9.9 GE/IE (Table 2).

Shoot Development of Germinated Embryos

GE of different cycles were cultured for shoot development on BM supplemented with 1 mg/l BAP. The formation of shoots is a slow process. After 1 month of culture about 25 % of the M. Col 22 GE had formed a stem and additional leaves. If these leaves had the cassava phenotype they were classified as normal if otherwise as deformed. Most of the deformed shoots will revert to normal with prolonged culture and especially when cuttings are made of deformed shoots. In Fig. 3 the shoot forming ability of GE of cycle 1 to 4 is given. The lower shoot conversion percentage of GE of cycle 2 and 4 compared to cycle 1 and 3 was caused by a shorter culture period of respectively 2 and 3 months. GE of cycle 7 developed shoots in the same frequency (data not shown). Besides good culture practice the developmental stage of the embryos influenced shoot conversion (Fig. 4). About 20 % of the torpedo shaped and 60 % of the GE developed into shoots.

Transformation of Germinated Embryos

Infection with the DNA agent Agrobacterium tumefaciens can be integrated in the somatic embryogenesis procedure at all steps. For example after cocultivation of fragmented GE with Agrobacterium tumefaciens, explants can be cultured on step 1 medium for the induction of new embryos or on step 3 medium for shoot development. Both strategies were applied but most attention was paid to step 1 transformation. Transformation experiments were conducted with LBA4404 containing a binary vector carrying the NPTII gene and either the GUS or GUS intron gene. About 10-50 percent of the explants had blue spots, indicative for GUS activity. Most of the transformed tissue is callus and as embryos are formed directly from parental tissue, the chance of regenerating transformed embryos is low. However about one GE with blue spots per 1000 initial GE was obtained. The transformed sections can be enlarged after culture of GE on kanamycin containing media.

Recently also DNA deliverance by particle bombardment was integrated in the culture of GE. Experiments are now going on to determine if the DNA is stably integrated in the genome and if it is possible to enlarge the transformed sections. For both Agrobacterium mediated and particle gun mediated transformation it should be possible to obtain complete transformed plants by cyclic culture of partly transgenic leaves on selective media as is described for walnut [1].

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Table 1. Influence of the light intensity during growth of donor plants on primary embryogenesis (step 1: 8 mg/l 2,4-D).

lightintensity ($\mu Em^{-2}s^{-1}$)	% responding explants (1)	GE per responding explant (2)	GE per initial explant (2)
40	38 b	4.6 b	1.7 b
28	54 ab	9.0 a	4.9 ab
8	64 b	10.3 a	6.6 a

48 leaf explants per treatment, means with the same letter in one column are not significantly different (p=0.1)

Table 2. Influence of 2,4-D pretreatment on primary embryogenesis and cyclic embryogenesis of Nigerian cassava clones.

	EMBRYOGENESIS			
Cassava clones	Embryogenesis : primary cyclic 2,4-D pretreatment : without Response : NE GE/IE		with NE GE/IE	GE/IE
TMS 30555	+	0	+ 0.7	6.2
TMS 50395	+	0	+ <0.1	5.3
TMS 60506	+	0	+ <0.1	0
TMS 90059	-	0	+ <0.1	7.2
TMS 30211	-	0	+ 0	-
TMS 60444	-	0	+ 1.1	9.9
TMS 30395	-	0	+ 0.1	6.7
TMS 90853	+	<0.1	+ 0.2	8.2
TMS 4(2)1244	+	<0.1	+ 0	5.4
TMS 30001	-	0	+ 0	-
TMS 30572	-	0	- 0	-

Average of three experiments (48-74 explants) NE nodular embryos, GE: germinated embryo.

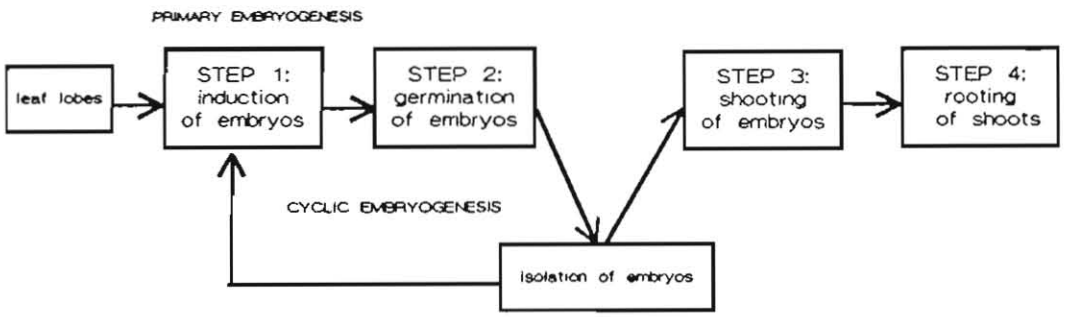

Fig. 1 Schematic representation of somatic embryogenesis in cassava.

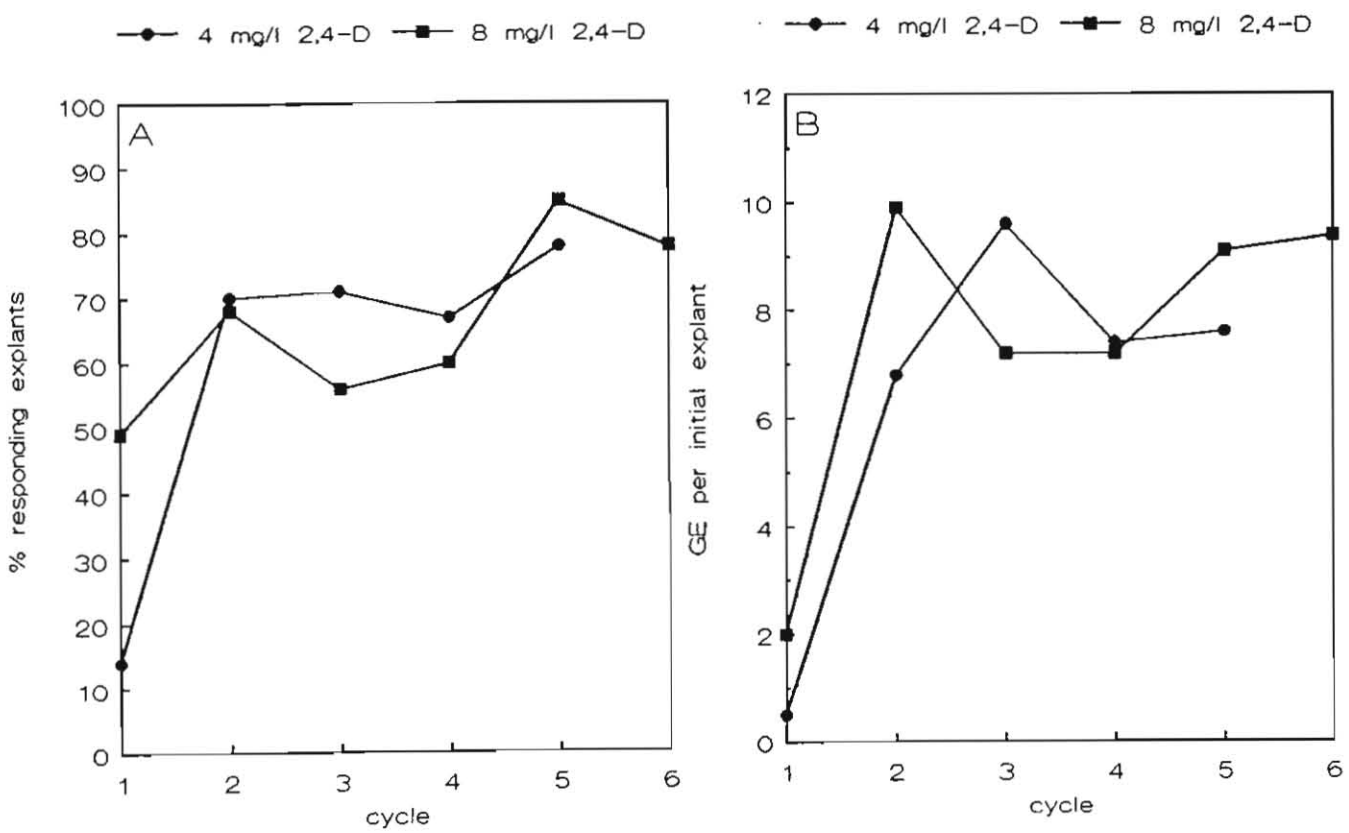

Fig. 2 Influence of cycle on percentage explants with GE (A) and the number of GE per initial explant (B).

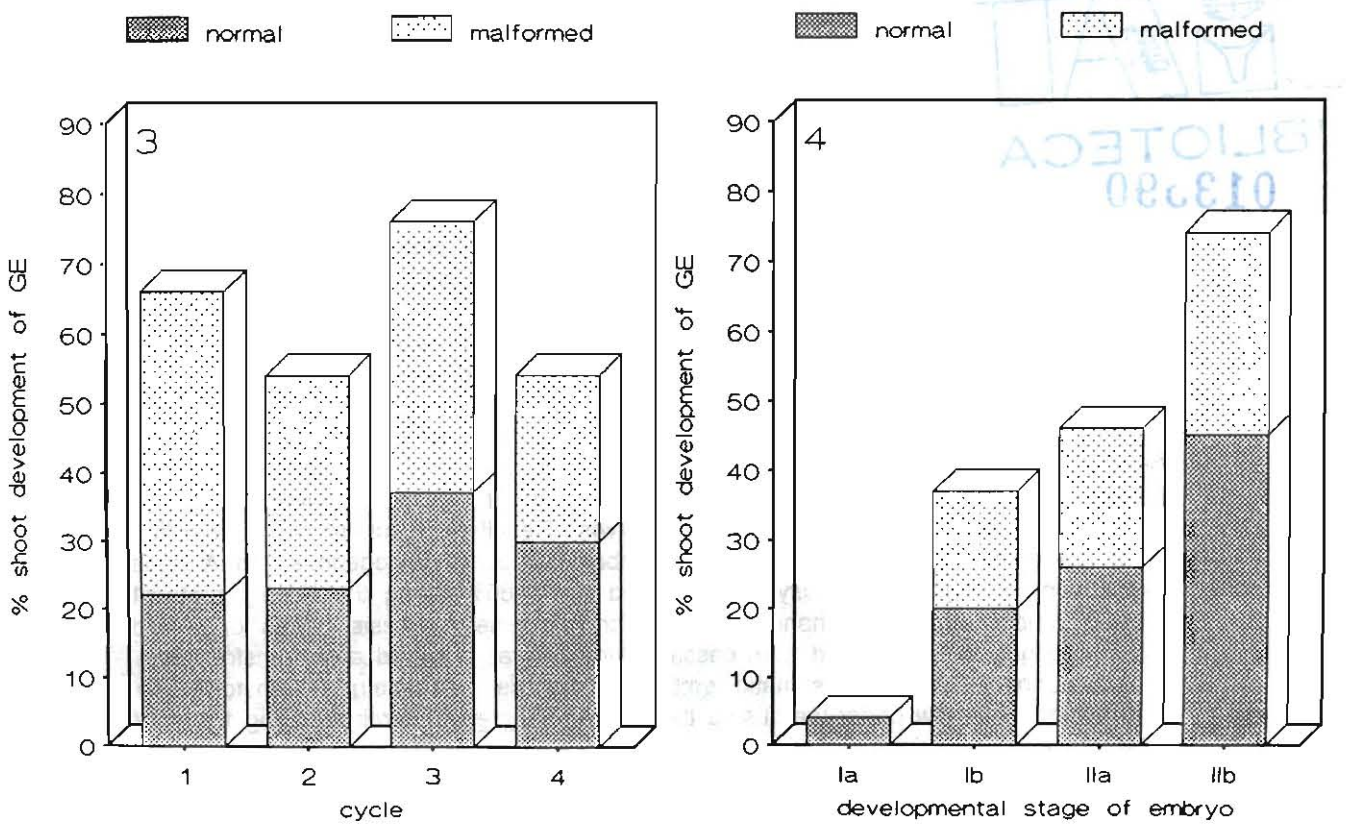

Fig. 3. Influence of cycle on shoot development of germinated embryos.

Fig. 4. Influence of developmental stage of embryo on shoot development.

TOWARDS THE DEVELOPMENT OF *Agrobacterium tumefaciens* AND PARTICLE BOMBARDMENT-MEDIATED CASSAVA TRANSFORMATION

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Our strategy for Agrobacterium-mediated cassava transformation involves the utilization of cotyledonary leaves from somatic embryos and infection with both wild and disarmed strains. Virulence tests for wild Agrobacterium strains as well as regeneration protocols have been developed; the plasmid used in all the experiments was the pGV 1040 containing the uid A, the bar and the npt II genes (provided by Plant Genetic Systems, Gent).

Sensitivity levels of cassava tissues to the commercial herbicide Basta, used as selection agent, were determined. Minimum lethal doses for the herbicide were 1 mg/l for immature leaves and 18 mg/l for somatic embryos. Results with kanamycin as selection agent were inconsistent. Only somatic embryos showed endogenous uid A gene expression; endogenous activity was reduced by 70-80% using the Kosugi protocol (1990), which includes methanol. Expression of opine synthesis genes of a wild Agrobacterium strain, isolated from cassava tumors, was obtained after transformation of cotyledonary leaves of somatic embryos with the wild strain. Transformation experiments are now under way using the wild Agrobacterium strain carrying the pGV 1040 plasmid.

A biolistic device was used to bombard early stage somatic embryos using the pGV 1040 construct harboring the same genes as in the Agrobacterium experiments. An average of 20 GUS expressing spots per 0,5 cm2 of embryogenic tissue was obtained. GUS expression in proliferating somatic embryos has been observed after one and two months from bombardment.

INTRODUCTION

The objective of our work is to develop a transformation system for cassava, using two strategies, the *Agrobacterium*-mediated transformation system and the particle delivery system. The plasmid vector used in both cases is the pGV1040[1] containing the bar[2], the npt II and the uid A[3] genes. In this paper we present some promising results obtained with both the *Agrobacterium tumefaciens* and the particle bombardment-mediated systems.

MATERIALS AND METHODS

Explants

In our *Agrobacterium*-mediated transformation experiments, cultivar MPER 183 (a cultivar sensitive to Agrobacterium infection) was used as source of cotyledonary leaves from somatic embryos for inoculations. These were used as initial explants due to their high response to embryogenesis and to their similarity with immature

leaves, which is an explant that could be infected in previous experiments. The primary source of somatic embryos were apical meristems of one month greenhouse grown plants, cultured in basal MS[4] containing 8 mg/l of 2,4-D, under low light conditions (100 $\mu E.s^{-1}.m^{-2}$) [5].

For particle bombardment we are using somatic embryos at the globular stage, one month in age, of the cultivar MCOL 1505. These somatic embryos were induced from apical meristems as mentioned for MPER 183. The clumps used were about 0.5 cm^2 in size.

Agrobacterium Strain

Experiments with 25 different *Agrobacterium* strains for the identification of highly infective strains in cassava were done both under greenhouse and *in vitro* conditions using five different cassava cultivars.

As a result of these experiments, the strain used for infection of explants was the *Agrobacterium tumefaciens* CIAT 1182, an Agropine/mannopine strain, carrying plasmid pGV1040 in a binary vector system.

In experiments with immature leaves of MPER183, strain 1182, could induce tumor formation on hormone-free medium. The presence of agropine and mannopine was demonstrated by paper electrophoresis (Fig. 1).

Inoculation and Cocultivation

Inoculations were done by adding drops of an overnight culture (OD_{550}: 0.8) of 1182pGV1040 containing 100 μM acetosyringone on each of the explants. The borders of the explants were cut to leave a surface exposed to *Agrobacterium* infection.

Cocultivation was carried out for 24 hours at 28°C in darkness. After this, explants were washed in basal liquid MS containing 500 mg/l Carbenicillin and 250 mg/l Cefotaxime, blotted dry on filter paper and cultured for embryo induction.

Particle Bombardment

Our system for bombardment is based on the original system developed by Sanford[6]. The bombardment cocktail contains tungsten particles, $CaCl_2$, spermidine, and the gene construct (pGV1040).

Tissues were bombarded at 22 cm and 680 mm Hg of vacuum. A mesh was used to disperse the particles, to prevent tissue damage.

Selection Conditions

A previous experiment was performed to determine selective conditions for PPT. It was found that LD_{50} for embryos is 16 mg/l (10-15 d) while for green tissues it is 1 mg/l. For our *Agrobacterium* experiments, we used 1 mg/l PPT before the embryo formation and 16 mg/l for the proliferation stage.

Embryo Induction

In this step, explants were cultured on basal MS containing 8 mg/l 2,4-D, 1 mg/l phosphinotricin (PPT), 500 mg/l Carbenicilline and 250 mg/l Cefotaxime, under low light conditions (100 $\mu E.S^{-1}.m^{-2}$). After 4-5 days tissues were transferred to the same medium containing 16 mg/l PPT, at the same light conditions. Once embryo induction occured, the tissues were maintained in the same medium in the dark for proliferation.

Regeneration

Torpedo stage embryos from the *Agrobacterium* experiments were transferred, for germination, to basal MS medium, without hormones, containing 500 mg/l carbenicillin, 250 mg/l cefotaxime and 16 mg/l PPT at high light conditions (300 $\mu E. S^{-1}.m^{-2}$). Germinated embryos were transferred to basal MS medium containing 0,5 mg/l BAP, without PPT or antibiotics.

Three months after bombardment, the embryos were induced to germinate at the same light conditions on basal MS with 16 mg/l PPT, Basal MS containing 0.5 mg/l BAP, without antibiotics and PPT. For elongation the embryos will be transferred to the same medium containing 1 mg/l PPT and 0.5 mg/l BAP.

RESULTS AND DISCUSSION

Experiments with *Agrobacterium*

A low frequency of embryo induction was obtained in the tissues infected with strain 1182 carrying pGV1040. Seven explants produced embryos 20 days after selection, from a total of 120 explants infected. Embryo formation started after 7-10 days in inoculated explants under selective conditions and after 3-5 days in control non-treated explants under non selective conditions. In general proliferation of infected explants was slower than in the control tissues, this could be due to the selection pressure produced by PPT. Kanamycin has not been used for selection of putative transformed embryos because of its seemingly deleterious action as a selective agent in previous experiments.

Three embryos have now been germinated and transferred to the BAP-containing media for elongation. PPT has not been included at these stages.

Regenerated plantlets will be tested for GUS expression using the Kosugi protocol, and DNA analyses will also be conducted.

The utilization of cotyledonary leaves from somatic embryos is a novel strategy to generate transformed cassava plants. Since embryos are difficult to infect with by *Agrobacterium*, we are using as explant cotyledonary leaves that are highly embryogenic and, as we demonstrated can be infected using the CIAT 1182 *Agrobacterium* strain.

Experiments with Particle Bombardment

Gus expression was monitored in bombarded tissues at 1, 30 and 60 days after bombardment using the Kosugi protocol for GUS detection [7]. This test decreased by about 10 times the endogenous GUS expression when compared with the Jefferson protoco l[8] (Table 1).

GUS expressing foci at 30 and 60 days were larger than observed at one day. The percentage of GUS expressing regions in the tissues at different times are presented in Table 2.

At present, we are in the proccess of regenerating the embryos under selective conditions, using PPT as selective agent.

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Table 1. Percentage of endogenous GUS activity in different types of cassava tissues, using the Kosugi and the Jefferson protocols. Only embryogenic tissues exhibited endogenous activity.

TISSUES	JEFFERSON	KOSUGI
MATURE/IMMATURE LEAVES STEMS ROOTS	0.0	0.0
SOMATIC EMBRYOS	30	3

Table 2. Gus expression detected in somatic embryos at different times after bombardment.

Days	Embryonic groups tested	GUS spots per embryonic group	% remaining GUS activity after 1st evaluation
1	140	15.42	---
30	203	1.37	8.9
60	268	0.81	5.2

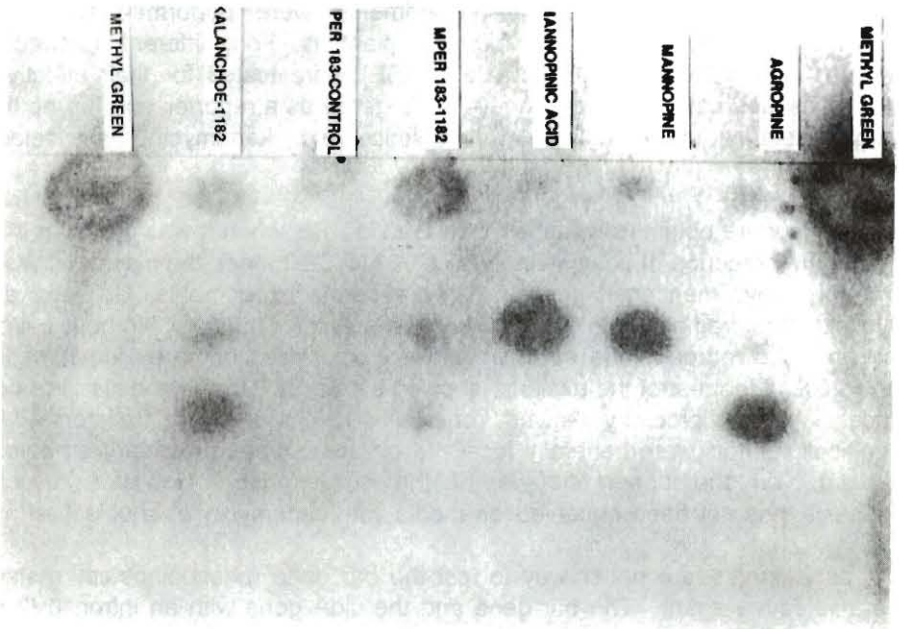

Fig. 1. Paper electrophoresis using silver staining, that shows the presence of opines in explants infected with the CIAT 1182 strain. *Kalanchoe sp.* was used as a positive control for transformation.

TRANSFORMATION OF CASSAVA (*Manihot esculenta* Crantz) EMBRYOGENIC TISSUES USING Agrobacterium tumefaciens.

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Agrobacterium-mediated transformation experiments were performed with somatic embryos derived from young leaf lobes of *in vitro* plantlets. Four different *Agrobacterium* strains (EHA101, GV3111-SE, LBA4404 and ASE) were tested for their infectivity on embryogenic tissues using the uidA gene(= GUS gene) as a reporter and the nptII gene (conferring resistance to aminoglycoside antibiotics, e.g., kanamycin) as a selectable marker.

The best results were obtained with the strain GV3111-SE when it was grown in Minimal A medium in the presence of acetosyringone, GV3111-SE carries the plasmid pMON505 containing the above mentioned genes. In two separate experiments, GUS-expression of embryo explants was assayed after 14 days on selection medium. In both cases 5% of the explants showed dark-blue spots which were composed of more than 10 cells. An average of 20% of the rest of the explants showed single GUS-positive cells. We believe the multicellular spots probably derived from cell divisions of single transformed cells. These multicellular foci were generally localized on areas were secondary embryos can develop, e.g., on the upper surface of the cotyledons. However, secondary embryogenesis has not been observed on media with kanamycin as a selective agent.

Currently, experiments are under way to test the bar gene (phosphinotricin resistance gene) as a selective agent. The bar gene and the uidA gene with an intron (IV2 intron of the ST-LS1 gene of potato) have been inserted into pMON977. This plasmid is being used for transformation of cassava embryogenic tissues via *Agrobacterium*. First results show that the GUS gene containing an intron can be expressed in cassava somatic embryos.

Abbreviations: ACMV (African Cassava Mosaic virus), AS (acetosyringone), BA (6-benzyladenine), CCMV (Common Cassava Mosaic Virus), GUS (ß-glucuronidase), GUSINT (intron-containing ß-glucuronidase gene),Km (kanamycin), LB (Luria-Broth medium), MinA (minimal-A modified medium), MS (Murashige and Skoog medium), X-gluc (5-bromo-4-chloro-3-indolyl-ß-glucuronic acid), 2,4-D (2,4dichlorophenoxyacetic acid), 35S (cauliflower mosaic virus 35S promoter), 35SpA (cauliflower mosaic virus 35S 3' end), 7S (3' end of the α-subunit gene of soybean ß-conglycinin).

INTRODUCTION

The main objective of our research is to use the coat protein-mediated protection strategy (for review see Beachy *et al.* 1990) to obtain transgenic cassava plants. We are attempting to develop transgenic cassava plants using the *Agrobacterium-mediated* transformation system to introduce into embryogenic cassava cells the *nptII* gene and the *uid4* gene as a scoreable marker. In the present paper we describe the results obtained with transformation of cassava embryogenic tissues using four *Agrobacterium* strains carrying different GUS constructs in pMON505 or pBinl9-derived vectors.

MATERIALS AND METHODS

Plant material and culture media: Explants used for transformation were green somatic embryos with differentiated cotyledons, as well as parts of young non-pigmented embryos, which were separated from 2 month old embryo clumps and cut into several pieces. The embryo clumps were derived from young leaf lobes of plantlets of cultivar MCol-1505 produced *in vitro.* These clumps had been maintained as embryogenic cell lines by subculturing them every four weeks onto fresh propagation medium (see below). An average of 8 petri dishes containing 9 clumps each contained approximately 4 g fresh weight of plant material. Culture conditions: 25°C; 16 h photoperiod in a walk-in growth chamber; diffuse light provided by fluorescent tubes (Sylvania cool white, 40W, 90-110 $\mu Mm^{-2}s^{-1}$ PAR)

MS-medium (Murashige and Skoog 1962) supplemented with 20 g/l sucrose and solidified with 2 g/l Phytagel (Sigma) was used as basal medium. The clump propagation medium was basal medium supplemented with 2 mg/l of 2,4-D. For all media the final concentration of Cu2+ was adjusted to 2μM with $CuSO_4$ since it was found to be beneficial for the growth *in vitro* of cassava tissues (see paper by Schopke *et al.).* The cocultivation medium comprised the clump propagation medium with 0.05 mg/l BA and 200 μM acetosyringone (AS). Non-selective and selective media were the same as clump propagation medium with 0.05 mg/l BA and 300 mg/l carbenicillin and, the latter, with 100 to 200 mg/l km.

Bacterial strains and culture media: The disarmed *Agrobacterium* octopine strains GV3111-SE (Fraley *et al. 1985)* and LBA4404 (de Framond et *al.* 1983; cited in Hood *et al.* 1986), and the disarmed nopaline strains EHA101 (Hood *et al.* 1986) and ASE-1 (ibid) were used for transformation experiments. They were grown at 28°C on solid LB plates with the corresponding antibiotics, or on a rotary shaker at 300 rpm in the case of liquid cultures. Liquid medium was MinA modified medium (Herrera-Estrella, Simpson 1988), pH 5.7, supplemented with AS 200 μM.

Plasmids: The vectors used for transformation experiments are summarized in Table 1. The 35S-GUSINT-35SpA construct was originally cloned into a pBinl9 derivative (Vancanneyt *et al.* 1990), from which it was subcloned into pMON505 (Horsch and Klee 1986) by standard recombinant DNA techniques (Sambrook et *al.* 1989; P. Chavarriaga, unpublished data). This construct contains the IV2 intron of the ST-LS1 gene of potato that allows expression of the uid4 gene only in eukaryotic cells. The 35S-GUS-7S construct was also cloned into pMON505 by standard recombinant DNA techniques (Franche *et al.* 1991). Both pBinl9 and pMON505 contain the *nptII* gene for selection of transformed plant cells.

Preparation of bacteria to inoculate explants: Two to three bacterial colonies were inoculated into 5 ml MinA medium containing the respective antibiotics, vortexed vigorously and incubated for 24 h on a rotary shaker at 300 rpm. Bacteria were pelleted at 4000 rpm (Beckman J-6B), the supernatant was discarded and resuspended in the same volume of MinA medium without antibiotics. The number

of bacteria was measured at 660 nm (LKB Ultrospec K) and adjusted to 4-8x10^8 cells/ml final concentration for inoculation.

Inoculation, cocultivation and selection: Explants were inoculated for a mini-nun of 10 min with the *Agrobacterium* suspension blotted on sterile filter paper and transferred to cocultivation medium where they remained for three days. Explants were then washed three times with sterile distilled water to eliminate excess bacteria, blotted, transferred to either selective or non-selective medium and kept for different periods of time (see Table 2). GUS assays were done after nonselection/selection periods as explained in Table 2.

GUS assays: Histological GUS assays were performed after incubating the explants for 16 h in a 0.1 M sodium phosphate buffer, pH 7.0, containing 1 mM X-gluc, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 0.1% Triton X-100 (Jefferson 1987). Explants were scored positive for expression of the uid4 gene if they had multicellular dark blue spots or more than 10 individual dark blue cells/explant.

RESULTS AND DISCUSSION

Virulence of the Agrobacterium *strains:* In an experiment that compared the transformation efficiency of four different *Agrobacterium* strains carrying the GUS construct 35S-GUSINT-35SpA, the highest number of GUS-positive explants (57%) was obtained with the octopine strain GV3111-SE (Table 2, experiment I). In this experiment all explants were scored for GUS expression after having been on selective medium with 200 mg/l kanamycin for 17 days. The same strain carrying the GUS construct 35S-GUS7S was tested for infectivity on embryogenic tissues. The GUS assays after 0,10 and 14 days of selection on 100 mg/l kanamycin (Table 2, experiment II) showed comparable results in terms of percentage of GUS-positive explants. With the data obtained from experiments II and III (Table 2), we noticed that the longer the explants remained on selection medium, the higher the final number of GUS-positive explants obtained. The striking difference between the two GUS constructs used in these experiments was the amount and size of multicellular dark blue foci observed in each case, for which possible explanations are given later.

Interestingly, the nopaline strain EHA101, which has been classified as supervirulent in crops such as soybean (Hood *et al.* 1987), was the least virulent in this experiment. There is evidence that the presence of AS at pH 5.8 reduces the virulence of strain A281 (wild type strain from which EHA101 was derived) on *Antirrhinum majus* (Godwin 1991). Although we used similar conditions to grow *Agrobacterium, i.e.* 200 μM AS in MinA medium adjusted to pH 5.7, additional experiments are necessary to elucidate the causes of the low infectivity of EHA101 on cassava embryogenic tissues.

Expression of the GUS constructs and localization of G.US-positive cells: We observed that the size and number of blue foci/explant produced on embryogenic tissues transformed with 35S-GUS-7S were reproducibly larger than those transformed with 35S-GUSINT-35SpA. With the latter construct, the blue color was often limited to single cells probably due to less diffusion of the blue product. Similar results have been obtained with the direct introduction, by biolistic methods, of the same constructs into cassava embryo clumps and cotyledons (unpublished observations).

Although the dark blue color observed on the explants was clearly different from the weak blue background produced as the result of endogenous GUS-activity of cassava embryogenic cells (Schöpke et al. 1992), we have to consider the contribution of this source to the total GUS activity observed.

The variation found in expression of the GUS constructs could be due, among many other factors, either to the different polyadenylation signals or the presence of the intron in the GUS coding sequence, or both. The expression of the uidA gene has been tested in cassava leaves using different promoters and polyadenylation signals. In the experiments carried out by Franche et al. (1991), the 35S promoter directed the transient expression of comparable levels of ß-glucuronidase independently of the polyadenylation signal. However, since the 35SpA 3' end was not tested in the above mentioned experiments, we could speculate that this polyadenylation signal might diminish the expression of the GUS gene. However, additional experiments are required in order to support this assumption. Although the presence of the IV2 intron in the GUS sequence does not dramatically influences its expression in *Arabidopsis* (Vancanneyt *et al.* 1990), we must consider this factor as another source of variation in the expression of the GUS gene.

In most experiments the multicellular blue foci, some of which possibly were derived from single transformation events, and individual cells were generally localized on areas where secondary embryos can develop, i.e., lower and upper surface of cotyledons. Root poles and hypocotyls were other areas where blue foci were also seen. It is important to note that scalpel-wounded somatic embryos can develop secondary embryos on the cotyledonary area under non-selective conditions (unpublished observations). After exposure to *Agrobacterium,* several attempts were made to induce secondary embryos on potentially transformed tissues. In all cases no secondary embryogenesis was observed, indicating that kanamycin might not be the best selectable marker for transformed embryogenic cells.

Current experiments are underway to test the *bar* gene, which confers resistance to the herbicide phosphinotricin, for transformation and selection of embryogenic tissues. The *bar* gene has been cloned into the binary vector pMON977 (The Monsanto Company, Saint Louis, MO, USA) and this plasmid introduced into *Agrobacterium* strain ABI (The Monsanto Company, Saint Louis, MO, USA) to be used in transformation experiments.

CONCLUSIONS

Under the experimental conditions reported here, out of four different strains tested, the octopine Agrobacterium strain GV3111-SE was the most virulent on cassava embryogenic tissues. Using this strain and the GUS construct 35S-GUS-7S we were able to show chimerical expression of the GUS gene in embryogenic tissues. The selection with 100-200 mg/l of kanamycin did not allow the production of embryogenic tissues from transformed cells, indicating that different selectable markers, or lower concentrations of kanamycin, should be tested.

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Table 1. Plasmids, GUS constructs and bacterial strains used for cassava transformation experiments.

Plasmid name	Original plasmid	GUS constructs	Agrobacterium strains
35SGUSINT	pBin19 derivative	p35S-GUSINT-35pA	LBA4404
GUS-1	pMON505	p35S-GUS-7S	GV3111-SE
GUS-2	pMON505	p35S-GUSINT-35SpA	GV3111-SE EHA101 ASE-1

Table 2. Results obtained with GUS assays after transformation of cassava embryogenic tissues using different *Agrobacterium* strains and plasmids. The data presented are the results of single experiments. The third column indicates the time the explants stayed on non-selective medium, followed by the time they were on selective medium and the amount of Km used in each case. The total number of explants infected with *Agrobacterium is* indicated in brackets in column 4.

Experiment number	Agrobacterium strain and plasmid	Non-selection (days); selection (days); km concentration (Mg/l)	Number of explants used for GUS assay	Number of explants positive for GUS expression
I	EHA101/GUS2	0;17; 200	150 (180)	7 (5%)
	ASE-1/GUS2	0;17; 200	130 (180)	40 (30%)
	GV3111-SE/GUS2	0;17; 200	70 (150)	40 (57%)
	LBA4404/35SGUSINT	0;17; 200	55 (115)	8 (14%)
II	GV3111-SE/GUS1	0;14; 100	130 (560)	53 (40%)
		4;10; 100	70 (130)	16 (20%)
		16; 0; 0	115 (245)	15 (13%)
III	GV3111-SE/GUS1	4;14; 100	40 (40)	5 (12%)
		4;30; 100	201 (200)	58 (29%)

CLONING AND CHARACTERIZATION OF STARCH BIOSYNTHETIC GENES OF CASSAVA (*MANIHOT ESCULENTA* Crantz)

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Reserve plant starches consist of 20-25% amylose and 75-80% amylopectin. In amylose D-glucose units are linked together by α-1,4 bonds, while amylopectin consists of α-1,4 linked D-glucose units which are cross-linked by α-1,6 bonds. The D-glucose units used to make the polymer are either ADP(or UDP)-glucose molecules which are synthesized by ADPG- or UDPG-pyrofosforylase. The α-1.4, bonds are synthesized by starch synthases, the α-1,6 bonds by branching enzymes. A tuber specific cDNA library of cassava (*Manihot esculenta* Crantz) was constructed and cDNAs encoding ADPG- and UDPg pyrofosforylase, granule-bound starch synthase (GBSS) and branching enzyme (BE) were cloned. The identity of the clones was confirmed by (partial) sequencing. Furthermore, in the case of BE a full length cDNA clone was able to restore branching enzyme activity in a branching enzyme deficient *E. coli.* For GBSS and BE it was shown that they are single or low copy genes. Study of expression patterns by Northern hybridization showed that these genes are highly expressed in tubers and in some cases (GBSS and BE) also detectable in other organs. Detailed analysis of the expression of BE and GBSS in different organs of *in vitro* grown cassava plants showed that they are differentially expressed; BE is more abundantly expressed in stems while GBSS is more expressed in leaves. Tuberous roots could not be obtained from *in vitro* grown cassava plants, which formed instead thickened stems. These thickened parts of the stem contained large amounts of starch but did not contain more transcript of either GBSS or BE than normal parts of the stem.

INTRODUCTION

Like many plant starches, cassava starch consists of about 20% amylose and 80% amylopectin[5]. In amylose, D-glucose units are linked together by α-1,4 bonds, while amylopectin consists of α-1,4 linked D-glucose units which are cross linked by α-1,6 bonds. The D-glucose units used to make up the polymer are either ADP (or UDP)- glucose molecules which are synthesised by ADPG- or (UDPG-) pyrophosphorylase (AGPase/UGPase). The α-1,4 bonds are synthesised by granule-bound starch synthase (GBSS) while the α-1,6 cross links present in amylopectin are synthesised by branching enzyme (BE). Both enzymes are present in multiple isoforms in several plant species[1,3,8].

Cassava is an important source of starch in the tropics and a major source of calories for some 500 million people[4]. Due to urbanization and increase in demand for processed food and feed products in many countries, cassava starch has entered the modern industrial market[2]. The physico-chemical properties of cassava starch have therefore become important considerations, because these characteristics determine the industrial use of any starch.

Molecular engineering can be used to change the properties of starch to make it more suitable for specific chemical and technological processes. In potato,

antisense constructs of GBSS cDNA have been used to alter its starch composition[9] and the gene coding for branching enzyme may be used to manipulate the branching properties of amylopectin. In order to achieve the afore mentioned goal cloning of the genes is essential. Our laboratory is engaged in cloning and characterizing the genes coding for various enzymes involved in starch biosynthesis in cassava with the aim of modifying both the quantity and quality of cassava starch.

RESULTS AND DISCUSSION

(a) Cloning and sequencing of genes. A cDNA library was constructed in λgt11 using poly (A+) mRNA from tubers of cassava genotype M.Col 22[6]. Approximately 150,000 plaques from the library were screened by plaque hybridization with [^{32}P]dCTP labelled cDNAs of potato as probe. This resulted in the isolation of several cDNAs encoding GBSS, BE, AGPase B, AGPase S and UGPase. Both from GBSS and BE full length cDNAs were obtained of which a physical map was made (Fig.1).

The GBSS clone G61 was completely and the branching enzyme clone B40 partially sequenced. They share 65% to 85% sequence homology with the corresponding genes of other species like potato, pea, maize, rice, wheat, etc. Clone B40 was also able to restore the branching enzyme activity in a branching enzyme deficient mutant *E. coli*, strain KV832.

Hybridization of the southern blots of genomic DNA from M.Col 22 with G61 and B40 suggested that there is only one copy of the BE gene[6], but possibly more than one (but few) GBSS gene in the cassava genome present.

Analysis and further characterization of AGPase and UGPase genes is in progress.

(b) Expression patterns. Northern analysis of cassava RNA showed that both the GBSS and BE were most abundantly expressed in the tubers of greenhouse grown plants (Fig.2). The transcripts were present in a much lower amount in petiole and stem, but hardly detectable in leaves and roots. The expression level of different stages of growth were similar with the exception of very young tubers and stems in which the expression was relatively low. When RNA from ten cassava genotypes was tested, it was found that cassava genotype Tjurug had more BE transcript than other genotypes, while GBSS was highly expressed in M.Col 22.

In *in vitro* grown plants, GBSS and BE genes are differently expressed: GBSS transcript is most abundantly present in leaf followed by petiole and stem in decreasing order, while the level of BE transcript was higher in stem than in petiole and leaf. Somatic embryos contained more GBSS and BE mRNA than undifferented callus tissue.

(c) Induction of expression by exogenous supply of sugars. Cassava plants were incubated in the dark for 24 h in water or in water supplemented with different sugars (40 g/L). Incubation in sucrose and glucose resulted in dramatic increases in transcript levels for both GBSS and BE compared to water.

(d) Starch content and mRNA level. Starch content was measured from different organs of cassava and from different stem tissues for a number of genotypes. The result shows that there is a correlation between the starch content in an organ and the level of transcript present. Low levels of mRNA in roots and leaves are accompanied by very low starch content in these tissues. A slightly higher mRNA level in stem tissue is reflected by a higher starch content. Tubers contain high levels of mRNA and very large amounts of starch. However, this relationship between starch content and mRNA level does not always hold true, some organs of *in vitro* grown plants contain more GBSS and BE mRNA than those of greenhouse plants, but they do not contain more starch.

(e) Attempts to obtain *in vitro* tubers. Cassava explants (top shoots or shoots with (1)callus proliferation (2)root primordia (3)full grown roots) were grown in growth media containing 80 g/L sucrose in combination with 2 mg/L Kinetin, Zeatin or α-naphthalene acetic acid (NAA). Under these conditions *in vitro* plants did not produce any tubers. Rather, after prolonged growth on synthetic medium, the plants shed all their leaves and their stems thickeded. Microscopic observation through cross section of these stems in combination with starch measurements showed that the thickened stems contained a very high amount of starch (68% dry weight basis; compared to 70% in tubers, 10% in normal stem and 2% in leaves). However, northern analysis showed that the thickened stems did not contain more GBSS and BE mRNA than normal stems.

CONCLUSIONS

After cloning some of the starch biosynthetic genes from cassava, possibilities have now arisen to manipulate the properties of cassava starch through genetic engineering. Our preliminary investigations already showed that antisense constructs of cassava GBSS cDNA can change the amylose content in potato. So, when a transformation method is developed in cassava (see article by Raemakers et al in this proceedings) all the requirements to genetically modify starch content and composition in cassava are fulfilled.

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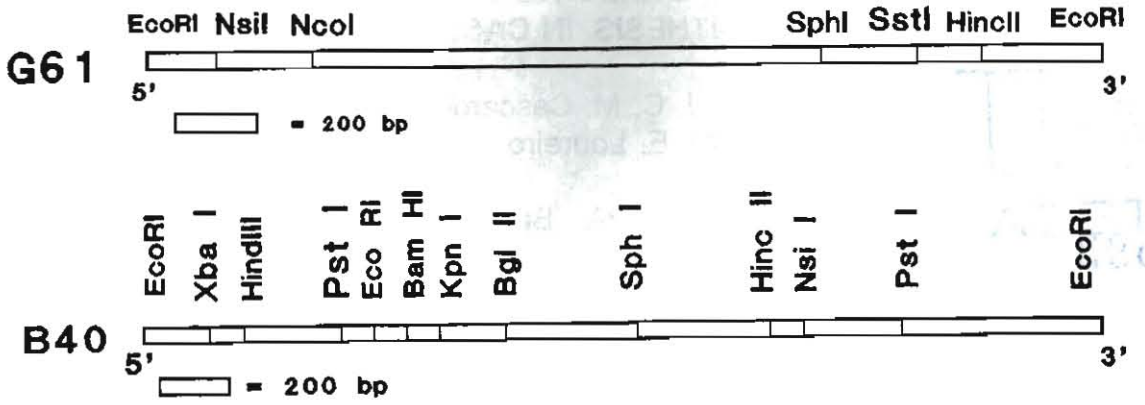

Fig.1: Restriction map of cassava GBSS and BE cDNAs (clone G61 and B40 respectively).

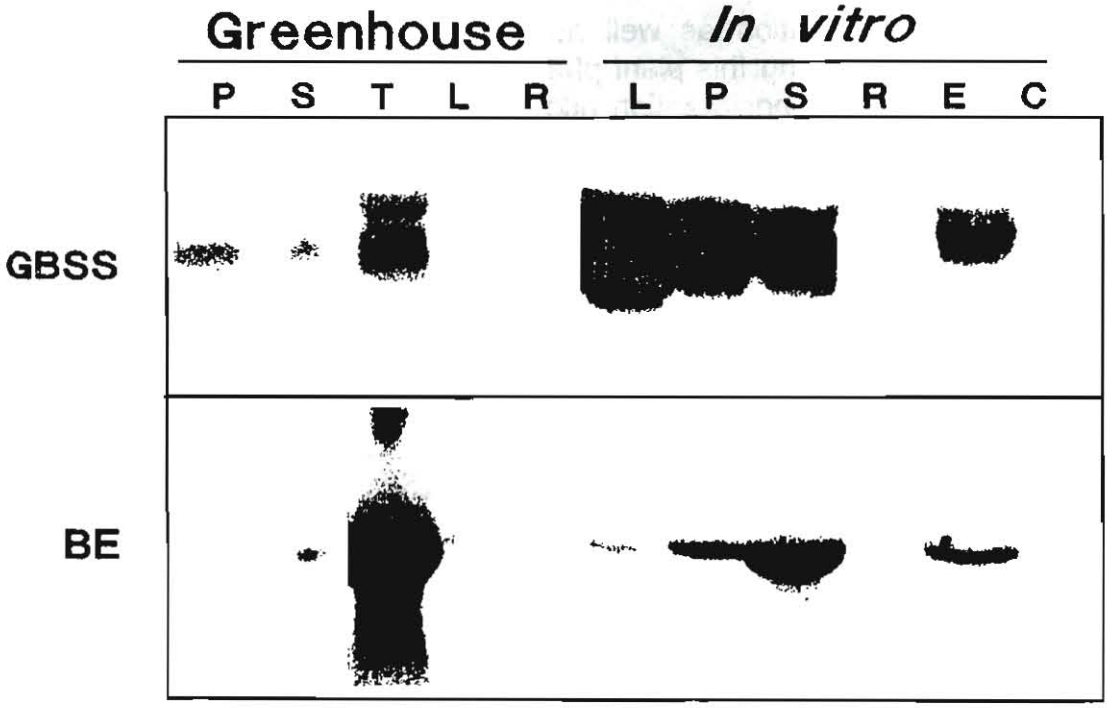

Fig.2: Tissue specific expression of GBSS and BE genes in greenhouse grown and *in vitro* cultured cassava plant. 60 μg of total RNA from different plant organs of cassava was run on a 1.4% agarose formaldehyde gel[7], blotted to Hybond nylon membrane and hybridized with [^{32}P]dCTP labelled cassava cDNA probe. P=petiole, S=stem, T=tuber, L=leaf, R=root, E=somatic embryo, C=callus.

STUDIES ON PROTEINS AND ENZYMES RELATED TO TUBERIZATION AND STARCH BIOSYNTHESIS IN CASSAVA ROOTS

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A method has been developed to isolate intact cassava root amyloplast through protoplast preparation. Protoplasts were obtained after incubating cassava root slices in a medium containing cellulase and pectolyase for more than 10 hours at room temperature. After purification iln a discontinuous Ficoll density gradient, the protoplasts were ruptured by forcing the suspension through modified disposable syringe. Amyloplast ilntactness was observed in a fluorescence microscopy and activity of amyloplast marker enzymes before and after rupture of the amyloplast membrane. Starch branching enzyme, ADPG-pyrophosphorylase, and starch synthetase were used as amyloplast marker enzymes. The results of the experiment are discussed in a broad starch biosynthesis and tuberization project that is undergoing in CENARGEN.

INTRODUCTION

Root "tuber" formation as well as starch deposition, involves differential expression of genes. During this plant phenomenon, growth patterns are changed, tissue differentiation and specialization occur, and organs may become a deposit of starch and proteins. Several plant endogenous factors as well as environmental factors appear to influence those processes. Despite the precise stimuli that induce tuberization and starch accumulation, the molecular changes during cell differentiating events need to be investigated in the cassava root.

The changes associated with tuber formation appear to be a switch in the plane of cell division, an increase in the mitotic index of the meristematic cambium and the rate of starch deposition. Whilst the activities of enzymes regulating starch biosynthesis increase substantially during the early stages of tuber development, these also occur in other tissues such as sotolons (Salanoubat and Belliard, 1989). Therefore these changes in gene expression should be considered an accompanying process during tuber formation. Since there is no basic information on cassava root tuber formation and enzyme related to starch synthesis we started a project to generate basic information in order to better understand these plant developmental process as well as to define a plant model system to study in cassava.

Our preliminary results indicate a quantitative and qualitative change in the polypeptide composition of proteins from cassava root during tuberization. In addition enzymes related to starch biosynthesis is also evaluated in amyloplast produced via protoplast preparation.

METHODS

Field grown cassava plants were harvested 90 days after planting and roots at different stages of development were used for amyloplast isolation and protein extraction. Root developmental stages were defined as Stage I - root of less than 1,0 cm diameter, Stage II - roots with 1,0 to 2,0 cm diameter, Stage III - roots of more than 2,0 cm diameter. About 5.0 g of fresh roots of three stages of root tuberization (I, II, III) were ground in a polytron in phosphate buffer. Samples were exhaustively dialysed against water, concentrated and kept in the freezer at -20°C.

Tuberized root (Stage III) was used to prepare protoplast in an osmotically buffered medium containing wall digesting enzymes. After releasing the protoplast from the root slices, they were filtered through a nylon mesh of 100μm and layered on a discontinuous Ficoll density gradient. Intact protoplasts sedimented into the 25% Ficoll layer after setting for more than 2.0 h on ice. Intact protoplast were washed in an osmotically buffered solution and lysed in a syringe made from a bent needle. The amyloplast (Echeverria et al. 1985) suspension was layered in a discontinuous Ficoll density gradient and sedimented for more than 3.0 h setting on ice.

Fractions of all steps in the preparation protoplast and amyloplast were visualized in an inverted fluorescent microscopy. Samples were spread in several glass slides and we added of drops of 3,3' *Dihexyloxacarbocyamine or iodine.* The slides were examined in a 1000 magnification and micrographs recorded on Kodak Ektachrome 400 films. *ADPglucose pyrophosphorylase* (EC 2.7.7.27) was assayed based on the formation of G1P, interconvertion of G1P into G6P, and the oxidation of NADP to NADPH in a coupled reaction system. Absorption at 340 nm on time rate was measured. *Starch synthase* (EC 2.4.1.21) was assayed based on ADP/ATP interconvertion, and the oxidation of NADP to NADPH absorption at 340 nm on time rate was measured. *Branching enzyme (Qenzyme)* (EC 2.4.1.18) was assayed based on the reducing end of branches (Nakamura et al. 1989). Absorption at 540 nm on time rate was measured.

RESULTS

Table 1 indicates the quantitative variation in protein content of root tuber at different stages of tuber development. Values of less than 1.0% protein are similar to values reported in cassava. Although the amount of protein did not vary between cultivars studied, there was a large variation on the protein profile when analysed in gel electrophoresis. It is observed in Figure 1 that cv. Pioneira showed a different protein profile in a 10% PAGE than cv. IAC 12-829. It is also observed that several proteins, which increase in cv. Pioneira as the tuber develops, are not present in cv. IAC 12-829. On the other hand two large proteins are present in both cultivars at any stage of tuber development.

We also developed a procedure to isolate root amyloplast via protoplast preparation in order to study compartmentalization of starch biosynthesis in the

cassava root. Our preliminary results indicated that amyloplast can be isolated in a discontinuous Ficoll gradient at interface of 30% and 20% Ficoll, as indicated (Table 2) by the activity of the marker enzymes (Rees and Entwistle, 1989) as well as observation in fluorescent microscopy (not showed).

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Table 1. Protein content in cassava root at different stage of tuberization.

Cultivars	Protein content (mg/g root FW)		
	Stage I	Stage II	Stage III
Pioneira	1.56	2.26	2.47
IAC 12-829	1.10	2.78	2.21

Table 2. Activities of different markers enzymes in different fractions during amyloplast isolation from cassava tuber.

Description	Enzyme activities ADPG pyrophosphorilase	Starch synthase	Branch enzyme
Ficoll Lower layer (30%)			
lysed	0.604	0	390.6
non lysed	0	0	59.42
latent	0.604	0	331.18
% intactness	100%	0	85%
Ficoll uper layer (20-25%)			
lysed	0.830	0	5002.0
non lysed	0	0	121.14
latent	0.830	0	4880.86
% intactness	100%	0	98%
Polytron extraction	0.226	7.60	169.47

* Activity is defined as formation of NADPH measured at 340 nm absorbance per unit of time (min) per ml of purified extract.

** Activity is defined as the amount of change in 540 nm absorbance per unit of time (min) per ml of purified extract.

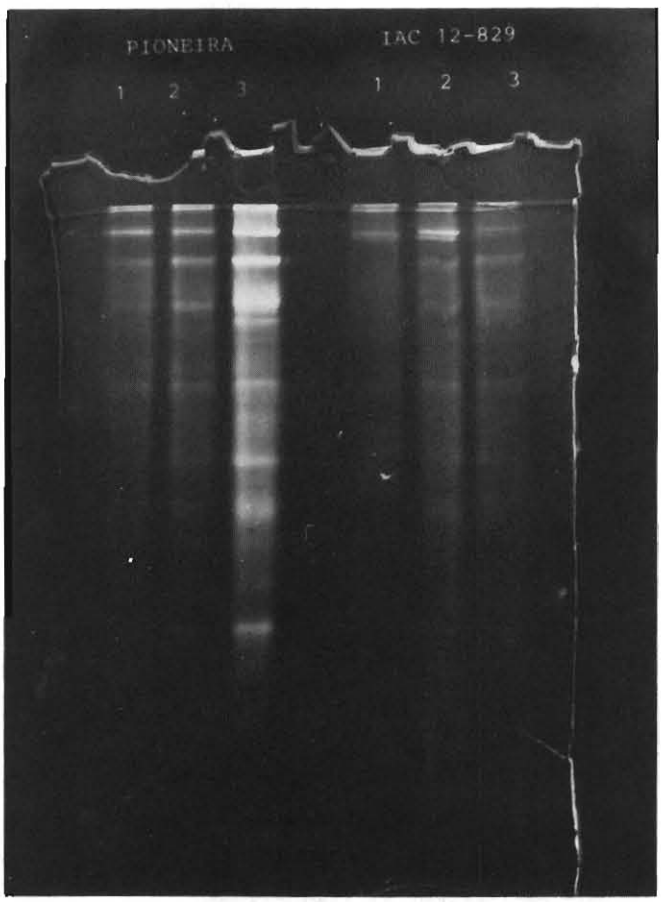

Figure 1. Comparison of protein crude extract from cassava root at different stages of development. Total proteins (25~g) were separated in native 10% PAGE and stained with comassie blue.

DIFFERENTIAL INHIBITION OF TRANSIENT GENE EXPRESSION IN CASSAVA ROOT AND LEAF TISSUES

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In order to determine whether the cauliflower mosaic virus 35S promoter could drive gene expression in different cassava tissues, we introduced a plasmid containing this promoter fused to the ß-glucuronidase reporter gene into cassava tissues by particle inflow injection. We observed several hundred blue spots or localized transformed regions expressing ß-glucuronidase activity in leaves but virtually none in roots. A possible explanation for this differential expression is that foreign DNA could be degraded by endogenous nucleases of the roots before it is expressed. Incubation of phage DNA with root extracts resulted in complete degradation of the DNA in as little as 10 minutes, unlike incubation with leaf extracts. Comparative analysis of the DNase activity in roots versus leaves indicates that there is substantially higher DNase activity in the root tissue than leaf tissue. We suggest that the differential abundance of nuclease activity in the two tissues accounts for the tissue specific differences in transient DNA transformation and gene expression in cassava.

INTRODUCTION

It is estimated that over 300 million people in tropical countries subsist on a cassava based diet, however, cassava roots have a low protein content and contain cyanogenic glycosides which can cause neurological disorders (3,9). These and other features may be amenable to modification via genetic transformation but first it is necessary to identify strong root specific promoters which can be used to drive the expression of introduced genetic material.

In order to identify such promoters, we introduced plasmids containing various promoters fused to the bacterial B-glucuronidase reporter gene (GUS) into cassava tissues via a particle inflow gun. Using a cauliflower mosaic virus 35S (CaMV 35S) promoter gene fusion, we routinely observed significant differences in the number of transiently transformed regions (blue spots) expressing GUS activity in cassava leaf and root tissues. Comparative analyses of the levels of DNase activity and ß-glucuronidase inhibitor(s) in roots and leaves indicated that there were significant differences in these activities between different tissues.

Based on these observations, we suggest that the tissue specific differences in the number of transforming events may be due either to differences in the ability to deliver DNA intact to the nuclei or to inhibition of ß-glucuronidase activity.

MATERIALS AND METHODS

Plant Material

Seeds and/or stem cuttings of cassava (Manihot esculenta Crantz) varieties Mcol 2215 and Mven 25 were obtained from the International Center for Tropical Agriculture (CIAT), Cali, Colombia. Plants were grown under greenhouse or in vitro conditions (10).

Transient DNA Transformation

Plasmid pBI221 (Clontech), containing the CaMV 35S promoter fused to the ß-glucuronidase gene and a NOS terminator sequence or plasmid pBinGSGUS containing the soybean glutamine synthetase promoter fused to the GUS gene (5) was precipitated onto DNA coated tungsten particles according to the procedure of Finer and McMullen (4). Tungsten particles (2.5 μl) were shot into cassava leaf or root tissues using a particle inflow gun at a helium pressure of 80 psi (4,7). In each case, an equal area (3 cm circle) of plant material was shot in a Petri dish containing standard culture medium for cassava (10).

Transient expression of GUS activity was visualized by staining with X-Gluc, according to the procedures described by Jefferson (7).

Crude Protein Extracts

Cassava root and leaf proteins were extracted from 1 gm of in vitro (sterile) grown plant tissue. The plant tissue was ground to a fine powder in liquid nitrogen in 3 ml of 50 mM HEPES, pH 7.5, 5,0 mM $MgCl_2$, and centrifuged twice at 23,000 x G for 15 min to remove cell debris (1). The supernatant was stored at -80 °C until use. Protein concentrations were determined by the method of Bradford (2).

DNase Activity

One μg of lambda phage DNA was incubated for various time intervals with cassava crude protein extract in 25 μl final volume of buffer containing 2.0 mM $MgCl_2$ and 25 mM HEPES pH 7.5 at room temperature. The DNA was then electrophoresed in a 1.2% (w/v) TBE agarose gel containing ethidium bromide (0.5 μg/ml) and photographed using a UV transilluminator.

DNase activity was also quantified by determination of precipitable DNA following incubation with crude protein extracts (8). Random primer ^{32}P-labelled DNA (30 ng) was incubated with 0.3 ug of crude cassava protein extract in 2 mM $MgCl_2$ and 25 mM HEPES, pH 7.5 at room temperature in a final volume of 25 μl. The reaction was stopped after 30 min incubation by the addition of 100 ul of a solution containing salmon sperm DNA (500 μg/ml) and 20 mM EDTA, pH 8.0, followed by addition of 14 μl of 100% (w/v) TCA. The solution was then incubated on ice for 15

min, pelleted at 13,500 x G for 15 min and washed with 1 ml of 10% (w/v) TCA followed by 1 ml of 70% (v/v) ethanol. The precipitated DNA was resuspended in 400 µl of water for quantification by liquid scintillation counting (1).

ß-glucuronidase Activity

Bacterial ß-glucuronidase (Sigma) was incubated with or without cassava tissue extracts in a reaction mixture containing: 500 ul of 75 mM potassium phosphate buffer, pH 6.8 plus 0.1% (w/v) BSA; 250 µl of 3.0 mM p-nitrophenol ß-D glucuronide in 50 mM HEPES, pH 7.5; 5 µl (4 units) of ß-glucuronidase and various amounts of tissue extracts in a final volume of 1,500 µl. Following 30 min incubation at 37 °C the reactions were stopped by addition of 5 ml of 0.2 M glycine, pH 10.4 and the nitrophenol produced was quantified spectrophotometrically at 400 nm. Control blanks were prepared the same way except that the glycine buffer was added prior to addition of the ß-glucuronidase solution. In some assays tissue extract was added before addition of the substrate. The tissue extract/ß-glucuronidase mixtures were then pre-incubated for various times, with and without PMSF, prior to initiation of the assay.

RESULTS AND DISCUSSION

In order to determine whether the CaMV 35S promoter would drive gene expression in different cassava tissues we shot a CaMV 35S-GUS reporter gene construct into cassava and quantified the number of regions transiently expressing ß-glucuronidase activity. We observed numerous localized regions of GUS activity in leaf tissue but virtually none in roots. Similarly, using a soybean, root specific promoter from the glutamine synthetase gene (5) we observed no expression of ß-glucuronidase activity in roots (Note: no GUS activity was observed in leaves either using this promoter). One possible explanation for why root specific transient gene expression may be reduced is degradation of the introduced DNA by endogenous nuclease activity (6). In order to determine whether there were tissue specific differences in total nuclease activity, crude tissue extracts were incubated with lambda DNA for various time intervals and DNA integrity was analyzed. In the presence of 0.2 ug of root protein lambda DNA was substantially degraded in as little as 10 min, whereas there was little evidence of DNA degradation in the presence of 20 fold higher levels of leaf protein (4 µg) even after 2 hours of incubation.

In order to quantify the level of DNase activity, we quantified the amount of precipitable ^{32}P-labeled DNA following incubation with crude tissue extracts. It was found that 26% of the labeled DNA was degraded when incubated with 0.3 µg of root crude protein extract. This level of DNase activity was 3.25 times higher than that found in leaf crude extract (1). This difference was even more apparent when we used reduced amounts of extracts(0.15 µg of protein). It is apparent that the DNase activity in roots can cause substantial damage to high molecular weight DNA (Note: similar effects were observed with pBI221 and pBinGSGUS plasmid DNA). At present, the cellular location of the DNase activity is not known, however, preliminary

studies indicate that the pH optimum for this activity is between 6.0 and 6.5, suggesting that it may not be cytoplasmic.

We also investigated the possibility that the lack of GUS expression in roots could be due to the presence of inhibitors of GUS or proteases. The addition of root extract equivalent to 13% of the total assay volume inhibited GUS activity by 34%. Expressed on a protein basis, 20 μg of root extract was 5 fold more effective in inhibiting ß-glucuronidase activity than was an equivalent amount of leaf extract. Since the protein concentration of root extracts was generally several times less than that of leaf extracts, expression of GUS activity on a volume basis is probably more reflective of the in vivo conditions than is expression on the basis of protein concentration. In addition, we also compared the effect of pre-incubation of ß-glucuronidase with tissue extracts on GUS activity. We observed no differences in GUS activity between 0 and 60 min pre-incubation with root extract either in the presence or absence of PMSF. These results suggest that GUS inhibition is rapid and that proteolytic (serine type) activity is probably not responsible for the inhibition of GUS activity by root extracts.

In conclusion, these results suggest that transient gene expression assays, particularly those using the B-glucuronidase reporter gene, may not be feasible for analysis of gene expression in cassava root tissue.

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TRANSIENT GENE EXPRESSION IN CASSAVA PROTOPLASTS

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The aim of the project is the development and improvement of methodologies for gene transfer in cassava. Isolated protoplasts provide a convenient system for studying the parameters influencing the transformation of protoplasts by direct DNA uptake. Transient expression studies have utilized a plasmid pAL carrying the ß-glucoronidase gene under control of 35S-CaMV promoter and nos polyadenilation region, following electroporation-mediated plasmid uptake. Protoplasts were isolated from cassave leaves (var. MCol22) growing in vitro, resluspended iln elevtroporation buffer (20 mM MES, pH 5.8, 10 mM CaCl2) and 20 µg ml-1 plasmid. A viability culrve of the protoplasts was ilnitially established utilizing different electroporation parameters. The optimum electroporation parameter gave an electric field of 715 V.cm-1 and a tilme constant =12.8 msec. The expression of the gene product was analyzed by a qualitative and quantitative ß-glucuronidase enzyme assay. The results obtained from the foundation to study direct gene transfer in cassava. In addition, may allow a rapid method to evaluate the functional expression of tissue-specific promoters. Nevertheless, experiments have been carried out utilizing particle bombardment using tissues with regeneration capability. The production of transgenic plants should facilitate, in the future, studies to the introduction and expression of important agronomically traits in cassava.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most important crop species in the world. Cassava is essentially used as basic food in tropical and subtropical areas. In addition, cassava could be used as source of energy and in animal food (CIAT, 1980).

In recent years, the transformation of higher plants has become a powerful tool in improving conventional breeding programs of agronomically important crops. The direct gene transfer provides a convenient system for plant genetic transformation without the use of Agrobacterium vectors.

Electroporation is a technique that allows the introduction of gene into plant cells (protoplasts) and their functional integration into the host genome (Fromm et al., 1985; Shillito *et al.*, 1985).

We describe here the development and improvement of a methodology for cassava protoplasts electroporation using the ß-glucuronidase as reporter gene.

MATERIAL AND METHODS

Protoplasts were isolated from cassava plantlets growing "in vitro" (var. MCOL 22). Leaves were sliced into small strips and put in Petri dishes containing 20 ml of an

enzymatic mixture solution. Plasmolysis was carried out for 16-18 hours in the dark, at 28°C. The digestion mixture was then filtered and protoplasts were collected by centrifugation. After the last wash, protoplasts were resuspended in the electroporation buffer (20 mM MES, pH 5.8; 10 mM CaCl2) to obtain a final density of 106 protoplastsl/ml, to which 20 μg/ml of pEA 18 plasmid were added. This plasmid carries the ß-glucuronidase (GUS) gene under the control of 35S-CaMV promoter and the nopaline synthase (nos) polyadenylation region.

The protoplast suspension was electroporated with 3 and 25 μF at voltages ranging from 250 to 2000 V/cm. The BIORAD electroporation apparatus was used in these experiments. Electroporated protoplasts were then transferred to culture medium (a modified To medium; Bourgin et al., 1979) and incubated at 28°C.

Protoplasts viability (Figure 1) was determined by staining with fluorescein diacetate (FDA). After 24 hours, the protoplasts were collected and the expression of the GUS gene product was analysed by a quantitative enzyme assay, according to Jefferson (1987).

RESULTS AND DISCUSSION

A viability curve of the protoplasts was initially established using different electroporation parameters (Figure 2). For a capacitance of 25 μF. with the voltage ranging from 250 to 2000 V/cm,l GUS transient expression is roughly proportional to the voltages, reaches a maximum at 1250 V/cm, and then decreases. When a capacitance of 3 μF was used, no GUS activity could be detected in any voltage tested. Thus, the optimum GUS transient expression was obtained with protoplasts electroporated at 1250 V/cm at 25 μF, in a constant time of 12.8 msec. However, under these conditions, the protoplasts viability decreases significantly (Figure 2).

The influence of DNA concentration on the efficiency of electroporation was also analysed. As expected, GUS transient expression signals increased proportionally with the amount of pEA18 plasmid added in the protoplasts suspension (Figure 3).

The results presented here proved that electroporation could be used as an efficient technique to introduce foreign genes on cassava protoplasts. It is also a rapid method to evaluate the functional expression of tissue-specific promoters. We are now optimising the regeneration capacity from protoplasts derived calli and the electroporation protocol, in order to obtain transformed plants. The production of transgenic plants should facilitate, in the future, studies of expression of agronomically important genes in cassava.

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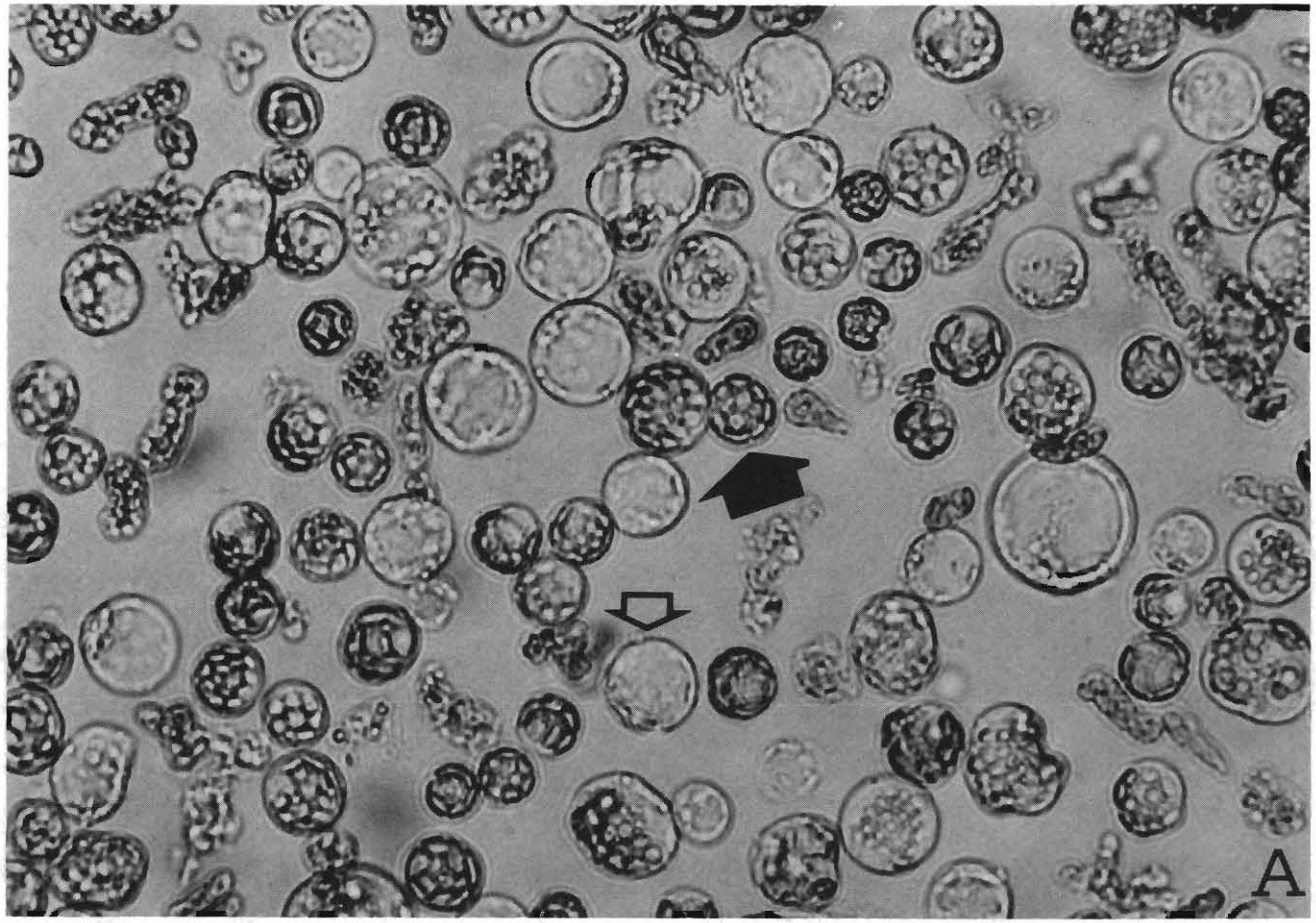


Fig. 1 A. Viability of protoplasts: Protoplasts from cassava leaves in visible light (200 x) (⬛) living protoplasts; (⬜) dead protoplasts.

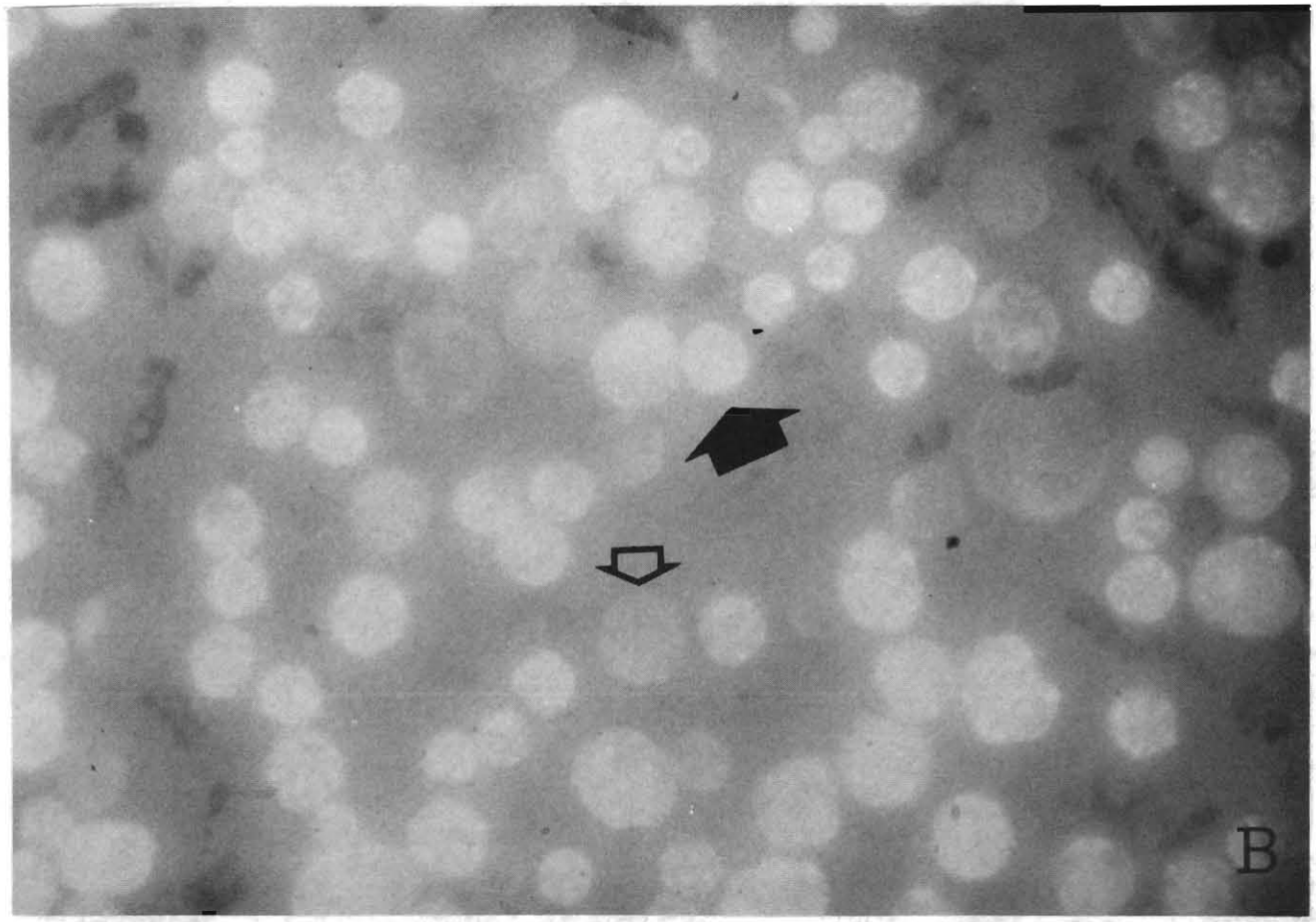


Fig. 1 B. Viability of protoplasts: Protoplasts stained with FDA and irradiated by ultra-violet light (200x). Living protoplasts show fluorescence (▬) when dead protoplasts do not (▭).

Fig. 2.

Cassava (Manihot esculenta CRANTZ)
Protoplasts viability
and GUS gene expression

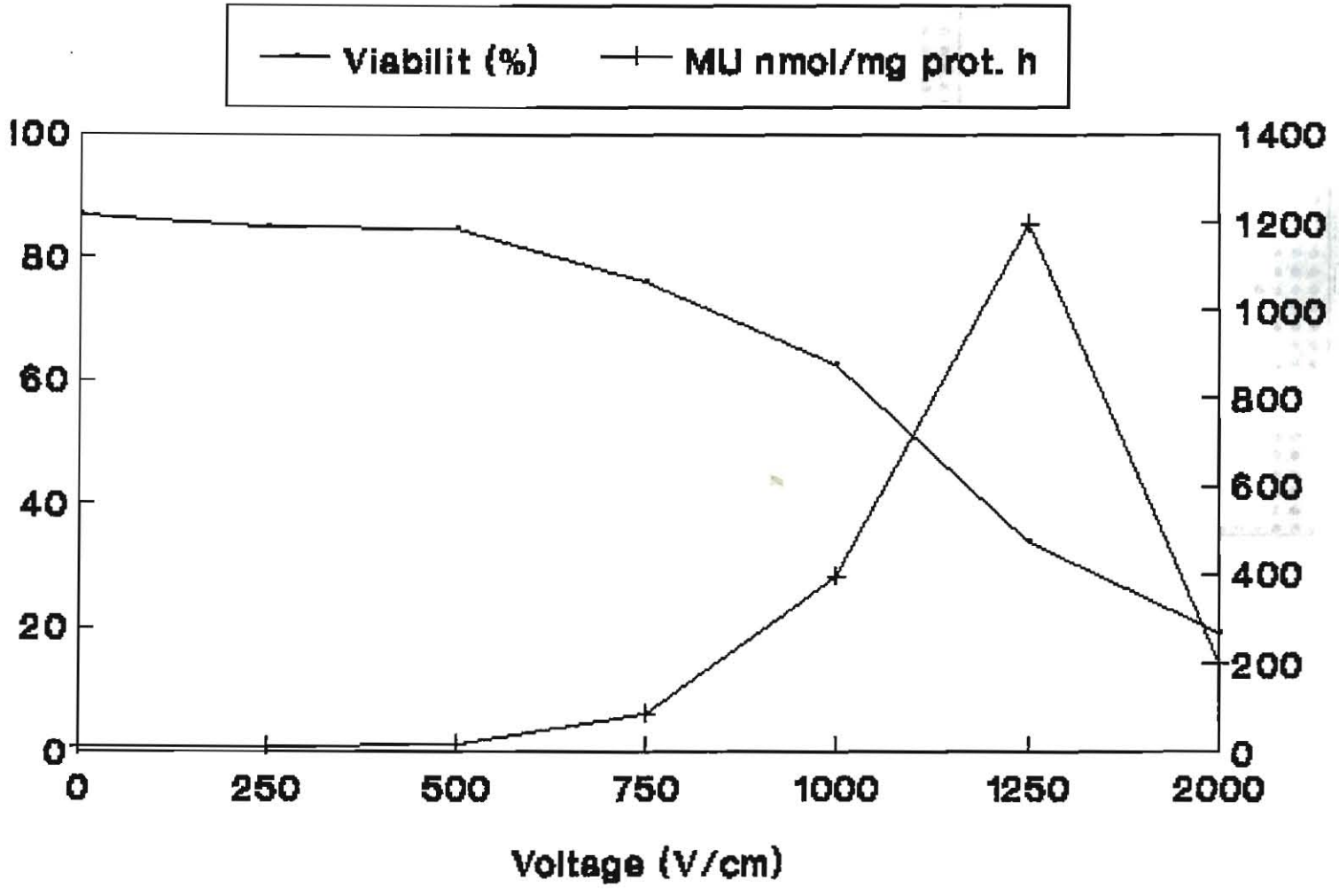
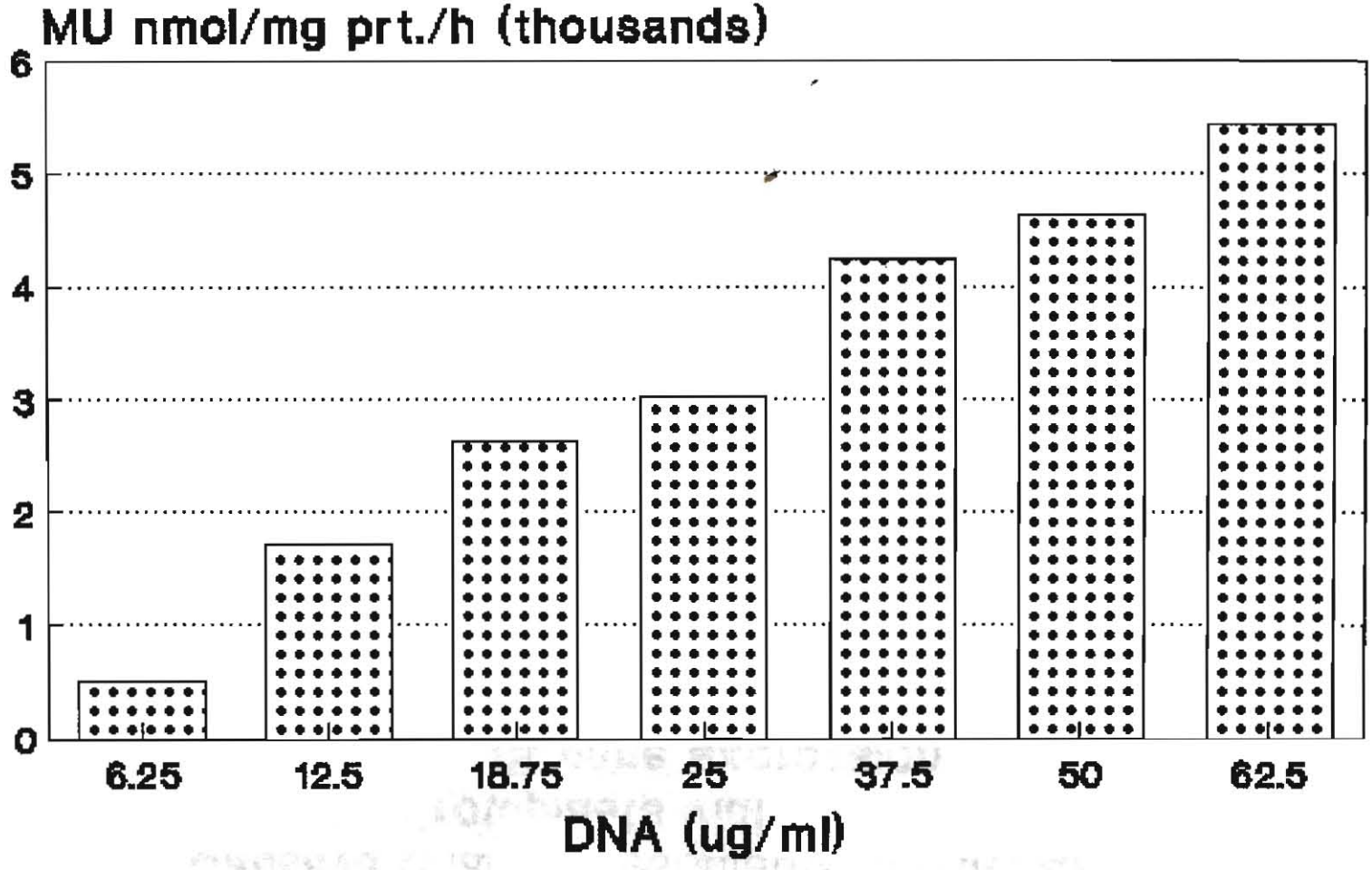


Fig. 3. **Cassava (Manihot esculenta CRANTZ.)**
DNA concentrations X GUS gene expression



1250 V/cm and 25 uF

OPPORTUNITIES FOR MANIPULATING THE AMOUNT AND COMPOSITION OF PROTEINS IN CASSAVA TUBEROUS ROOTS

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INTRODUCTION

The cassava tuber is formed by secondary thickening of the root parenchyma, and consists predominantly of starch (about 85% on a dry wt. basis) (Cock, 1985a). It is therefore consumed principally as a source of calories, and it has been estimated that about 70 million people throughout the tropics obtain more than 500 calories a day solely from cassava products (Cock, 1985a). The tuber contains only about 1-2% protein, and is also low in the essential amino acids valine, isoleucine, cysteine, methionine, tyrosine and phenylalanine (Yeoh and Chew, 1977). As a result the consumption of cassava as a sole source of protein can result in protein deficiency (Cock 1985b).

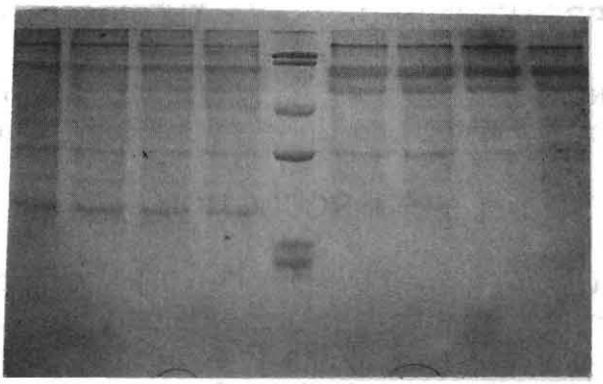
Although most cassava is used for direct human consumption (either fresh or in products such as gari and fufu), cassava flour is also used as a partial substitute for wheat flour in breadmaking. However, if the amount used exceeds 30% of the total the quality of the bread becomes very poor (Almazon, 1990).

We are studying the tuber proteins of cassava in order to manipulate their amount and composition to improve the quality for human nutrition and for substituting for wheat flour in breadmaking.

EXTRACTION AND CHARACTERIZATION OF TUBER PROTEINS

Two varieties of cassava were grown in a glasshouse at Long Ashton Research Station. These were CMC40, a low cyanide type and MCol1684, a high cyanide type. Tubers were peeled, chipped, lyophilized and milled to pass a 0.7 mm sieve. The total nitrogen contents of the two meals, determined by stable isotope ratio analysis (mass spectroscopy), were 0.356 (cv 1684) and 0.303 (cv CMC 40) % dry wt. Assuming a conversion factor of 6.25 these correspond to 2.22% and 1.89% protein respectively, although this does not allow for nitrogen present in cyanogenic glycosides and other non-protein components.

Preliminary experiments demonstrated that most of the proteins were extracted with water or dilute phosphate buffer (50mM), with a number of components separated by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE). These ranged in M_r from about 20,000 to 90,000, with considerable variation in the proportions of different components present in the two cultivars (Fig.1).

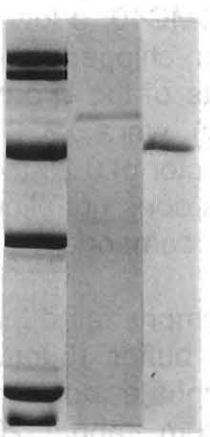


a b c d e f g h i

Fig. 1 SDS-PAGE of total water-soluble proteins from tubers of cassava cv. CMC40 (a-d) and MCol1684 (f-i). Track e is M_r standards: 12,300, 17,200, 30,000, 42,700, 66,200 and 76,000.

In order to purify individual components, flour of cv. CMC40 was extracted with 50 mM phosphate buffer, pH7, containing 10 mM phenylmethyl sulphonyl fluoride (PMSF) (to inhibit serine proteases) and 10% (w/v) polyvinyl polypyrrolidone (to absorb phenolics). The supernatant was concentrated, dialysed and lyophilized.

Purification of individual components was achieved using a combination of several procedures, including anion exchange chromatography (on DE52), gel filtration chromatography (on Sephadex G-50 or G75), reversed-phase high performance liquid chromatography (on a Vydac C18 column) and preparative SDS-PAGE (using a Bio-Rad Prep Cell apparatus). This enabled a number of individual components to be purified (Fig.2) and transferred to ProBlot membrane for microsequencing.



a b c

Fig. 2 Purification of proteins from tubers of cassava cv. CMC40 by ion exchange chromatography on DE52 cellulose.

a, Mr standards (see Fig. 1 legend); b,c, purified proteins

Although it was possible to purify some individual components, the amounts were very small and the procedures were hindered by high levels of starch and other contaminants. In addition, the absence of any major components raised the question of whether any of the proteins were true storage proteins.

LIGHT MICROSCOPY OF CASSAVA TUBERS

In order to determine whether storage protein deposits were present sections of developing tubers of cv CMC 40 were stained for carbohydrate with Schiff reagent and for protein with Toluidine Blue and observed by light microscopy. This failed to demonstrate any protein deposits, indicating that true storage proteins were either not present or were present in amounts too small to form dense deposits.

STRATEGIES FOR MANIPULATING CASSAVA TUBER PROTEINS

The apparent absence of major storage proteins from cassava tubers will not necessarily limit our ability to improve the amount and composition of cassava tuber proteins by genetic engineering. A major consideration is, of course, where the protein content of the tuber is determined by source activity, sink activity or a combination of these. Limitation by sink activity would imply that increasing the sink "pull" by increasing the number of genes encoding tuber-specific proteins would result in greater transport of nitrogenous compounds and higher total protein. Alternatively it is possible that the activity of tuber protein gene expression is itself limited by availability of amino acids, in which case it would be necessary to improve the uptake and transport of nitrogenous compounds in order to increase total tuber protein.

Assuming that protein content in cassava is limited by the expression of genes encoding tuber - specific proteins, it should be possible to increase the total amount and improve the composition by inserting genes from other plants. One of the aims of our project is to isolate strong tuber - specific promoters for such genes.

An alternative approach would be to use available promoters from other systems, such as patatin or sporamin (the storage proteins of potato stem tubers and sweet potato root tubers respectively). Neither of these proteins is completely tuber-specific, as patatin is synthesised in stems and petioles under conditions that prevent tuberisation (Paiva et al, 1983), while sporamin is synthesised in stems during *in vitro* propagation (Hattori and Nakamura, 1988).

The nutritional quality of tubers could be improved by inserting genes for the lysine - rich chymotryptic inhibitor CI-2 from barley (Peterson et al, 1991) or the methionine and cysteine - rich 2S albumin from Brazil nut (Gander et al, 1991). However, in both cases the proteins are readily soluble in water, and could be lost during traditional processing to remove cyanogenic glucosides.

Improvement in the quality for mixing with wheat flours could possibly be achieved by inserting genes for wheat gluten proteins, especially the high molecular weight subunits of glutenin which appear to be largely responsible for the formation of elastic gluten polymers. A number of such genes are available (see Shewry et al, 1991), and the water insolubility of the proteins would eliminate the need for specific targetting.

The final limitation is, of course, our inability to stably transform cassava. Consideration of this topic is outside the scope of the present article, but once such methods become available it will be possible to put the above strategies to the test.

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QUALITY EVALUATION OF THE CASSAVA CORE COLLECTION AT CIAT

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A core collection of 630 cassava clones has been established at CIAT, representative of the complete germplasm collection of 5169 clones. All available clones in the core collection (560) were analysed for dry matter and total cyanogen contents, and starch was extracted for determination of amylose % values. Root parenchyma dry matter and total cyanogen contents had maximum and minimum values of 49-13% and 1041-7 mg kg^{-1} (fresh weight basis) respectively. Mean and median parenchyma total cyanogen contents were 102 and 55 mg kg^{-1} (fresh weight basis) respectively. Significant positive correlations were found between dry matter contents in peel and parenchyma, and between total cyanogens in peel and parenchyma. Maximum and minimum amylose % values of 28 and 15% were found.

Cluster analysis identified clones containing both high root dry matter (>43%) and low parenchyma total cyanogen contents (<20 mg kg^{-1}). Significant differences in starch functionality were found between clones with high and low total cyanogen contents.

INTRODUCTION

Cassava (Manihot esculenta Crantz) is the fourth most important source of calories for human consumption in the tropics, and is the most important root crop. The crop is mainly used for human food, either fresh or processed into a wide variety of products. Its importance for animal feed and as an industrial raw material is increasing. The crop is characterized by efficient production of carbohydrate in the form of starch (80-90% of root parenchyma by dry weight), by a rapid post harvest deterioration and the presence in all plant organs of cyanogenic compounds. Quality factors such as dry matter and cyanogen content are important in determining the acceptability and end use of different varieties.

CIAT holds the world collection of cassava germplasm, consisting of 5169 accessions. The collection has been characterized previously for root cyanide content using the qualitative picrate test (Williams and Edwards 1980), which has been demonstrated as insufficiently accurate for routine use (CIAT 1991). The collection has been evaluated for root dry matter content using a specific gravity estimation method (Grossman and de Freitas 1951), but not for starch content or any starch functional properties. In 1991 a core cassava collection was formed, comprising 630 accessions, which would represent the genetic variability of the entire collection as completely as possible. The accessions were selected based on morphological and biochemical characteristics, as well as geographical origin and pest and disease resistance. Some elite hybrids were also included. The core collection will be completely characterized for salient quality characteristics. This paper presents results for total root cyanogens, dry matter content and amylose percentage of the starch. The starch functional properties of some accessions are also presented.

A hypothesis has been proposed (Lathrap, 1973; Spath, 1971; Nye 1991 and Dufour, pers. com.) regarding the possible relationship between cassava root cyanogen content and the texture of processed products.They argue that cassava is processed in Amazonia principally to provide a storable, energy dense product suitable for transportation. Varietal selection for high quality processed products, (farinha, cassabe etc) has also resulted in a high level of root cyanogens. A strong preference therefore exists for high cyanide varieties for processing, despite the cultivation of low cyanide varieties in the same areas. If low and high cyanogen containing varieties produce different quality processed products, this could be detectable as differences in their starch functional properties.

MATERIALS AND METHODS

The core collection of the word cassava germplasm bank has 630 accessions. Of these, 566 (90%) were available for this characterization exercise. The remaining 64 accessions are held in vitro, and will be characterized at a later date.

All cassava roots were harvested at CIAT Palmira when plants were between 10 and 12 months of age. (ie at optimum maturity) for the ecozone in which CIAT is located). Approximately 15 clones were harvested each day. The germplasm bank contains six plants of each clone. A total of six roots were harvested per clone, usually two per plant from three plants, to provide a sample of approximately 3kg weight. Roots were packed into polyethylene bags and transported directly to the sample preparation area. Roots were held in the bags until removal for sample preparation. The maximum time between harvest and sample preparation was 6h. Roots were washed and peeled. Three slices were taken from the distal, central and proximal regions of each root, these were pooled and diced rapidly. Subsamples were taken for determination of total cyanogens, dry matter content, and other analyses not reported here (proximal analysis). In addition, starch was extracted from the remaining root parenchyma tissues, and dried at ambient temperature under ventilation, for later analysis of amylose and other starch functional properties. Samples were prepared similarly from the root peel.

Dry matter content was determined by drying 50g at 60°C for 24h; total cyanogens by using the enzymatic method of (Cooke, 1978) as modified by O'Brien et al.(1991). Amylose % was determined by the ISO method (IS0, 1987). On some samples, starch gelatinization and viscosity characteristics were determined using a Brabender viscoamylograph, a 5% starch paste (initial temperature 25°C) was heated at 1.5°C min^{-1} to 90°C and held for 20 min, before cooling at 1.5°C min^{-1} to 50°C, and holding for a further 15 minutes.

All statistical analyses were carried out using SAS (SAS, 1990). Basic information on means, standard deviations and quartile ranges was supplemented by univariate procedures. Correlation coefficients were calculated between all variables. Total cyanogen data were log transformed prior to factor and cluster analysis because of non-normal distribution.

RESULTS

Table 1 presents basic descriptive statistics for root parenchyma and peel separately, as well as the ratio between the two. The maximum dry matter content of the parenchyma, 48.9% is the highest ever reported for cassava. 15 clones had contents over 45%. The mean and minimum values of 34.2 and 13.0% are in accordance with previous reports (Wheatley and Chuzel, 1992). The dry matter content of the peel was generally lower than that of the parenchyma, although the maximum value of 46.1% is also higher than expected. The ratio of peel to parenchyma dry matter contents was below 1.0 for 84% of the clones analysed.

The total cyanogen contents are presented in fresh and dry weight base. On a dry matter basis, parenchyma total cyanogen contents ranged from 17 to 4126 mg kg^{-1}, or 7 to 1041 mg kg^{-1} on a fresh weight basis. These ranges are comparable with those reported previously in the (eg 35-3500 mg kg^{-1} dry weight basis, Wheatley and Chuzel 1992). The mean of 101.9 and median of 55 mg kg^{-1} fresh weight basis found in this study are both lower than expected. Figure 1 shows the frequency distribution of total cyanogens (fresh weight basis). This is highly skewed and unimodal, with no separation between high and low cyanogen containing varieties. The distribution of parenchyma dry matter % and amylose % is normal.

Both the maximum and minimum values for root total cyanogens in the peel are higher than for the parenchyma, reaching 8415 and 1983 mg kg^{-1} on dry and fresh weight bases respectively. The ratio between peel and parenchyma is generally above 1, with the maximum of 77 on a dry weight basis. Nevertheless, 7 clones were identified in which the total cyanogen content of the peel was lower than that of the parenchyma: these clones all had total cyanogen contents in the parenchyma over 200 on a fresh weight basis, except one (M Col 2526, 95mg kg^{-1}). The clones with the highest peel: parenchyma ratio were all of low total cyanogen content in the parenchyma (below 40mg kg^{-1} for the top 10 clones).

The amylose percentages in starch extracted from the parenchyma of each clone ranged from a maximum of 28 to a minimum of 15%. This relatively restricted range is comparable with previous literature reports based on the analysis of a more restricted range of clones.

A Pearson correlation coefficient matrix was calculated for all variables (Table 2). Because of the high number of clones (566) coefficients of only 0.150 were highly significant. Nevertheless, a clear positive correlation was found between root parenchyma and peel dry matter content. There was a strong negative relationship between the peel:parenchyma ratio and the parenchyma dry matter content. The same relation was also found for peel and parenchyma total cyanogen contents. In general there were no strong relationships between amylose % and other parenchyma variables, although there were some significant correlations (eg, with dry matter content).

Factor analysis was used to reduce the large number of variables to three factors, which encompass the variation found in the individual variables. For the purposes of this analysis, variables were selected which would be important to cassava used for processing (dry matter and cyanide contents of the peel and parenchyma, and amylose %). The five variables, comprising total cyanogens and dry matter content in peel and parenchyma, and parenchyma amylose %, were therefore subjected to principal component analysis, to identify the important factors involved. Three factors were identified which together accounted for 84.1% of the total variation occurring in the five selected variables (37.2, 32.0 and 14.9% for factors 1,2 and 3 respectively. Table 3 shows the distribution of the five variables among the three factors. Factor 1 is dominated by root cyanogens, Factor 2 by root dry matter content and factor 3 by amylose %. In all cases the relation between dominant variables and factors is positive.

The three factors generated in the previous analysis were used with equal weighting, as the basis for identifying and defining clusters of clones with similar characteristics. On the basis of the hiererarchical organisation of clusters and the R^2 values of each level in the hierarchy, 10 clusters were selected for further evaluation. Figure 2 provides a three dimensional representation of the centroids of each cluster with respect to the axes provided by the three factors. From this it can be seen that some clusters represent interesting combinations of characteristics, eg relatively high in root dry matter content but low in total cyanogens.

The clusters identified in this and other analyses, will be used to identify potential clones of interest to breeding programs and to select research materials for in depth study of specific root quality characteristics. For example, CIAT will undertake an evaluation of the starch functional properties of the core collection, based on taking representative clones from each of the clusters.

The 15 clones with the highest and lowest total cyanogen contents from the core collection were selected for detailed starch functionality studies (Table 4). Significant differences were found between mean values of the high and low cyanogen clones for all starch viscosity variables determined, plus the derived variables ease of cooking, gel instability and gelification index. Only the mean gelatinization temperature was similar for high and low cyanogen clones. The high cyanogen clones had lower starch viscosity values than low cyanogen clones. For example, M Col 2360 had a maximum viscosity of 168 and a viscosity at 90°C of 130 B.U., compared with mean values of 10 and 392 B.U. for low cyanogen clones. These results, although taken from a restricted number of clones, do strongly suggest that differences in starch viscosity characteristics, and hence in behavior during traditional processing, are significantly different between high and low cyganogen cultivars. It is interesting that most of the high cyanogen cultivars analysed for their starch properties were collected in the Amazonian region of Colombia and Brazil. In many crops, there is a strong relationship between starch functional properties and amylose % of the starch. In the case of cassava, this has not been shown: there was no correlation between amylose % and any of the starch

functional characteristics evaluated using the Brabender, and there was no significant difference in mean amylose % between the high and low cyanogen lones evaluated here.

CONCLUSIONS

The results presented in this paper are the first report of the total cyanogen contents and amylose percentage values from a representative selection of global cassava germplasm. The results demonstrate the genetic potential which exists to select germplasm low in total cyanogens for subsequent genetic improvement. The fact that over 50% of the core collection has total cyanogen contents below 55 mg kg^{-1} fresh weight of parenchyma is encouraging for the potential of traditional breeding to produce useful low cyanide hybrids. More importantly, there is no relationship between root dry matter and cyanide content, and several clones were identified which showed the useful combination of low total cyanogens and high parenchyma dry matter content. The low range of amylose percentage values found is interesting, but the relationship between starch functionality and amylose % is not clear, nor is the stability of amylose % in different crop environments. Wild species should also be screened for this characteristic.

This germplasm characterisation was undertaken using a small sample of roots from plants harvested at one locality at one time. The results, therefore, are not presented as absolute values for each cultivar, but are useful for general comparison purposes, and will be complemented by additional data from other seasons and locations.

Most of the clones high in total cyanogens originated in the Amazonian basin region of Brazil, Venezuela and Colombia. The preferences of Amazonian populations for high cyanide varieties may be related to the starch functional properties of these varieties. In this cassava consuming population, the sophisticated traditional processing methods used are entirely adequate to reduce the total cyanide contents of processed cassava products to innocuous levels (Dufour, 1988). This contrasts with the African situation where cyanide toxicity exists (Rosling, 1986). Future efforts to develop low cyanide varieties for populations currently cultivating and consuming products from high cyanide cultivars, should be aware of the potential importance of maintaining starch quality in new varieties, with regard to the products produced locally from this cassava.

As cassava is used more as an industrial raw material, varieties will be required with different starch functional properties. If some of these can only be found in the high cyanide varieties, problems of environmental contamination will become increase (eg waste water from starch extraction). In this case, separating the starch functionality from cyanogen content will become a valuable research endeavor.

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Table 1. Descriptive statistical analysis of total cyanogen content, dry matter % and CIAT amylose % of root parenchyma and peel from the core collection of the cassava germplasm.

Variable	N	Mean	Standard Deviation	Maximum	Median	Minimum	Range
Parenchyma							
Dry matter (%)	566	34.2	6.2	48.9	34.7	13.0	35.9
Total cyanogens (mg/kg, dry weight)	566	314.5	416.8	4126.0	164.0	17.0	4109.0
Total cyanogens (mg/kg, fresh weight)	566	101.9	124.2	1041.0	55.0	7.0	1034.0
Amylose (% starch)	503	22.3	2.1	28.8	22.3	15.3	13.4
Peel							
Dry matter	566	27.0	4.5	46.1	26.9	15.4	30.7
Total cyanogens (mg/kg, dry weight)	566	1871.2	1102.9	8415.0	1638.0	204.0	8211.0
Total cyanogens (mg/kg, fresh weight)	566	498.3	287.2	1983.0	437.0	55.0	1928.0
Peel: Parenchyma							
Dry matter	566	0.8	0.1	1.4	0.8	0.5	0.1
Total cyanogens (dry weight)	566	11.0	7.4	77.6	10.5	0.5	77.2
Total cyanogens (fresh weight)	566	8.4	5.0	42.7	8.3	0.5	42.1

Table 2. Correlation coefficient matrix between cassava root dry matter %, amylose % and total cyanogen contents in parenchyma and peel.

	Parenchyma			Peel		Peel: Parenchyma	
	Dry Matter	**Total Cyanogens**	**Amylose**	**Dry Matter**	**Total Cyanogens**	**Dry Matter**	**Total Cyanogens**
Parenchyma							
Dry Matter	1.000	-0.005	-0.342***	0.585***	0.313***	-0.579***	0.258***
Total Cyanogens		1.000	-0.232***	0.161***	0.643***	0.162***	-0.681***
Amylose			1.000	-0.342***	-0.187***	0.052	0.103
Peel							
Dry Matter				1.00	0.184***	0.296***	-0.032
Total Cyanogens					1.00	-0.192***	0.027
Peel: Parenchyma							
Dry Matter						1.00	-0.318***
Total Cyanogens							1.00

***, significant at P=0.001.

Table 3. Results of factor analysis using dry matter %, total cyanogen content and amylose % as variables.

	Rotated Factor Pattern		
	Factor 1	**Factor 2**	**Factor 3**
Dry matter %, parenchyma	0.046	0.957	-0.076
Dry matter %, peel	-0.134	0.707	-0.445
Total cyanogens, parenchyma[1]	0.807	-0.236	-0.341
Total cyanogens, peel[1]	0.936	0.138	0.140
Amylose %	-0.090	-0.229	0.876

[1] Dry matter basis only.

Table 4. Mean values of amylose % and starch functionality characteristics for 15 clones of extreme low and high total parenchyma cyanogen content.

	Low CN group	**High CN group**	**Significant Difference (P=)**
Total cyanogen (mg kg^{-1}) parenchyma, fresh basis	13	461	0.001
Amylose %	22.9	21.6	n.s.
Gelatinization temp. °C	64.1	64.7	n..s
Maximum viscosity	509.5	359.5	0.0001
Viscosity at 90°C	391.5	230.2	0.0001
Viscosity after 20 min at 90°C	232.6	133.5	0.0001
Viscosity at 50°C	391.3	198.7	0.0001
Ease of cooking	10.2	6.3	0.0001
Gel instability	276.9	222.1	0.014
Gelification Index	158.7	65.3	0.0001

Notes: Ease of cooking = Time to Vmax - Time to gelatinization,
Gel instability = Vmax - V90/20
Gelification Index = V50-V90/20

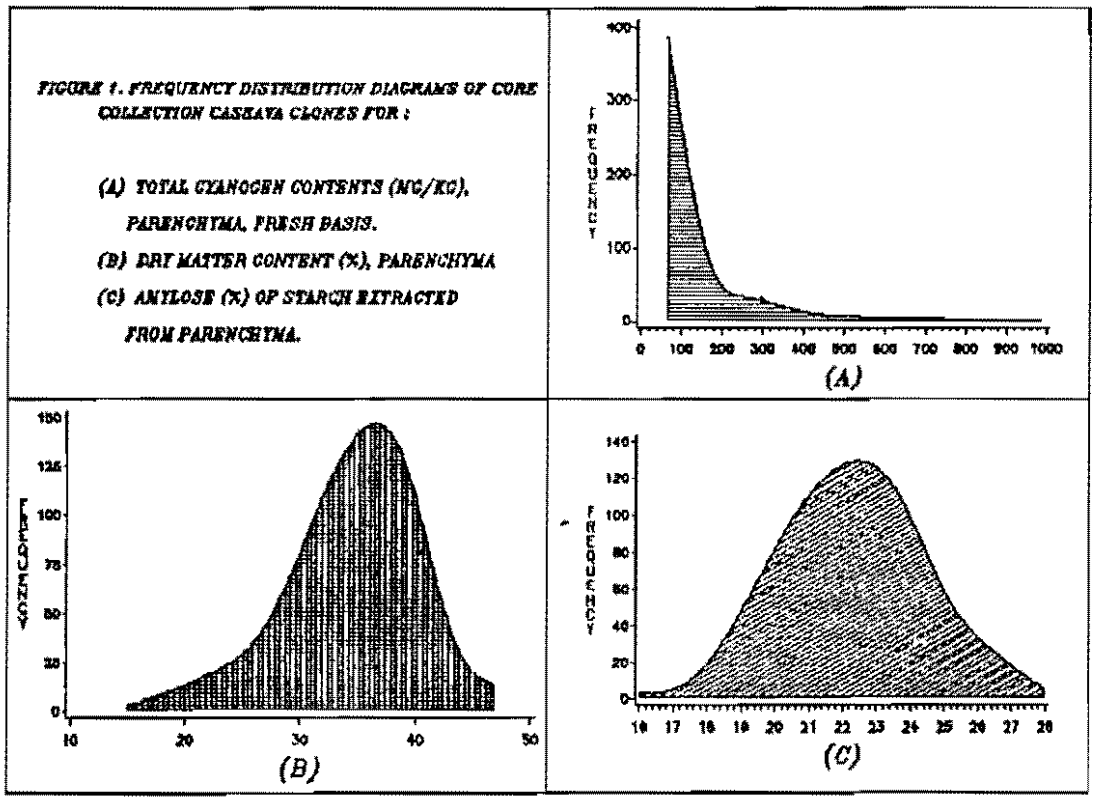
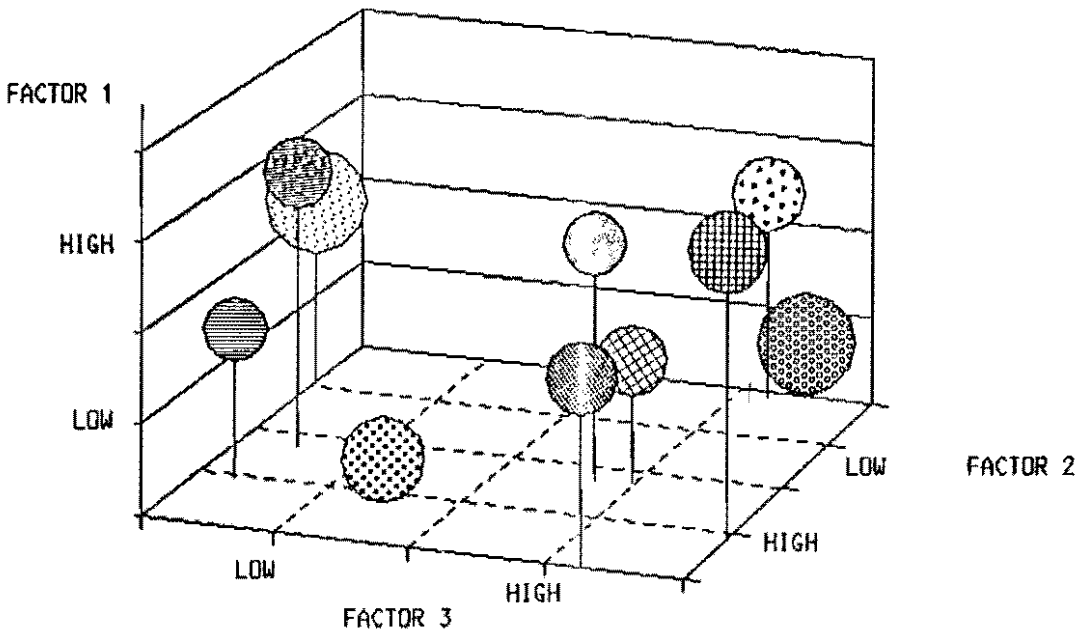


FIGURE 2. REPRESENTATION OF 10 CLUSTERS OF CLONES, USING THREE FACTORS AS AXES. (FACTOR 1: TOTAL CYANOGEN. FACTOR 2: DRY MATTER %. FACTOR 3 AMYLOSE %)



NOTE: THE RADIUS OF EACH SPHERE REPRESENTS THE MEAN DISPERSION OF EACH CLUSTER

STARCH PROPERTIES IN RELATION TO COOKING QUALITY OF CASSAVA

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Cassava tubers exhibit wide variation in cooking quality depending on varietal differences and environmental conditions. There have been few studies on the relationship between cooking quality and properties of starch extracted from different varieties. Hence studies were undertaken at CTCRI, University of Nottingham and NRI on starch extracted from five varieties of differing quality. The physicochemical and rheological properties were examined in detail. Only minor differences were observed in the total and soluble amylose contents. Though the average granule size was nearly similar among the varieties, the granule size distribution was quite different for var. H-1687. The Brabender Viscographic pattern was also quite distinct for this variety. The swelling volume of starch of var. M4 was invariably the lowest among the varieties. There was no difference in the XRD patterns, but the DSC patterns wee noticeably different for starch of H-97 and M4. Though the results do not give any conclusive relationship between cooking quality and starch properties, the existence of differences can be useful in unravelling any possible relationship.

INTRODUCTION

Though cassava, of late, is assuming more and more importance as an industrial crop, it continues to be a secondary staple in many parts of the world. In Kerala, for example, around 70% of the produce is still used for human consumption. However, the consumers are very choosy about the taste and quality of cooked tubers. This is amply clear from the fact that the variety M4 released into Kerala around forty years ago continues to enjoy pride of place as the most popular variety for culinary purposes. The cooked tubers are soft and mealy in texture and have a characteristic flavour and taste. Work done in CTCRI and elsewhere has shown that biochemical constitution alone does not decide the cooking quality of the tubers. There have been only few studies on the properties of starch from different varieties in relation to quality, though it is a very important area for consideration (Raja et al; 1978, Emilia Abraham et al, 1978, Moorthy, 1985). Recently Asaoka et al (1992) have examined the physicochemical properties of starch from four cultivars of cassava of differing quality grown in Colombia. Their results indicate only minor variation in starch properties and no clear relationship between cooking quality and starch properties was obtained. This paper describes the results obtained in a study of properties of starch from five varieties grown in India having different cooking quality.

EXPERIMENTAL

Five varieties of cassava (H-97, H-165, H-856, H-1687 and M4) cultivated on CTCRI's farm in accordance with standard practices, were harvested at the 9 month stage. Starch was extracted by the standard procedure. The granule size was

determined by light microscopy while the granular size distribution was obtained on a Coulter counter.

The amylose content was determined colorimetrically. DSC of the samples was run on a Perkin Elmer DSC-2 equipment using indium as standard. After the run, the pans were punctured and dried in an oven to obtain weight of starch actually present, in order to calculate enthalpy of gelatinisation. Pasting characteristics were monitored on a Brabender Viscoamylograph. A 5% suspension of the starch was heated from 50 to 95°C at a rate of 1.5°C min^{-1}, maintained at 95°C for 10 min, and lowered to 50°C at the same rate. Swelling volumes were obtained by the procedure of Schoch (1964). A Phillips XRD instrument was used for getting the X-ray diffraction patterns.

RESULTS AND DISCUSSION

The average granule size of starch of the different varieties was not very different, though among them H-165 starch had the highest value. In the Coulter counter measurement, a distinct difference in the granule distribution pattern of variety H-1687 was noticed. Whereas H-97, H-165 and H-856 had almost similar distribution of granules in the range 6-30 m, the variety H-1687 had more granules in the range of 13-16 m and relatively less in the range 6-13. Variety M4 starch was also slightly different in its distribution pattern, but the difference was not so prominent as for H-1687. It is interesting that these two varieties have relatively better cooking quality. Asaoka et al (1992) have not observed such a noticeable variation among the varieties she studied. Though our results do not establish any definite relationship between granule size and quality, it points out the necessity of examining the granule size distribution of more varieties.

The amylose content determined colorimetrically showed minor variation among the varieties Table 1. Interestingly, M4 having the best quality had the highest value among them, and H-165 poorest in quality had the least amylose content. However the difference cannot be considered significant enough to draw any definite conclusions. Olorunda et al (1981) had observed that higher amylose contents, led to higher mealiness. More studies may be required for arriving at a definite conclusion. GPC profiles of the five varieties also confirm the results that there is only minor variation in the amylose content among the varieties. Asaoka et al (1992) did not observe any significant difference in the amylose contents in the Colombian varieties either. GPC profiles on the debranched amylopectin fractions were broadly similar to one another showing that the structure of amylopectin of the varieties is mostly similar.

DSC data of the different varieties are given in Table 2. The results indicated that there was a difference of 3-4°C in the T_{onset} among the varieties, with the highest value being obtained for H-97 and the lowest for H-165. Though the T_{end} also exhibited almost similar trend, M-4 starch had a wider temperature range compared to other varieties, which is very easily perceptible in the DSC graphs. Asaoka et al.

(1992) also identified one cultivar having higher gelatinisation temperatures compared to the others; but could not correlate this character with cooking quality. Our earlier results also indicated a higher gelatinisation temperature for M4, which has better quality. Another feature observed is that the gelatinisation temperature we obtained was 10-15°C higher than the values reported by Asaoka and 5°C higher than that obtained by Yamada et al (1987) for Thailand starch. All these results point out the necessity for comparing the starches from different countries to identify genetic and environmental factors leading to quality parameters. The enthalpy of gelatinisation of the samples varied from 2.63 to 3.43 Cal g^{-1}, the highest being for H-97. There were no significant differences among the varieties but the values were higher than those reported by Asaoka et al (1992).

The XRD pattern of all the five varieties were similar, and also similar to the ones obtained for the Colombian varieties. The absolute crystallinity values were also within short ranges, viz. between 10.5 and 14.4. Similar values were obtained by Asaoka et al (1992) and our values are also much lower than those reported earlier by Zobel (1988). The results indicate that there was not much difference among the varieties in terms of crystallinity.

Interesting results emanated from the emylography of the starches. One of the most striking observations was the high peak viscosity, high viscosity breakdown and only minor set-back viscosity for starch of variety H-165. While the viscosity breakdown for H-1687 was low, it underwent high set-back during cooling. M4 was somewhat similar to H-1687, though its set-back viscosity was not so high. The other two varieties had only low viscosity breakdown and set back viscosities. Here again, we found that H-1687 and M4 starches behaved slightly differently from the other varieties. Olorunda et al (1981) have tried to predict mealiness in cassava on the basis of viscosity and suggested that mealier cassava cultivars had slightly higher peak viscosities. However, our results do not show a similar trend, in that H-165 which had the highest peak viscosity has poor quality. Asaoka et al (1992) could not obtain any correlation between quality and viscosity properties.

The swelling volumes of the different cultivars are presented in Table 1. M4 starch had the lowest value among the different cultivars while H-165 had the highest. The data with these two varieties revealed some relationship between quality and swelling volumes but not for the other varieties. Asaoka et al (1992) could not correlate the cooking quality with swelling power.

Thus the results point out that though it may not be possible to assign any particular property of starch in deciding cooking quality, many factors like granule size, amylose content, and swelling patterns may be contributing in one way or other. Only a concerted and systematic analysis of a large number of samples of starch from varieties with very wide variation in quality will give concrete information on the factors determining quality.

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Table 1. Granule size, amylose content and swelling volumes of starch.

VARIETY	GRANULE SIZE m	TOTAL AMYLOSE %	SOLUBLE AMYLOSE %	SWELLING VOLUME ml g^{-1}
H.165	8.1-47.5	22.6	5.1	38.6
H.97	5.4-43.2	23.4	5.7	34.8
H.1687	5.4-40.5	25.1	6.2	35.4
H.856	5.9-42.5	24.1	6.1	35.0
M-4	5.4-39.5	26.2	5.9	30.0

Table 2. DSC data on the starches of different varieties.

Var.	T_{onset} °C	T_{max} °C	T_{end} °C	H Cal g^{-1}
H.97	69.36	72.29	77.13	3.43
H.165	65.35	69.22	74.86	3.27
H.856	65.62	70.14	74.94	2.65
H.1687	67.12	71.45	75.39	3.15
M-4	68.20	73.24	78.54	2.95

CAROTENOID PROFILE AND TANNIN CONTENT OF SOME CASSAVA CULTIVARS

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The carotenoid content of cassava leaves and tubers has been determined by open column chromatography. Mature leaves contain more carotenoids than immature leaves. Lutein is present in the greatest amount (86-239) followed by ß-carotene (13-78 mg/kg FW) with small amounts of _-carotene and ß-cryptoxanthin in some cases. With cassava tubers, the content of ß-carotene (0.1-14) exceeded that of lutein (0.1-0.5 mg/kg FW) with only traces of other carotenoids present. A deep yellow cultivar (V-2) from Vanuatu gave the highest amount and would be useful for further genetic studies. The content of tannin and iron binding phenolics in cassava leaves showed a range of values with Ceiba and SM1-150 having a low content. SM1-150 cultivar is useful because of the low cyanide and average carotenoid content of its tubers and the high carotenoid and low tannin content of its leaves.

INTRODUCTION

Vitamin A deficiency is one of the major public health nutritional problems in many developing countries and is an important cause of preventable blindness with an estimated 20,000 - 100,000 young children going blind each year (Tee, 1992). Since animal foods, the main sources of preformed vitamin A, are beyond the reach of most people in the third world, there is a heavy dependence on carotenoids (the precursors of vitamin A) in plant foods including vegetables, fruits and yellow cassava. Carotenoids, with or without vitamin A activity are involved in immunoenhancement (Bendich, 1991), treatment and prevention of cancer (Mathews-Roth, 1991) and morbidity and mortality in children of the third world (Tee, 1992).

Cassava is a primary staple food for about 200-300 million people of those developing countries in which xerophthalmia caused by vitamin A deficiency and the under 5 mortality rate are high (UNICEF, 1988). Yellow cassava tubers are a good source of carotenoids, which could enhance life and productivity in the third world. Cassava leaves, are consumed in many countries such as Senegal and Zaire and could be a major source of protein and carotenoids. However, plants contain tannins, which inhibit protein and carbohydrate digestion. Osuntogun et al, (1987) investigated the tannin content of cassava leaf protein concentrates prepared by several methods, but very little information is available on the comparative content of tannin in cassava cultivars.

MATERIALS AND METHODS

Cassava leaves and tubers from 5 cassava cultivars were harvested from plants grown in the greenhouse at the Australian National University. Immature cassava leaves are those not fully extended, and compose of the last three leaves

to the apex and are usually coloured brown/red. The just mature are those leaves next to the immature ones. ß-carotene was obtained from Sigma, _ and gamma (") carotene and ß-Apo-8-carotenal were obtained from Hoffman-La Roche.

Carotenoid Profile: Leaves (1.0g) and tubers (5-20g) were extracted and the open colum chromatography run essentially according to Rodriquez-Amaya et al, (1988). Each carotenoid was identified by (a) order of elution (b) visible absorption spectrum in petroleum spirit, ethanol and chloroform and (c) chemical reactions such as iodine catalysed isomerization and the epoxide test. Quantification was done using the molar extinction coefficients according to Davies et al, (1976).

Tannin: Tannin content was extracted by 1% HCl in methanol and estimated by the modified vanillin-HCl method of Price et al, (1978). Iron binding phenolics were estimated by the method of Brune et al, (1991) with slight modifications. Phenolics were extracted with acetone /0.1 M acetate buffer pH 4.4 (1:1) for 1 hour instead of dimethyl formamide / acetate buffer for 16 hours and urea was omitted entirely in the reaction mixture.

RESULTS AND DISCUSSION

Carotenoids: Lutein (a non-vitamin A precursor) is the most abundant carotenoid ranging from 86 to 286 mg/kg in all the leaves. ß-carotene is the highest pro-vitamin A carotenoid with the brown coloured mature leaves from M Aus 7 recording the highest concentration while the green coloured Ceiba leaves contained the least (Table 1). The carotenoid content is generally higher in mature than immature leaves despite the brown red colouration of the latter. The high values indicate that cassava leaf is a good source of carotenoids.

ß - carotene is also present in the largest amount in the tubers (Table 2), and ranged from 0.1 in M Aus 10 to 14 mg/kg in V-2. This wide variation shows that the deep yellow cassava tubers have greatly enhanced carotenoid contents and improved nutritional value. ß-cryptoxanthin was present in measurable quantities in only SM1-150 and V-2. In contrast to the leaves, lutein was generally lower than ß-carotene except in the slightly cream coloured M Aus 10 where the lutein concentration is 3 times that of ß-carotene. McDowell and Oduro (1983) using the HPLC reported ß-carotene values ranging from 1.0 to 11.3mg/kg dry weight (see Table 2), whereas, our results are on a fresh weight basis. Assuming a 65% moisture content (Bradbury and Holloway 1988) for the cassava tubers analysed in this report, the ß-carotene content would range from about 0.3 to 41 mg/kg dry weight. Hence TMS 71693 has about the same carotenoid content as Jamaica (yellow heart small) cultivar. V-2 is by far the best source of pro-vitamin A carotenoid.

The 51 mg/kg FW recorded for the mature leaves of SMI - 150 is similar to the 57.2mg/kg FW for ß-carotene reported by Tee and Lim (1991) for an unidentified tapioca shoot using the HPLC method. The lutein value reported by Tee and Lim

(1991) for cassava shoot was however insignificant compared with values now reported for cassava cultivars.

The expectation that high carotenoid content in the leaves could translate to high tuber carotenoid was not realised. Indeed the M Aus cultivar with the highest ß-carotene content had very little ß-carotene in the tuber.

Tannin: Tannin and other phenolics are defensive plant secondary metabolic products. They affect the nutritive value of vegetables by forming a complex with proteins (both substrates and enzymes) thereby inhibiting digestion and absorption (Osuntogun et al, 1987). They also bind iron, making it unavailable (Brune et al, 1991) and recent evidence suggests that condensed tannins may cleave DNA in the presence of copper ions (Shirahata et al, 1989). The tannin content of cassava leaves estimated in this study is high (72mg catechin equivalent) in TMS 71693, 71673 and M Aus while it is acceptable in both immature and mature leaves of SM1-150 and Ceiba cultivars (Table 3). Osuntogun et al (1987) evaluated leaf protein concentrates (LPC) from cassava and other tropical plants and observed that cassava had the highest tannin content (13.5mg/g FW), and had the poorest (negative) protein efficiency ratio (PER).

The amount of iron-binding phenolics was highest in the leaves of TMS and lowest in Ceiba. In developing countries where pernicious anaemia is endemic, ingestion of cassava leaves with high iron binding phenolics may aggravate the situation.

SMI - 150 holds a lot of promise for incorporation into breeding programs. The tuber is low in cyanide (Bradbury et al, 1991), average in carotenoid, and the leaves are high in carotenoids and low in tannins.

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Table 1: Carotenoid content (mg/kg fresh weight) of leaves of 5 cassava cultivars using open column chromatography. (standard deviation in parenthesis)

Samples	-carotene	ß-carotene	ß-cryptoxanthin	lutein	Leaf Colour
TMS 71693					
immature	1.7	23 (2)	2.5	158 (16)	brown
mature	-	33.(2)	2.0	239 (22)	green
TMS 71673					
immature	2.2 (0.1)	23 (4)	1.0 (0.2)	109 (23)	brown/red
mature	0.8 (0.1)	30 (2)	4.3	239 (13)	green
SM1 - 150					
immature	-	23 (3)	-	206 (35)	brown
mature	-	51 (7)	-	286 (19)	green
Ceiba					
immature	-	13 (2)	-	104 (25)	green
mature	-	27 (2)	-	185 (16)	green
M Aus					
immature	-	44 (2)	-	86 (14)	dark/brown
mature	-	78 (5)	-	128 (22)	brown
	Results from Tee & Lim (1991) for comparison				
Lettuce	-	1	-	73	
Spinach	-	32	-	42	
Tapioca shoots	-	57	-	17	
S. andro gynous	-	134	-	299	
Sestania	-	136	-	202	

Table 2: Carotenoid content (mg/kg fresh weight) of tubers of 5 cassava cultivars as determined by the open column chromatography and uv/visible spectrophotometer*. (standard deviation in parenthesis)

Samples	ß-carotene	ß-cryptoxanthin	lutein	Tuber Colour
TMS 71693	0.9 (0.2)		0.3 (0.0)	yellow
TMS 71673	0.3 (0.0)		0.1 (0.0)	cream
SM1 - 150	0.6 (0.0)	0.1 (0.0)	0.5 (0.1)	deep yellow
V - 2**	14 (4)	2.1 (0.2)	2.3 (0.8)	deep yellow
M Aus 10	0.1 (0.0)		0.3 (0.0)	cream/white

Results from McDowell and Oduro (1983) for comparison

Dry weight basis

Colombia 0-100A	5.8
Colombia 0-137	1.0
Colombia 0-799	10.5
Colombia M. col. 1816	1.7
Jamaica Yellow heart (large)	11.3
Jamaica yellow heart (small)	2.9

* *other carotenoids present in trace amount include, -carotene ß-carotene 5,6-5'6' diepoxide*

** *V-2 Vanuatu accession 2 cultivar*

Table 3: Tannin and Iron binding phenolics' content of some cassava leaves. (mg/g fresh weight) (standard deviation in parenthesis)

Samples	Tannin content mg catechin equivalent	Iron binding phenolics*	
		mg catechin equivalent	mg Tannic acid equivalent
TMS 71693			
immature	54.6 (1.7)	0.6	15.0
mature	78.4 (11.4)	1.2	28.4
TMS 71673			
immature	72.1 (3.2)	0.6	13.3
mature	60.0 (4.5)	0.8	18.5
SM1 - 150			
immature	18.5 (2.1)	0.4	8.8
mature	15.0 (0.5)	0.4	8.5
Ceiba			
immature	15.9 (2.9)	0.3	5.5
mature	10.4 (0.5)	0.1	3.3
M Aus			
immature	65.8 (9.3)	0.4	8.3
mature	28.4 (1.9)	0.4	9.0

* average of 2 determinations

CASSAVA PROCESSING AND BIOTECHNOLOGY

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Fermentation is the single most important mode of processing cassava in Africa. Biotechnology holds the potential to enhance the beneficial effects of cassava fermentation by developing and improving starter cultures. The removal of cyanogenic glucosides from cassava is a major benefit from cassava processing but is achieved to a greater or lesser extent depending on the type of process used. Molecular biology techniques are needed to assist conventional plant breeding in the development of cassava varieties containing little or no cyanogenic glucosides.

INTRODUCTION

A recent survey of the modes of utilization of cassava in Africa has revealed the overwhelming importance of fermentation in the processing of cassava. Nearly three out of four cassava-based foods encountered in the 250 villages of the study were fermented products (Westby, 1991). Cassava fermentation has been studied in our laboratory with the aim of understanding the microbiological and biochemical processes involved particularly in the elimination of cyanogenic glucosides from cassava and in the improvement of its nutritional value, food quality characteristics, and consumer acceptability. Biotechnology holds the potential to enhance the beneficial effects of cassava processing either by improving the fermentation process or by modifying the functional characteristics of the crop by changing its genotype through genetic manipulation.

STARTER CULTURES FOR FERMENTATION

Three types of fermentation are generally distinguished: a submerged fermentation, in which cassava roots, whole or in large pieces, are steeped in water for a period of 3 to 5 days; a mash fermentation, in which a mash is obtained by grating or rasping fresh cassava roots, and the mash is left to ferment in a container for several days; and a low-moisture fermentation whereby peeled cassava roots are heaped together and fungal growth is allowed to develop at the surface of the roots. Nearly all fermentations rely on the fortuitous presence of microbes on the roots and/or in the water, and on the prevailing favorable conditions for a production of the desired product. In some instances, a small amount of a previous batch is kept and used to inoculate the next, but the fermentation is allowed to follow its natural course with little or no attempt to control it. As a result, the flavor, aroma and texture of the fermented product varies with the season, location, and producer.

In recent years, several studies have identified the microorganisms associated with submerged cassava fermentations (Bokanga, 1989; Nwankwo, at al., 1989; Okafor et al., 1984), and with the cassava mash fermentation (Okafor and Uzuegbu, 1987; Ngaba and Lee, 1979; Abe and Lindsay, 1978). Lactic acid bacteria are

numerically the most important microbial group. *Lactobacillus plantarum, Streptococcus faecium* and *Leuconostoc mesenteroides* are the most frequently encountered species. They are mainly responsible for the rapid acidification that characterizes cassava fermentations. Spore forming bacteria such as *Bacillus* sp. seem to be important in inducing the retting of cassava root tissues during the submerged fermentation of whole roots. Other microorganisms, such as *Corynebacterium* sp., enterobacteriaceae, yeast and molds have been reported (Akinrele, 1964; Collard and Levi, 1959), but they are usually present in low numbers and their role is not clearly understood.

In a natural fermentation, a few microbial species are usually essential for bringing about most of the desirable changes, while other species may not contribute to the quality of the fermented product and in some instances may lower the quality of the food, or may represent a health hazard. For instance, in the submerged fermentation of cassava for the production of "kwanga" (or "chikwanghe"), a popular fermented cassava food in Central aFrica, 15 microbial species belonging to 10 genera have been isolated (Bokanga, 1989). Two of these species, *Streptococcus faecium* and *Bacillus licheniformis* when used as a mixture of pure cultures to aseptically inoculate a submerged cassava fermentation, a product having all the characteristics of "kwanga", is obtained. The detoxification of cassava, a major benefit from the fermentation of this food, is equally achieved in both the traditional and the pure culture process, although the fermentation time is reduced in half when pure cultures are used. The strong odors that are often associated with many fermented cassava products are absent, probably due to the lack of enterobacteriaceae and pseudomonads.

Some microorganisms however, including enterobacteriaceae, although present in low numbers, may impart to the fermented food a special flavor, a particular texture, or some valuable nutrient (e.g. vitamin B_{12} provided by *Klebsiella* in Indonesian "tempe") of great interest to the consumer. On the other hand, two species isolated in naturally fermenting cassava roots for "kwanga" production, *Staphylococcus haemolytica* and *Shigella* sp. contain strains which are known pathogens for humans; their presence in foods should be avoided. The use of starter cultures in traditional fermentations has the potential to reduce to a minimum the contamination by pathogenic and food spoilage organisms, and to maintain or improve the quality of fermented products. It will facilitate quality control and the formulation of quality standards, thus promoting the commercialization of fermented products.

CASSAVA FERMENTATION AND DETOXIFICATION

The major drawback for the utilization of cassava as food is that it contains the cyanogenic glucosides linamarin and, to a lesser extent, lotaustralin, which upon hydrolysis may release hydrocyanic acid (HCN). The cyanogenic glucosides are stored within vacuoles inside the cells of all cassava plant tissues. The cell walls of these tissues harbor the enzyme linamarase, a ß-glucosidase capable of hydrolyzing

linamarin to glucose and acetone cyanohydrin. At pH above 5, acetone cyanohydrin will spontaneously decompose to acetone and HCN which is rapidly lost by volatilization (boiling point of HCN is 25.7°C). A second enzyme, hydroxynitrile lyase, has also been shown to catalyze the decomposition of cyanohydrins. In processed cassava products it is possible to find intact linamarin, acetone cyanohydrin and HCN, three compounds usually referred to as cyanogens.

Most processes to which fresh cassava is submitted reduces its cyanogen content, but to a different extent. The submerged fermentation is the most efficient process of reducing the levels of cyanogens in cassava: reduction rates of 95 to 100% are often reported (Bokanga et al., 1990; Ezeala and Okoro, 1987; Ayernor, 1985). The removal of cyanogens from cassava during the submerged fermentation is probably the result of several factors among which: 1) textural changes in the plant tissues that make it possible for vacuole-bound cyanogenic glucosides to come into contact with membrane-bound linamarase, and for hydrolyzed and intact compounds to leach out; 2) increase in glucosidase activity in cassava tissue; and 3) the ability of the microorganisms to utilize cyanogenic glucosides and their breakdown products.

The detoxification of cassava in the mash fermentation follows a different mechanism. The grating of cassava roots to obtain the mash disrupts the structural integrity of plant cells thus allowing the cyanogenic glucosides from storage vacuoles to come in contact with the enzyme linamarase on the cell wall. The subsequent fermentation contributes very little to the breakdown of the glucosides (Vasconcelos et al., 1990). In fact, the low pH (around 4.0) rapidly achieved during fermentation is inhibitory to linamarase activity and stabilizes cyanohydrins, thus slowing down linamarin hydrolysis and cyanohydrin breakdown.

After fermentation, the cassava mash is usually dehydrated by pressing and quickly roasting the dewatered mash on a heated surface. The result is a free-flowing granulated product known as "gari" in West Africa. The residual amounts of cyanogens in gari obtained from fermented and unfermented cassava mash are equally low. However, in the gari from unfermented mash, the residual cyanogens are solely made up of intact cyanogenic glucosides, while in the gari from fermented mash the residual cyanogens are mostly cyanohydrins. In all cases, HCN is virtually absent.

CASSAVA DETOXIFICATION IN OTHER PROCESSING METHODS

Other methods of converting cassava into foods do not include a fermentation step. Cassava roots may be boiled and consumed like potatoes; or they may be dried and milled into flour. The heat of boiling and the loss of water inactivate enzymes and, therefore, reduce the degree of hydrolysis of cyanogenic glucosides by linamarase. On the average, 50 and 75% of the glucosides remain in the food after boiling and drying respectively (Nambisan and Sundaresan, 1985). It is therefore important that the CNP of fresh cassava roots intended for boiling and drying be the lowest possible.

Plant breeding programs in many agricultural research centers have among their objectives the lowering of the CNP of cassava. Progress has been slow due to a weak understanding of the genetic control of cyanogenesis in cassava and of the effect of the environment, season, age of the plant, and cultural conditions on the accumulation and distribution of cyanogenic glucosides in cassava plant tissues.

It is only recently that the biosynthesis of cyanogenic glucosides in cassava has come under study (Koch et al., 1992). The identification and characterization of the enzymes catalyzing the biosynthetic pathway and the structural and regulatory genes involved will undoubtedly permit the development of acyanogenic cassava lines through the use of molecular biology techniques. It will also lead to the development of more efficient, DNA-based screening tools which will guide the selection of the best genotypes to use as parental materials in traditional breeding schemes for reducing the CNP of cassava.

DIRECTIONS FOR FUTURE RESEARCH

For many years to come, cassava will continue to play a major role in the diet of millions of people in the Tropics. In Africa, fermentation will probably remain the most important mode of processing cassava into edible foods, because of its low cost and the desirable flavors it imparts to foods. The development of starter cultures will facilitate process standardization and quality control, and will set the stage of strain improvement programs through conventional microbiology and genetic engineering techniques, for better quality fermented products. The study of fermentation microorganisms can also lead to the identification of microbial strains particularly suited for the oversecretion of valuable biological products such as enzymes, amino acids, vitamins, antibiotics and flavor enhancers. These microorganisms could be, like in Japan, at the origin of very profitable businesses.

Biotechnology research is also needed to improve the cassava plant and introduce in its genome important characteristics for the value of cassava as food and which so far it has been difficult to confer to cassava (e.g. absence of cyanogens). While one approach could be to inactivate the biosynthetic pathway of cyanogenic glucosides, another would be to increase the content and/or activity of the enzymes linamarase and hydroxynitrile lyase in the plant tissues and in fermentation microorganisms. This would ensure that, during processing, all the cyanogenic glucosides are hydrolyzed and the cyanohydrin broken down to HCN which would subsequently be lost by evaporation.

Genetic modification of cassava by recombinant DNA technology requires effective and reproducible transformation and regeneration systems which are yet to be developed. Increased research efforts should be geared toward eliminating these bottlenecks for cassava improvement research.

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BIOTECHNOLOGICAL APPROACHES FOR CASSAVA UTILIZATION IN INDIA

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Biotechnological approaches have been made in India recently for the diversification of cassava for alternate uses in food, feed and industry. In this paper status of work done in India along with some of the significant results obtained are discussed. Solid state fermentation process with *Trichoderma pseudokoningii* showed maximum protein build up of 14.08% in the case of treatments where cassava flour and cassava starch factory wastes were mixed equally. The possibility of economic broiler farming by switching over to a cassava waste feed from the conventional feeds were brought out from the study. Liquid fermentation experiments on myco-protein production using cassava starch are also discussed. Various processes developed for the recovery of ethanol from cassava showed that approximately 400-420 liters of ethanol could be recovered from one tonne of dried chips by adopting various processes. Novel techniques like cell immobilization for continuous fermentation have been also attempted. Fermentation experiments for the production of other commodity chemicals like citric acid, itaconic acid, high fructose syrup and vitamin C using cassava starch showed promising results. Experiments on the detoxification of cyanide by *Rhizopus oryzae* showed the possibility of a biotechnological process for the elimination of cyanide from cassava starch factory effluents.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz.) plays a vital role in the food, feed and industrial economy of India. Almost 70 per cent of the of the product from Kerala, the predominant cassava producing state in India goes for human consumption, while in other parts of the country cassava finds place mainly as an industrial raw material.The utilization pattern of cassava over the past few years indicates its limited scope in future as a direct food item. Development of new methods of utilization will be a major avenue to promote cassava cultivation in India. Biotechnical approaches made recently for utilization of cassava in food, feed and industry have shown promising results and better prospects in future for cassava in India. Bioconversion of cassava starch and cassava wastes following solid state and submerged fermentation technology to yield proteins and commodity chemicals using appropriate micro-organisms were attempted and the results obtained are discussed in this paper.

BIOCONVERSION OF CASSAVA STARCH AND WASTES FOR MICROBIAL PROTEINS

Being a cheap carbohydrate source capable of supplying adequate calories , cassava has extensive potential as an animal feed.However, due to certain limitations like its low protein content and lack of certain aminoacids such as methionine, it is often rated as inferior to maize or wheat. To find a solution tro this and to widen the spheres of its utilization as animal feed biotechnological studies

have been initiated at CTCRI. An approach to this line is to enrich the cassava flour with proteins from micro-organisms.

1. Solid state fermentation of cassava flour and wastes with fungal cultures.

A solid state fermentation process for the protein enrichment of cassava flour and cassava starch factory wastes using the fungus, Trichoderma pseudokoningii Rifai was developed at CTCRI, India. The various treatments studied and results obtained are given in Table 1. The results showed that using this process it is possible to convert the substrate, using minimum of nutrients (0.15%(NH4)2SO4) to a protein enriched animal feed. The highest increase in protein content observed was 14.32g/100 g dry matter (DM) from an initial 1.28 g/100 g dry matter, where cassava flour was the sole ingredient.

The laboratory technology was evaluated with regard to its potential use in the large scale production of SCP enriched poultry feed at the Central Tuber Crops Research Institute, Trivandrum.

The solid state fermentation developed will suit to the village conditions and feeding trials with poultry birds showed the potentials that existed for commercial broiler farming to switch over to a cassava waste based feed from commercial feed[2].

2. Proteins from starch factory wastes

Another solid state fermentation process was developed to enhance the protein content of dried starch factory wastes using a strain of Aspergillus niger. The initial biomass protein in the material was 1.60% (w/w). A steep increase in the protein content was observed during the first three days, so that the material contained 7.0% protein by the third day. During the next two days of incubation the protein content only increased by an additional 0.7%.[3]

Protein enrichment studies with Saccharomycopsis fibuliger Y-2388 and its nor-leucine resistant mutant showed the efficiency of these strains for protein enrichment of cassava pulp[4].

3. Mycoproteins from gelatinised starch

Several fungal cultures were screened for their efficiency to utilize cassava starch and cassava wastes in a liquid medium, to be used as a protein supplement in cassava based animal feed preparations[5]. Aspergillus niger was able to produce maximum protein (163 mg) in hundred ml medium. Rhizopus delemer also produced almost an equal amount of protein (162 mg/100 ml). Trichoderma viride could produce protein in the range of 44-56 per cent in a starch medium containing 2.5% starch. When cassava rind was used as the sole source of carbon, the culture Rhozopus oryzae produced maximum protein yield of 199 mg/100 ml followed by Aspergillus oryzae which produced 137 mg/100 ml[6].

BIOCONVERSION OF CASSAVA STARCH FOR COMMODITY CHEMICALS

1. Ethanol

The projected demand of ethanol to the tune of 800-900 million litres during 1995-200 AD from the existing 600-700 million litres produced from molasses which is in short supply may create a situation to depend on cassava as an alternate source of ethanol. Techniques for the recovery of ethanol from cassava flour were standardised at CTCRI, Trivandrum and CFTRI, Mysore[7,8 and 10]. Fresh cassava roots or flour can be used for the production of ethanol. The first step is gelatinisation and conversion of starch to simpler sugars by a process called saccharification accomplished with the help of sacchaifying agents like mild acids, amylase enzymes and substances containing amylase enzymes. Fermentation of saccharified starch could produce an yield of ethanol varying from 400-420 litres/tonne of cassava flour.

Ethanol production from fresh tubers, flour and starch of cassava was studied by simultaneous saccharification and fermentation procedures at CFTRI (Mysore). In situ saccharification and fermentation techniques for ethanol production from cassava flour was also attemped[11]. Attempts have been also made to modernize and improve the
efficiency of fermentation by adopting novel techniques like immobilization of yeast cells on various carriers like alginate beads, Kieselghur, brick powder and silicagel. However such techniques remain as the future possibilities in the alcoholic fermentation technology based on cassava starch[12].

2. Itaconic acid

The possibilities of producing itaconic acid using, saccharified cassava starch was investigated and after 15 days of fermentation of 4% cassava starch with Aspergillus itaconicus the percentage of itaconic acid recovered was 34.5 compared to 37.1 in sucrose medium[13].

3. Citric acid

Attempts are also under way at CTCRI to produce citric acid from cassava starch factory waste hydrolysate using strains of Aspergillus niger.

4. Vitamin C

Vitamin C is an important pharmaceutical product obtained from starch. Starch is hydrolysed to glucose which is reduced to sorbitol and then treated with Acetobacter suboxidans to give sorbose. The sorbose is converted to ascorbic acid (Vitamin C) by a series of chemical reactions. Recently chemical reactions have been replaced by a microbiological process which saves lot of time and expense. Of late some pharmaceutical firms in India have started production of vitamin C from cassava starch.

5. Antibiotics

Cassava starch liquefied by-amylase was found as good source of carbon for extra cellular alkaline proteinase. Out of 45 strains screened, Streptomyces moderatus was found to have maximum enzyme activity. Cassava starch at 6% concentration was found to be quite suitable as carbon source for mutant streptomyces fradiae for antibiotic production[14].

6. Cyclodextrins

Cyclodextrins have gained considerable importance recently as basic material in various applications. Attempts have been made to use cassava starch for production of cyclodextrin. The enzyme used was cyclodextrin glyceryl transferase. The yield was approximately 10% W/W based on starch used[15].

7. Sweetener industry

A. Starch is made of glucose units and hence can be broken down to glucose by acid or enzyme. In India, both maize and cassava starches are used for production of glucose and though there is no particular preference for either of starches for production of glucose, cassava starch, finds an important outlet for use in glucose production. The saccharification is carried out either by using acid or enzymes.

B. Various fungal enzymes have been compared for their ability to hydrolyse starch to glucose. Stable amylolytic enzymes produced by Aspergillus oryzae, A. awamori and Rhizopus niveus were used to study the content and nature of hydrolysis of cassava starch. It was found that after hydrolysis by A. awamori, and R. niveus only glucose was found in the syrup. A.oryzae hydrolysed starch to maltose and glucose and hence may be used for producing high maltose syrup. If hydrolysis with A. oryzae was maintained for 72-96 hours, the percentage of hydrolysis could be increased.

C. The effect of incubation of -amylase on viscosity of cassava flour was studied in detail. It was observed that at same temperature, higher concentration of enzyme led to more reduction in viscosity for the three concentrations of flour used. The results also showed that less than 0.1 ml enzyme/100 g flour would be enough to bring about liquefaction of flour[18].

D. High fructose syrup has been an important break through in sweetener technology. Cassava starch has been tried for production of high fructose syrup. Isomerisation to the extent of 20-22% could be achieved. The optimum concentration of glucose was found to be 50-60% and temperature 60-65°C. However, since the conversion was

very poor, immobilised glucose isomerase was tried on both acid hydrolysed and enzyme hydrolysed starch. Enzyme hydrolysis was carried out using termamyl and amyloglucosidase. After the glucose syrup decoloured by animal charcoal, concentration of syrup was increased to 40-42% and pH adjusted to 8.2. Isomerisation was carried out using 12 g sweetzyme and 0.5 g $MgSO_4$ per 100 ml syrup for 6 hour at 61°C. It was decolourised with animal charcoal, passed through ion-exchange columns and concentrated to get a colourless syrup and it was 39.8% for enzyme hydrolysed syrup[19].

8. Microbial techniques for the separation of flour from cassava

A mixture culture inoculum provided microbial treatment enhanced extraction of flour from cassava tubers appreciably. The role of polysaccharide and pectin degrading enzymes were studied during the microbial activity[20].

UTILIZATION AND TREATMENT OF WASTES FROM CASSAVA PROCESSING INDUSTRY

1. Single cell proteins

Two kinds of effluents, the primary and secondary effluents from the large scale starch factory and the secondary effluent from the small-scale starch factory were the main sources of pollution. The primary effluent is a thick slurry with a high pollution load as evidenced by a BOD content of 13,200-14,300 mg/l and a COD content of 38,220-48,820 mg/l. The secondary effluent was rather a diluted one with a comparatively low BOD of 3,600-7,050 mg/l and a COD of 3,800-12,050 mg/l. At the early stages of discharge the effluents showed the presence of cyanide. Among the various yeasts[9] and yeast like organisms chosen for the aerobic treatment of cassava starch factory effluents, Endomycopsis magnusi and Endomycopsis fibuliger were found to be efficient in utilizing starch present in the effluent, though they had a comparatively slow growth. Co-culturing of Candida utilis and the Endomycopsis fibuliger was found to be a better method for the hydrolysis of starch and assimilation of the resultant free reducing sugar. The conversion of carbohydrate waste into SCP was rapid when fermentation was carried out under monitored conditions i.e. in a laboratory fermentor with proper mixing, aeration, pH and temperature control. About 94% of the COD and 91% of the BOD were removed by aerobic treatment. The protein content of the dried residue obtained from the fermentor at 28 hr of incubation was 22%, which was found to be maximum during the incubation of 0 hr to 60 hr.[21] In both submerged and solid state fermentation, enrichment of the substrate with nutrients promoted the growth of the organisms and production of protein.

2. Biogas

Anaerobic digestion of cassava starch factory effluent in batch digesters produced 13.2 l. of biogas per kg dry matter with an average methane content of

59%. The anaerobic process could reduce 63% COD during two months of incubation. Semi-continuous digestion of cassava starch factory effluents produced about 325 l of biogas per kg dry matter. During the hydraulic retention time of two days in the anaerobic digester 46% solids were digested under ambient temperature[22].

3. Ethanol

Techniques have been also standardised to produce ethanol from cassava starch factory wastes (Thippi)[22 and 23]. 1000 kg of the tippi slury (30% solids) when saccharified with enzymes produced 994 kg of glucodextrin. When further processed by fermentation it led to about 55 kg (69.52 L) ethanol.

4. Detoxification of cyanide by Rhizopus oryzae

Since cyanoglucoside impart toxicity to cassava tubers and leaves there by limiting the utilization of these as human food or animal feed, a study was made to detoxify the HCN in cassava tubers using R. oryzae. It was found that R. oryzae can effectively degrade the cyanoglucosides of cassava. R. oryzae degraded linamarin to cyanide by elaborating the enxyme rhodanese. The rhodanese of R. oryzae was found to be an inducible enzyme and was induced effectively by 1% KCN. This finding is of utmost importance since the culture can be effectively used for the detoxification of cyanogenic glycosides from cassava and also for the elimination of cyanide from industrial waste water as the rhodanese of R. oryzae will be induced under such environments[25 and 26].

CONCLUSION

Application of biotechnology in cassava utilization chiefly involve the use of micro-organisms for fermentation. Increased understanding of the genetics of environmental factors influencing the production of primary and secondary metabolites in the microorganisms have facilitated the use of genetic engineering methods to produce strains leading to enhanced yields. The new techniques available will usher in a refreshing transformation of the whole concept of how to deal wih various problems in cassava utilization and treatment of wastes and effluents discharged from the cassava processing industries.

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Table 1. Total protein (g/100 g DM) changes during fermentation of different mixtures of cassava flour.

Cassava mixture flour/wastes	Fermentation period (days) 0	6	12	18	24
0/100	1.26	4.06	4.45	5.09	6.18
25/75	1.34	4.38	6.88	6.00	6.60
50/50	1.32	7.75	8.50	5.57	7.48
75/25	1.30	8.01	8.84	10.82	10.67
100/0	1.28	7.68	9.55	14.32	13.10

Source: Balagopalan and Padmaja, 1988

GENETIC IMPROVEMENT OF CASSAVA FOR ENHANCED POST-HARVEST STORABILITY: AN FAO INITIATIVE

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Cassava root is prone to rapid post-harvest deterioration which seriously reduces its industrial and marketing potential and causes significant storage losses. To alleviate these problems, FAO is exploring how modern biotechnological approaches may lead to the development of cassava with improved storage life. An expert consultation, co-sponsored by The Rockefeller Foundation, was held in December 1991 at FAO headquarters to review current knowledge about the physiological, biochemical and pathological process of cassava deterioration. A framework for strategic research on this topic was developed during the meeting by the participants. The consultation endorsed the merit of addressing this problem and recommended that FAO, in collaboration with other appropriate institutions, should develop a mechanism to facilitate a concerted and comprehensive research initiative to address this critical area. The major components of the proposed program of action include:

1) Support to development of a reliable genotype-independent transformation method for cassava.
2) Collection of additional data on aspects of underlying biochemical mechanisms of deterioration with the view to produce transgenic plants with altered accumulation of phenolics and other compounds which play key roles in deterioration.
3) Further examination of existing genetic variability, including that in wild relatives for storability and the development of improved screening methods for this trait, for application in conventional breeding and to support biotechnology approaches.
4) Determining if molecular markers can be identified to facilitate the recognition of parents and progeny carrying the improved storability trait.
5) Conducting socio-economic studies to determine which parameters of deteriorated cassava (i.e. streaking, hardening, flavor etc.,) are of the greatest importance to consumers.

This effort by FAO to catalyze strategic research is intended to be complementary to, and coordinated with, the Cassava Biotechnology Network. FAO is currently preparing the proceedings of the expert consultation and a program document with sub-projects for donor support.

INTRODUCTION

The Food Agricultural Organization of the United Nations (FAO) is delighted to see the creation of cooperative programmes such as those that should evolve from this network; programmes with well focused goals; programmes designed to solve real problems. The network should also provide an important forum for communication among scientist working on common goals. Improved communications are needed to accelerate research and especially development.

For me, it is a pleasure to represent the Plant Production and Protection Division of FAO at this Meeting, to become more familiar with the action programmes

underway, and to share with you some information about a new initiative being promoted by FAO, i.e. The Genetic Improvement of Cassava for enhanced post-harvest storability.

For those of you now totally familiar with FAO and its mandates, I would like to make several points clear:

1) FAO is not a major source of direct funding for research or development.

2) FAO does promote and where appropriate recommend national and international action with respect to scientific, technological, social and economic research related to nutrition, food and agriculture.

What is the problem?: Cassava roots begin to deteriorate as quickly as 24 hours after harvest and most varieties deteriorate within four or five days. Poor storability puts constraints on how the crop can be handled both in traditional marketing processing systems but also limits the crop's suitability for modern processing options.

Extending the storability of cassava will expand the basket of choices that can be made by the farmers, transporters, processors and consumers.

ACTION

A group of plant scientists met at FAO, Rome on December 11-13, 1991 at a workshop on post harvest deterioration of cassava sponsored by FAO and co-sponsored by the Rockefeller Foundation. The purpose of this meeting was to provide a forum for scientists to review the biochemistry and physiology associated with post harvest deterioration of cassava and explore the possibilities of developing cassava varieties with superior storability, with special emphasis on application of modern molecular biology.

The export consultation, while recognizing that there are major gaps in the knowledge base about post-harvest cassava deterioration, considered that the problem was tractable. The inherent long-term storability of the crop is demonstrated by the fact that it can be left in the soil for a year, while harvested material commonly degrades within two to three days.

An important component of the umbrella programme is to more fully understand, in existing utilization systems, what changes in the deterioration root are deleterious to the quality of the different end-products. Thus, the research action programme will be directed, in part, by socio-economic information.

Other components will include:

- Developing protocols for assessing existing varietal (genetic) variability and to elucidate biochemical/physical reasons for such variability.

- Identifying biochemical mechanisms of the rapid deterioration of cassava root to alternately direct transgenic work.

- Developing functional genetic cassettes for transforming cassava to realize varieties with enhanced storability.

A COMBINED FERMENTATION SYSTEM FOR PRODUCING BOTH ETHANOL AND PROTEIN-ENRICHED FEED FROM CASSAVA

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Cassava (*Manihot esculenta*) is mainly grown by small subsistance farmers in the rural areas of Southern Africa. In order to assist these small farmers in our third world situation to optimally utilize their cassava crops, we investigated the development of value-added products from cassava through the application of modern biotechnology. Our approach was to use the about 70% starch in the cassava root as a sole energy and carbon source in microbial fermentation systems to produce industrially-important products. To convert the starch in the cassava root to fermentable sugars, the ground and dried, non-toxic cassava was gelatinized in phosphate buffer, diluted in buffer to a final concentration of 5% solids and exposed to the heat-stable amylase in the supernatant of an overnight culture of *Bacillus stearothermophillus* or inoculated with viable cells of the same organism. This fermentation was performed at 55°C for 60 and 35 hrs., respectively, and conversion of starch to glucose was constantly monitored throughout this process. The cells and solids were filtered from the fermentation broth. The liquid phase was pasteurized and inoculated with *Zymomonas mobilis*. This mixture was fermented for four days in a non-aerated batch culture fermentation system with mild agitation to facilitate maximal conversion of fermentable sugars to ethanol. Ethanol was subsequently collected by distillation. The cells and undigested cassava solids collected before ethanol production were autoclaved and diluted with pasteurized cheese whey. The resultant mixture was inoculated with *Propionibacterium freundenreichii* and fermented anoxically without agitation at 30°C for a further two days to produce single cell proteins. Protein contents of the different fermentation mixtures were monitored throughout the whole process. The solids were again filtered off, air-dried to about 12-16% remaining mixture and added to commercial chicken feed in quantities of 5-20% final weight. The performance of this protein-enriched feed was determined by additional weight gain in three week old chickens, as compared with chickens feed on commercially available chicken fees only. The socio-economic impact of the transfer to small farmers of the biotechnology developed in the above processes will be subsequently determined.

INTRODUCTION

The severe continual cyclic droughts in Southern Africa have yet again caused the maize crop to fall and the search for an alternative source of carbohydrate as high energy staple food to stave off the reality of emerging famine, especially for the small subsistence farmers in the rural areas, has focused renewed attention on the potential of the 'orphan' crop cassava or Manihot esculenta Crantz. This crop is in the top rank of biomass producers and since it is the highest known yielder of starch (5), its additional importance as industrial raw material (13) and animal feed mix has been realised and it consequently plays a vital role in the economy of many nations in the world (4).

Our present study is part of a much bigger project aimed at the optimium exploitation of modern biotechnological techniques to achieve economically identifiable goals in cassava utilisation through the development of value-added

products from this high-starch tuber crop. The search for a renewable source of fuel as a substitute for non-renewable fossil sources has accentuated the advantages of the manufacture of alcohol from cassava starch (5) and offers a profitable outlet for cassava production by peasant farmers in our third world situation. The use of microorganisms for production of dietary protein for animal feed from cassava starch is also well documented (1;3) and according to Phalaraksh *et al.* (1) cassava-fed pullets were more tolerant to heat-stress and showed lower loss in terms of mortality than those fed corn or maize.

MATERIALS AND METHODS

Source of microorganisms and maintenance

Bacillus strearothermophilus ATCC 31783, *Propionibacterium freudenreichii* ATCC 13673 and *Zymomonas mobilis* ATCC 31822 were purchased from the American Type Culture Collection. *B. Stearothermophilus* and *P. freudenreichii* were maintained on the media and under the conditions prescribed by ATCC and cultured in nutrient broth for seed inocula or amylase production. Preservation of the *Z. mobilis* strain and the preparation of a pre-seed inoculum had been described by Rogers *et al.* (12).

The amyoiolytic thermotolerant mold, *Rhizopus oligosporus*, was obtained from the culture collection of the Council for Scientific and Industrial Research (Pretoria, South Africa) and was maintained on Potato Dextrose Agar plates at 4°C. Spores were harvested by aseptically flooding 72 h old plates with 3 ml (per plate) distilled water + 0.1% Tween to obtain a spore suspension of 10^5 - 10^6 viable counts ml^{-1}.

Production of the cassava meal

Cassava meal was produced by chipping the freshly-harvested whole cassava root and sun-drying the chips on a concrete slab to about 14% (w/w) remaining moisture. The chips were then milled to a relatively fine flour. The flour/meal was stored in sealed containers at 4°C until required.

Preparation of alpha-amylase

400 ml Nutrient broth with 2.5% (w/v) alkali-treated, cooked cassava meal (pH 7.0) in a 1-litre Erienmeyer flask was inoculated 1:100 with an overnight culture of *B. Strearothermophilus* and incubated at 55°C for 32 h with constant agitation (150 rpm) (13). The cells were harvested from the culture broth by centrifugation and discarded. Partial purification of the extracellular amylase in the supernatant was achieved by the addition of solid ammonium sulphate to a final concentration of 60% (w/v) with constant stirring at room temperature. The precipitate was collected by centrifugation (12 000 X g, 20 min, 4°C), dissolved in 20 ml 0.2 M sodium phosphate buffer (pH 7) and dialysed against two changes of distilled water at 4°C.

Pretreatment of cassava meal

158 g of the cassava meal was steeped in 500 ml 0.25 N NaOH for 12 h (14). The excess liquid was filtered off by suction and the alkali-treated cassava meal was spread on a fine wire mesh of a perforated steel tray, covered with aluminium foil and heated to 110°C for 45 min in a horizontal autoclave. The gelatinised cassava was adjusted to pH 7 by addition of 2M HCl and the 20 ml partially-purified alpha-amylase enzyme was stirred through the mixture and left at 45°C for 2 h. The volume of this liquefied slurry was subsequently made up to 1 litre by the addition of 0.2M citrate buffer. The pH of this medium was adjusted to 3.8 and it was supplemented with 0.01 g $MnSO_4$ and 12 g urea (3). The medium was inoculated with 2% (v/v) of the *Rhizopus* spore suspension and fermentation was conducted for 36 h at 40°C with continuous shaking in a reciprocating shaker water bath (100 strokes min^{-1}).

The starch content of the cassava meal was measured by the slightly modified technique of Smith and Roe described by Garg & Doelle (3) and D-glucose (as reducing sugar) was determined enzymatically by UV absorbance at 340 nm, using the Boehringer Mannheim kit (Cat. no. 716 251).

Alcohol fermentation

The *Rhizopus* mycelia and undigested cassava solids were filtered from the fermentation broth. The liquid phase was again made up to 1 litre with the citrate buffer, adjusted to 1 g l^{-1} ammonium sulphate and a pH of 5 and pasteurised. This fermentation broth was subsequently inoculated 1:10 with a saturated culture of *Z. mobilis* and the mixture was fermented to 20 h at 30°C in a non-aerated batch culture fermentation system with mild agitation to facilitate maximal conversion of fermentable sugars to ethanol. Samples were filtered through Whatman No. 1 filter paper and ethanol in the filtrate was determined enzymatically by UV absorbance at 340 nm by using the Boehringer Mannheim kit (Cat. no. 176 290). Ethanol was subsequently collected by distillation through two successive columns (to a concentration of 96.8%).

Preparation of protein-enriched chicken feed

The *Rhizopus* mycella and filtered solids from the starch saccharification broth were resuspended in 500 ml cheese whey, 50 ml white grape juice, 3.5 g l^{-1} Na_2HPO_4 and 1.5 g l^{-1} KH_2PO_4 and the volume was made up to 1 litre with distilled water. The pH was adjusted to 7.2 and this broth was inoculated 1:10 with an overnight culture of *P. freudenreichii*. Fermentation was performed anoxically without agitation for 28 h at 30°C. The fermented cassava flour was dewatered by filtration and air-drying for three days at room temperature. Protein concentration of the fermented cassava meal (FCM) was determined by the Kjeidahi method (1) and was monitored throughout the whole process.

Broiler feed trial

Feather-sexed 1-day old male broilers (Ross line) were fed on diets of equal protein content (190 g protein kg^{-1}) in which maize had been partially replaced by fermented cassava flour mash at concentrations of 50, 100 and 150 g kg^{-1}. The chickens were kept in environmentally-controlled battery brooders set at diurnally cycling from 21° to 30°C and constant lighting and humidity. Food and water were supplied *ad libitum* and the trial lasted 21 days. Chickens were weighed before and after the trials and the performance of this protein-enriched feed was determined by additional weight gain in broilers, as compared with chickens fed on commercially available chicken feeds only.

RESULTS AND DISCUSSION

The cassava meal we used in our experiments contained 82% starch (w/w) (on a dry weight basis), which is less than the reported in the literature for cassava flour prepared from peeled roots (1). The effect of the specific pre-treatment of the cassava meal employed by us on the conversion of the starch to fermentable sugars is given in Table 1. Ninety-nine percent of the hydrocyanic acid is supposedly destroyed during the drying and cooking processes (1). During fermentation of cooked, enzyme-liquefied cassava meal by *R. oligosporus* a high concentration of glucose accumulates in the fermenting slurry which remains unutilized by the fungus itself (3).

The alkali treatment did not hydrolyze the cassava starch, but changed the starch granule structurally (14) and increased viscosity remarkably. Shin *et al.* (14) found that alkali serves as a macerating agent in the place of pectin depolymerase and we found that alkali treatment plus cooking completely ruptured root cells and released the starch
granules bound to the lignocellulosic compounds of the roots to form a gel (2; 7). The subsequent amylase treatment effectively liquefied the cassava slurry to make it less viscous enabled us to use such high concentrations of cassava meal in the fermentations without too many problems with agitation.

The main advantage of cassava over other energy crops is the presence of high fermentable sugars after saccharification. *Z. mobilis* grows and ferments glucose very fast and also has a higher specific rate of glucose uptake and ethanol production than *Saccharomyces carlsbergensis* (12). Ethanol production by *Z. mobilis* is not growth-associated and the improved ethanol yield of *Z. mobilis* to *S. carisbergensis* results from the different carbohydrate metabolisms (Entner-Doudoroff pathway vs glycolysis pathway) and subsequent lower biomass formation by *Z. mobilis* (12).

We obtained 46.4 mg ml^{-1} ethanol (from 100 mg ml^{-1} glucose in 158 gl^{-1} cassava meal, Table 1), which indicated 91% of theoretical conversion. This is lower than the 93-97% reported for pure glucose media by several researchers and may

be due to the inhibitory effect of maltose present in the fermentation broth on alcohol conversion (10). The *Z. mobilis* culture in our experiment did not function under product inhibition of growth (product <50 gl^{-1}; 12) or glucose limitation and further experiments will be performed in tower fermentation systems to optimise ethanol production and induce a tolerance to ethanol (12) in *Z. mobilis*.

Cassava flour is low in protein and may cause protein deficiency diseases in animals if used as only feed stuff. The simplest and cheapest way of increasing protein content of cassava meal is to ferment the tuber mash with bacteria and fungi to produce single-cell proteins (1;6). Although cassava flour is rich in calcium, phosphorous, iron and vitamin C and also contains significant quantities of thiamine, lysine, riboflavin and nocotinic acid (1), methionine (11) and vitamin B12 is limited and needed to be supplemented (1). According to Muller *et al*, biotin supplementation was found to improve broiler performance fed on cassava (1). The protein-enrichment of fermented cassava meal obtained in our experiments is given in Table 2.

We obtained a 450 % protein enrichment over the initial protein concentration after fermentation of the cassava mash with *Rhizopus* and the addition of the whey. Mycella of *Rhizopus* contains a high true protein content (3). Fermentation with *P. freudenreichii* did not contribute much to protein content (8%), but this organism is known to produce vitamin B12 from cheese whey by fermentation. The initial protein content of the cassava meal is much higher than reported by other researchers (12% vs 1-2%) and may be due to the very heavy contaminations of the meal by spore-forming soil bacteria because of the relatively crude production technique.

Alternative dietary ingredients for feeding poultry became necessary because of the rising costs of maize-based feed (9). We performed a very limited short-termed feeding trial on broilers with FCM, just to establish a trend and the results are given in Table 3.

There seems to be a definite optimum concentration of FCM added to broiler diets to achieve an increase in carcass yield, similar to what other researchers found (1). The percentage increase in carcass yield is higher with 10% FCM than with 15%. Balagopalan *et al.* (1) suggested that broilers should be fed a low level of cassava up to the fourth week. As cassava replaced maize, the content of metabolizable energy declined. Birds fed on diets with increased cassava replacement (more bulk) and resultant lower energy content, also consumed more food in an attempt to meet their energy requirements (9; 11).

It is also known that animal feed with more than 20% fibre content might not be tolerated by some monogastric animals (8). The production of our cassava meal was relatively crude in the sense that the roots were not peeled before chipping and drying, with the consequence that the crude fibre content of the cassava meal was much higher than reported for cassava flour produced from peeled roots (1). We

have to perform a vastly extended feed trial to optimise the replacement and enrichment of maize-based poultry feed with FCM.

The socio-economic impact of the transfer to small farmers of the biotechnology developed in the above processes will subsequently be determined.

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Table 1. Effect of pre-treatment on the degree of saccharification of cassava starch.

Treatment	% total starch converted	Glucose yield	% conversion of starch to glucose g 1^{-1}
Pretreatment with 0.25 N NaOH	<1	NT	NT
Alkali treatment + cooking	23	3.3	11
Alkali treatment + cooking + liquefaction	89	67	59
Alkali treatment, cooking, liquefaction + saccharification	99.2	100.2	78

[a] Ethanol yield from 12.96 g starch 100 ml^{-1} (on dry wt basis), as described in the text.
NT = not tested

Table 2. Protein-enriched of fermented cassava meal (FCM) in g 100 g-1.

Unfermented liquefied and saccharified cassava meal	12
After *Rhizopus* fermentation and addition of whey	50
After *Propionibacterium* fermentation	54

Table 3. Effect of the addition of protein-enriched cassava flour to broiler diets.

	Dietary treatment			
Period: 1-21 days	Control	5% FCM	10% FCM	15% FCM
Final weight, g	635	662	701	632
Weight gain, g[a]	590	617	656	578
Additional protein added, g kg^{-1}	0	27	54	81
% Weight increase above control	0	4.5	11	-3

[a] initial weight of chickens = 45 +/ -6 g

CURRENT AND FUTURE INITIATIVES IN CASSAVA RESEARCH IN SOUTH AFRICA

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INTRODUCTION

The present severe economic slump in South Africa is intimately linked to the worst drought in many years and the rapidly rising population. Food shortages and malnutrition amongst especially the rural populace are gaining ever-increasing momentum under these conditions (it has been estimated that at least 30% of the population presently suffer from malnutrition). South Africa has a mix of both first and third world agriculture, with units ranging from very large commercial operations of thousands of hectares down to subsistence plots of 1 ha or less.

Cassava (*Manihot esculenta* Crantz) is a high-biomass, low-protein, high-energy producing tuber crop consisting mainly of starch, which is available all year round, is extremely tolerant to climatic stress (1) and is therefore ideally suited to production in areas where there are sporadic droughts. The fact that the roots can be stored in the soil without special provision is also of importance in these areas. People eat more than 60% of all cassava produced, and about a third of the harvest feeds animals, while the rest is transformed into secondary products (5). It is already grown by many subsistence farmers in South Africa and could fill an important niche in alleviating the malnutrition problem in the rural areas of South Africa. However, cassava's status as a peasant crop has inhibited all significant development of the crop, illustrating a serious lack of communication between agriculture in the developed and less-developed regions of the world, with the South African situation being very symptomatic of this problem.

HISTORY OF CASSAVA IN SOUTH AFRICA

Cassava was introduced into Africa by the Portuguese slavers during the later helf of the 16th century. From the areas of introduction on both the East and West coasts, the crop spread throughout the tropical areas of Africa (6). The crop was apparently introduced into South Africa by southward-migrating Tonga tribesmen, who settled in the Eastern Transvaal and Northern Natal. The first recorded cassava plantings took place with the major tribal movements of the 1830's and 1860's (3). It is well known by the older members of the local populace in rural areas (eg. it is known as *ndumbula* by the Zulus). However, as living conditions improved, cassava lost its appeal as the staple "food of the people" and the younger generations no longer know this useful reserve food.

Interest in the commercial exploitation of this tuber crop was first stimulated in 1948 due to its potential as a source of industrial starch. Yield trials and chemical

analyses of the roots were carried out by the then African Products Manufacturing Company. Interest subsequently waned because of the unfavourable economic climate in the aftermath of the Second World War and major change in ruling political parties. The plummeting sugar price again led to a brief resurgence of interest in the production of commercial starch in the 1960's when trials were carried by Natal Estates Ltd.

Interest in cassava was again stimulated during 1974 when the Anglo American Corporation forecasted the strong possibility of a major shortage of basic energy foods in South Africa by the turn of the century. During 1975/76 was established that production in South Africa was not hampered by any serious limitations and cultivar evaluations took place at several sites throughout the sub-tropical areas of the country. The adaptation of the crop to local conditions was monitored, and it was concluded that this crop could successfully be cultivated in the hotter areas of South Africa. Since 1979 larger areas were planted to cassava, genetically-improved cultivars were imported from CIAT, Colombia, and yield trials were carried out. A cassava breeding station was established at Mtunzini in Northern Natal, and a great deal of research was performed on the etiology and control of two cassava diseases, cassava mosaic disease and cassava bacterial blight (3).

The Economic Development Corporation, later the SA Development Trust Corporation (STK), started research on the crop during 1979. Their interest at that stage was primarily centred around the conversion of the high starch content of the roots into ethanol by fermentation to serve as a substitute for petrol. High-starch-containing, hardy cultivars were obtained from IITA, CIAT, Thailand, Australia and Brazil. Both cultivar evaluation and root development trials were carried out. The latter work resulted in the official registration of two herbicides for use for weed control of the cassava crops. Some local breeding work was also undertaken, which resulted in the development of some promising highly-adapted cultivars.

All cassava research done by the Anglo American Corporation was terminated during 1989, due to the worsening economic climate in South Africa. At present no major research work is being carried out by the private sector, apart from the continuation of limited cultivar evaluation trials by STK, primarily to maintain the imported and locally-developed genetic material. However, with the tremendous advances in techniques for managing and growing cassava, coupled with the endless possibilities for creation of new improved plant species offered to us by DNA recombinant technology, consideration of the potential social and economic impact of these technologies on the development of the 'orphan' crop, cassava, is once again imperative.

RESEARCH PRIORITIES

The majority of research work carried out on this crop in South Africa up to this stage has been concentrated on optimum agronomic propagation of the crop under local conditions. This has established that root bulking effectively ceases

during the period from May to September, and the best time for harvesting appears to be from 18 to 24 months after planting. We plan to initiate a project on the molecular fingerprinting of locally-adapted cassava cultivars in the near future to assess genetic variability, to identify germplasm accessions and to aid in future plant breedings projects. Current research recently initiated by our group centres around the evaluation of the crop for both animal and human consumption, and the application of biotechnology to utilize this high starch producing tuber for industrial gain. We have reported on the single-cell protein enrichment of cassava meal for food and the production of ethanol from cassava starch. Several other fermentation projects on the production of value-added enzymes from cassava starch are currently under way.

African Cassava Mosaic Disease (CMD) is a major debilitating disease throughout Africa, and South Africa is no exception. Trench & Martin (7) reported that high incidences of the disease were found in subsistence plantings in both Natal and the Transvaal. Up to the present time no official country wide survey of the distribution of the disease has been carried out. Large scale outbreaks of the disease in newly planted areas can often be attributed to contamination of locally-propagated and imported planting material. Presently tissue culture techniques are being evaluated in order to produce pathogen-free research material from CMD-infected mother plants. Research aimed at identifying the causative agent of CMD is also done in South Africa, in accordance with similar research done in all the major cassava research institutes in the world. The ultimate aim is to 1) construct a probe for the quick screening and identification of infected plant material, in order to control the incidence of the disease by good preventative nursery practices; 2) to isolate and clone a coat protein gene of CMD under inducive control of a cassava plant immune system gene promotor, to enable the plant to attain permanent resistance to CMD infections.

To aid in this particular project and all the other genetic manipulation experiments suggested further on, an efficient system for preparation of protoplasts and subsequent regeneration to whole plants will be established. Existing tissue culture techniques will be adapted to our available research facilities. Gene transfer will initially be done by electroporation, using the plasmid pGV1040 from Plant Genetic Systems, Belgium, with the *bar, nptII* and GUS selectable genes. Transformation of leaf and tuber discs with *Agrobacterium* vectors will also be tested. If we can raise the necessary funds in future to buy the equipment, we will attempt gene transfer by the very successful technique of high velocity microprojectile bombardment.

During 1989 the cassava mealy bug (*Phenacoccus manihoti*) was identified in South Africa for the first time after being introduced into Congo-Zaire from Latin America in 1973 (2). This pest spread rapidly and has no natural enemies in South Africa. Infection of the cassava plants can cause up to 90% crop loss, and it is difficult to control economically by chemical pesticides. Although a parasitoid wasp (genus *Epidinocarsis*) and predatory ladybirds had been imported and is proving to

be successful in controlling this pest, it will be a long time (if ever) before they spread throughout the cassava-producing areas, the reasons being that cassava is produced in relatively small patches and that these areas are far apart. Research is currently under way to establish what attracts the insect in order to use this knowledge in making the plant less attractive to the insect by genetic manipulation (eg. production of "unpalatable" plants). It has been observed that CMD-infected plants initially appear to be less susceptible to this pest than healthy plants. Although this could be a cultivar trait, virus-induced proteins in these plants may first be investigated as starting material in the search for an insect "repellant". Several prokaryotic genes that produce potentially useful insecticides have been identified and selective cloning of these genes will be investigated for enhancing plant resistance.

Although cassava roots are a good source of energy due to their high starch content, they are very low in protein (4). This is a serious drawback in the utilisation of the tubers as a food replacement. Although the leaves can be used together with the tubers in order to increase the protein content of the dish, this may be problematic under drought conditions when the plant drops the majority of its foliage. The enrichment of the protein content of particularly the roots of the plants by means of genetic manipulation, is currently receiving attention. Our approach will mainly entail protoplast fusions with high-protein plant species and selective enrichment of existing proteins by site-specific mutagenesis.

Callus cultures will also be used to identify stress proteins involved in both temperature (high and low) and salinity stress. The related genes will then be used in multimer cloning into existing cultivars in order to enhance the salt, drought, and especially cold, tolerance of the crop for expansion of possible production areas. South Africa also has a particular problem with aluminium toxicity as a result of the low pH in the majority of arable soils, which allows the aluminium ions to go into solution and be taken up by the plant roots. The identification of genes for aluminium tolerance and their transfer to high-yielding cultivars is an important priority.

The matter of cyanogenesis always causes negative sentiments when utilisation of the cassava plant for human or animal consumption is considered. Reduction of the formation of the particualar glucosides involved will be attempted by means of deletion mutation of the gene level, but this might have undesirable implications with regard to pest resistance and damage by rodents and other mammals which might feed on the plants and roots.

In order to make cassava more attractive for commercial production it is necessary to reduce input costs, particularly expensive labour costs. Weeding is a labour-intensive activity which must be carried out until the canopy closes, and with the plant losing its leaves every year, these costs become a major factor in production. In South Africa the only two legally-registered herbicides for this particular crop cannot be used on sets that have sprouted or are actively proliferating, as such practices result in crop damage. The only logical alternative is therefore to look into the possibility of eliminating phytotoxicity by genetically manipulating the crop for

resistance to a single broad-spectrum herbicide that exhibits reasonable residual activity. Collaboration with Monsanto will be sought in this venture, although we have already identified a prokaryotic gene that confers resistance to copper. This gene may be cloned into cassava and tested for resistance to several commercially available copper-based herbicides.

CONCLUSION

Tyson (8) speculated that a sequence of extended wet and dry spells may materialise in the early twenty-first century. Vast areas of Southern Africa, including the subtropical regions, are semi-arid. Future planning should be based on this assumption of recurring dry spells. The consequences of these extended dry spells in South Africa entail arratic crop yield, widespread crop failures, loss in GDP and per capita food production, as well as increasing food prices.

Biotechnology offers a very powerful addition to the traditional tools of crop improvement. Though still in its infancy in South Africa because of the extremely poor economic climate in the country and a severe lack of research funds, this technology applied to the high-potential crop cassava may do much to alleviate the malnutrition problem in rural South Africa and soon will begin to contribute to the steady rise in agricultural productivity needed to meet projected food needs for an exploding population in the coming decades. Research priorities will continually be reviewed, and refined by means of socio-economic analysis focused on the needs of particularly the small farmer.

However, in order to produce a well-adapted, high-yielding drought-, insect- and salt-resistant cassava cultivar, we need closer collaboration especially other scientists working in cassava in Africa, and we are hopeful for a such links to be established in the immediate future.

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CHARACTERIZATION OF BACTERIAL AMYLOLYTIC ACTIVITIES DURING CASSAVA SOLID STATE FERMENTATION

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Cassava sour starch is a naturally fermented product, which is used in the elaboration of several traditional, flour-based products, like pandeyuca and rosquillas, specially in Colombia and Brazil. Fermentation and drying under the sun confer to the product its specific baking properties (panification power, flavor and aroma). The main problem encountered in the production process, is the fluctuation in product quality.

We have set out to study this very interesting solid state fermentation, whose only substrate is cassava starch. The fermentation process takes mainly place under anaerobic and low pH conditions. The production of lactic acid correlates well with the quality of the product. We are presently studying the enzymatic activities of the lactic amylolytic flora (LAB), homo-and heterofermentative.

Several LAB strains were isolated on selective MRS starch medium with aniline blue. After Gram, catalase, sugar metabolism (API), and mobility analysis, the best 12 growing bacterial strains were selected for further analysis. They all seem to belong to the genus Lactobacillus. Amylolytic activities of these selected strains were chromatographically enriched from the medium, and their enzymatic parameters (Km. Vmax) determined, as well as their pH and temperature optima. The 12 strains show varying degrees of amylolytic activities. this has been clearly demonstrated on activity polyacrylamide gels, on which the different levels of activity as well as the isozyme patterns were observed. Protein fingerprinting using SDS PAGE was performed on these strains, again demonstrating the non-identity of the strains.

Judging by the amylolytic activities and cell count numbers during the fermentation process, it seems likely that Lactobacillus spp are the dominating genus throughout the process.

INTRODUCTION

Cassava sour starch is a naturally fermented product, which is used in the elaboration of several traditional, flour-based products, like pandeyuca and rosquillas, specially in Colombia and Brazil. Fermentation and drying under the sun confer to the product its specific baking properties (panification power, flavor and aroma). The main problem encountered in the production process, is the fluctuation in product quality.

Sour starch is a product of some economic importance, especially in alimentary industry. It can replace wheat flour to a great extent, which could greatly reduce wheat imports. The product has a high potential for broader industrial applicability, but present production methodology is rudimentary. The process is slow (20-30 days). Low efficiency and irregular quality are a consequence of the lack of control parameters. In Colombia, most production is carried out in rural units, the so-called rallanderías, in the Northern Cauca.

Preliminary results show that market acceptability of sour starch is related to partial amylolytic digestion of the starch grains by bacterial exoamylolytic activities. Strains of the genus *Lactobacillus, Leuconostoc* and *Streptococcus* constitute the bulk of bacteria found in the fermentation process.

SAR/CIRAD (France) has initiated a project to define the fermentation parameters, improve production practices, and eventually develop starter inocula. The Universidad del Valle of Cali, the Cassava Utilization Section (CIAT), ORSTOM (France) and the Molecular Biochemistry Laboratory (Biotechnology Research Unit, CIAT) are involved in this project. The isolation and microbiological characterization of strains from the natural fermentation process were carried out at UniValle. We are characterizing selected strains with respect to their amylolytic enzymes.

Most of the fermentation process takes place under anaerobic and low pH conditions. Acidification of the medium is a selective process, in which most other competing bacteria are killed. The production of lactic acid correlates well with the quality of the product. 75 lactic acid bacterial strains (LAB) were isolated on selective MRS starch medium with aniline blue. After Gram, catalase, sugar metabolism (API), and mobility analysis, the best 12 growing bacterial strains were selected for further analysis. Most belong to the genus Lactobacillus and are of homofermentative character.

METHODOLOGY

Isolation and characterization of amylolytic enzymes. Lactic bacteria were grown in MRS-starch broth under aerobic conditions for 72 h at 30°C. Proteins were precipitated from the supernatant with 4 vol of acetone and separated on Laemmli polyacrylamide gels. Amylolytic activities were specifically identified by incubation in citrate-phosphate buffer containing 1% starch and posteriorly stained with iodine. Isolated proteins were also analyzed on denaturing and non-denaturing gels using silver stain. Amylolytic activities were quantified using 0.3% starch as substrate and dinitrosalicylic acid (DNS) as a specific reagent for reducing sugars.

Isolation of α-amylases from the fermentation mass. Fermenting starch was extracted with acetate buffer (50 mM pH 6.5), which was then precipitated with 80% ammonium sulphate. The precipitated proteins were separated by anion exchange chromatography (DEAE-Biogel Agarose). The amylolytic activity containing peak was concentrated with polyethylene glycol.

Fingerprinting using total protein patterns. Bacterial cells were cracked by ultrasonic treatment and separated by electrophoresis on denaturing polyacrylamide gels. Protein bands were revealed by silver stain.

RESULTS

Amylolytic activities of the selected strains were chromatographically enriched from the medium, and their enzymatic parameters (K_m, V_{max}) determined, as well as their pH and temperature optima (Fig.1 and Table 1). The isolated amylolytic activities were thermostable and acid tolerant, two qualities that make them specially interesting for industrial applications. The 12 strains show varying degrees of amylolytic activities. This has been clearly demonstrated on activity polyacrylamide gels, on which the different levels of activity as well as the isozyme patterns were observed. Protein fingerprinting using SDS PAGE was performed on these strains, again demonstrating the non-identity of the strains.

The techniques for characterization of LAB are well established now. Our next goal is to characterize the whole collection of 75 strains isolated from the natural fermentation process.

We are conducting HPLC analysis of the organic acids produced by the LAB under different growth conditions. The production of organic acids during the natural fermentation process is also being monitored.

Physiological fermentation parameters of the isolated strains have to be established under varying conditions to determine the potential industrial applications of the strains. Growth under optimal conditions is needed to establish parameters like biomass conversion and maximal growth rates, but the growth conditions for the induction of specific enzymatic activities has to be established in each case. Small scale natural fermentation with selected strains will be also conducted to establish the performance of potential starter inocula.

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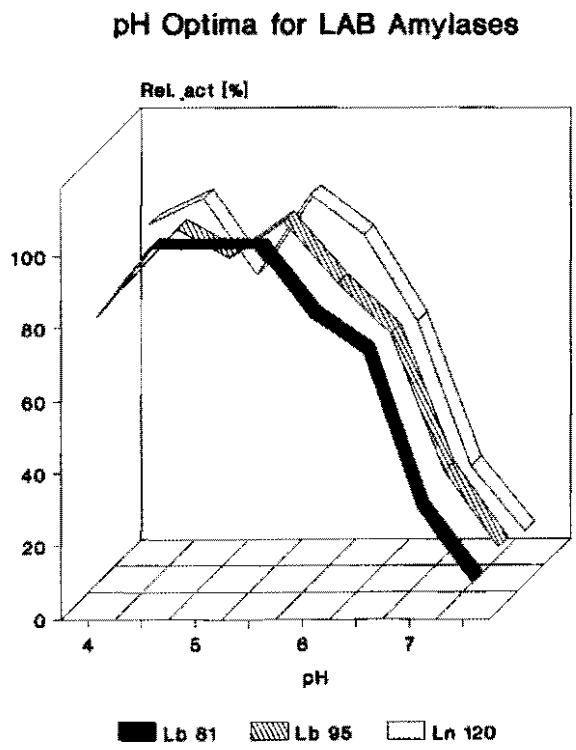
Table 1. Amylolytic activity of selected lactic acid bacterial strains.

Strain	Amylose	Starch	Gel	pH	T °C
Lb 01	+	+	+	6.0	
05	-	-	-		
07	+	+	+	6.0-6.4	
12	-	-	-		
81	+	+	+	4.4-5.4	60
95	+	+	+	4.4-5.4	60
105	+	+	-		
160		+	+	4.0	
214		+	+	6.4-7.4	
120		+	+	4.4-5.4	80
B.cereus		+	+	7.0	



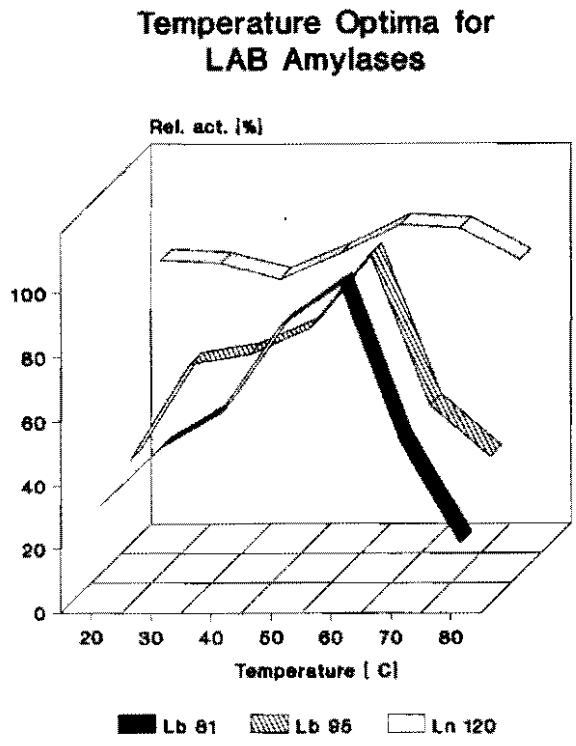


Figure 1. pH and temperature optima for lactic acid bacterial strains amylases.

PHOTOSYNTHESIS OF CASSAVA AND ITS RELATION TO CROP PRODUCTIVITY

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Cassava (Manihot esculenta Crantz) is a hot-climate crop that is also grown in cool areas of the highland tropics, where yields are reduced. The purpose of this study was to evaluate the effect of growth temperature on photosynthesis of two cassava cultivars from contrasting habitat and to determine the relationship between yield and photosynthesis in a set of 15 field-grown cassava cultivars under rainfed conditions. Irrespective of the original habitat of cultivars, photosynthesis was substantially reduced in leaves developed in a cool climate as compared with warm-climate leaves. Cool-climate leaves partially recovered their photosynthetic capacity after 7 d acclimatization in a warm climate. The hot-climate cultivar showed a broad optimum temperature from 30 to 40°C, while the cool-climate cultivar showed an upward shift in optimum temperature in the acclimatized and warm-climate leaves. In field-grown cassava, maximum net photosynthesis of upper canopy leaves was greater than 40 μmol CO_2 $m^{-2}s^{-1}$ when measured in high rainfall season and the rates did not show light saturation up to 1800 μmol $m^{-2}s^{-1}$ PAR. The seasonal average net photosynthesis of upper canopy leaves was significantly correlated with both root yield and harvestable biomass. It was concluded that selection for high photosynthesis in parental materials may lead to high yields when combined with other yield determinants.

In the developing countries, population continues to grow at a high rate (>2.5% per year), which has led to acute food shortage. In order to be able to feed their ever-increasing population, these countries need to increase food production at a sustainable level utilizing the existing available resources. Prime land and water needed for intensive high-input agriculture are scarce, and further increases in food production will probably come from expansion in areas with marginal edaphic and climatic conditions. Food crops that tolerate these marginal conditions and are capable of producing reasonable yields are of paramount importance in this case. Cassava (Manihot esculenta Crantz) is a major staple food crop for more than 500 million people in tropical Africa, Asia and Latin America (Cock, 1985). It is highly productive under favorable conditions (El-Sharkawy et al., 1990; Ramanujam, 1990) and produces reasonably well in adverse environments (Connor et al; 1981; Howeler, 1991; Kawano et al, 1978; El-Sharkawy and Cock 1987a). One of the most important physiological processes that control primary productivity and crop yield is leaf photosynthesis (Zelitch, 1982; Beadle et al., 1985; Boerma and Ashley, 1988). Therefore, research at CIAT has focused on the characterization of the photosynthetic capacity of cassava germplasm in relation to productivity (El-Sharkawy and Cock, 1990; El-Sharkawy et al, 1990).

The objectives of this paper were (1) to evaluate the photosynthetic capacity of two cassava cultivars from contrasting habitats as affected by environmental conditions; and (2) to determine the relationship between productivity and

photosynthesis in field-grown cassava under rainfed conditions using a number of cultivars from CIAT core collection.

MATERIALS AND METHODS

Two cultivars from contrasting habitats were used to test the effect of growth temperature on leaf photosynthesis. They were M Bra 12 (habitat: hot-humid) and M Col 2059 (habitat: cool-humid). Plants were grown in 40-L pots (filled with a mixture by weight of 40% top soil, 33% compost, and 27% sand) in the open at a high-altitude site (elevation 2000 m, mean annual temperature 17°C). The pots were well watered and adequately fertilized. At 2 mo after planting, the pots were brought to the laboratory (elevation 965 m, mean annual temperature 24°C) where leaf gas exchanges (CO_2 uptake and H_2O loss) were determined using a multichannel open-end infrared CO_2 analyzer (Model 225, Analytical Development Co., Ltd., Hoddesdon, England) and a dew-point hygrometer (Model 660, EG&G Instruments, Cambridge, MA). One week before gas exchange measurements, leaves were removed at every other node to balance source (leaves) with the confined and limited sink (roots). The response of net photosynthesis to leaf temperature were determined in normal air (330 ± 10 μLL^{-1} CO_2) and at near-saturating light of 1800 $\mu mol\ m^{-2}s^{-1}$ in the photosynthetic active range. The temperature inside the chambers was controlled by circulating water into the two sides of the chambers through a refrigerated-heated water bath. Leaf temperature was measured by copper-constantan thermocouples touching the lower surface. Measurements were conducted on attached and fully expanded young leaves from different plants. After gas exchange measurements, plants were left outdoors at the warmer site to acclimate to the higher temperature, and gas exchange was measured again after 7 d on leaves of the same age as those previously measured before acclimation. Four weeks later, the fully expanded and newly developed leaves at the warmer site were used for further gas exchange measurements. The responses of net photosynthesis to light were also determined at near optimum temperature (30°C) for leaves that developed in the two different environments.

To determine the relationship between productivity and leaf photosynthesis, 15 cultivars (as listed in Table 1, below) were grown in the field under rainfed conditions at Santander de Quilichao Experiment Station, Department of Cauca, Colombia (elevation 990 m; 3°30'N; 76°31'W; mean annual temperature 24°c).

Stem cuttings (0.2m) were planted on 7 May, 1990 in ridges at 1 m by 1 m distance in 5 by 5 m plots with four replications in a randomized complete-block design. All plots received 50:75:100 kg ha^{-1} N-P-K at 30 d after planting and were kept weed-free. Total rainfall (1148 mm) was slightly less than pan evaporation (1304 mm); however, there were two dry periods (June-September and January-February) when rainfall was much less than evaporation, and one period with excess water (October-December).

Total biomass (excluding fallen leaves and fibrous roots) and storage-root yield were determined by harvesting the eight central plants in each plot on 4-7 March, 1991. Five kg samples each of roots and shoots were cut and oven-dried at 75°C for 75 h to determine dry weight.

Measurements of leaf gas exchange (CO_2 uptake and H_2O loss) were made with a LCA-2 portable infrared gas analyzer (Analytical Development Co., Hoddesdon, England) on several occasions from 23 July to 26 November, 1990. Across all replications, 30 fully expanded upper canopy leaves were measured per cultivar. All measurements were made from 0800 to 1100 h local time with a solar irradiance of 1200-2000 μmol $m^{-2}s^{-1}$. Normal air (325 $\pm$ 10 μLL^{-1} CO_2) was drawn from above canopy using a vertically mounted 4-m glass-fiber probe connected to a pump. A small leaf chamber (Parkinson Broad Leaf Model, Analytical Development Co., Hoddesdon, England), connected to the portable infrared gas analyzer, was clamped over the middle portion (6.25×10^{-4} m^2 surface area) of the central lobe of the measured leaves, and was held toward the sun for 30 to 60 s to obtain steady-state gas exchange. Air temperature in the cuvette varied between 27 and 32°C, depending on time and date of measurement. Measurements were made during both low-and high-rainfall periods with the majority of measurements (80%) made in the former. Responses of leaf photosynthesis to light were also determined for some cultivars during the high rainfall period.

RESULTS AND DISCUSSION

In both cassava cultivars from contrasting habitats, photosynthesis was substantially reduced in leaves that had developed in the cool climate as compared to leaves developed in the warm climate (Fig. 1). Leaves that developed in the cool climate and then acclimated in a warm climate for 7 d partially recovered their photosynthetic capacities; however, these rates were much lower than those measured for leaves developed in a warm climate. Maximum rates in all sets of leaves were higher in the hot climate cultivar (MBra 12) than in the cool-climate cultivar (M Col 2059). A temperature optimum from 30 to 40°C was observed in the hot-climate cultivar for all sets of leaves, while the cool-climate cultivar showed an apparent upward shift in optimum temperature in both the acclimated and warm-climate leaves. During 7 d of acclimation in warm climate, changes in nonstomatal components of photosynthesis (e.g. photosystems I and II, and CO_2 fixation reactions) are more likely than changes in physical stomatal characteristics (Berry and Björkman, 1980). Moreover, the photosynthetic rates in cool-climate leaves were much lower at all light levels and had a lower saturation light than both the acclimated and warm-climate leaves (Fig. 2). The differences in light saturated rates among these sets of leaves may be attributed mainly to differences in CO_2 fixation capacity (Björkman et al., 1980). It is noteworthy that warm-climate leaves were not light-saturated up to 1800 μmol $m^{-2}s^{-1}$. The same phenomenon was observed in field-grown cassava in a warm climate when photosynthesis was measured in the high rainfall period (Fig. 3). Maximum net photosynthesis of field-grown cassava was greater than 40 μmol CO_2 $m^{-2}s^{-1}$. These data indicate that the photosynthetic

capacity of cassava is high and can be fully expressed only in hot-humid climate with high solar radiation. Thus when grown in environments -either natural or artificial - that deviate from these fundamental climatic requirements, its photosynthetic capacity would not be fully expressed. Studies that showed much lower photosynthetic rates (15-20 μmol CO_2 $m^{-2}s^{-1}$) in greenhouse or growth-chamber-grown plants (Aslam et al., 1977; Mahon et al., 1977 a,b; Edwards et al., 1990) are of limited value if such rates are to be interpreted in relation to the real potential of cassava. It is likely that lower photosynthetic rates of cassava grown in greenhouses or in growth chambers were due to lower activities of photosynthetic enzymes (Björkman et al., 1980) and/or changes in leaf anatomy (Nobel, 1980; Nobel and Hartsock, 1981) because of exposure to suboptimal irradiance levels and air temperature during leaf development.

Compared to C_4 crops such as maize and sorghum and to warm-climate C_3 crops such as cotton and sunflower (El-Sharkawy and Hesketh, 1965; El-Sharkawy et al., 1965; Muramoto et al., 1965), cassava is efficient in carbon assimilation, considering its thin and hypostomatous leaves (El-Sharkawy et al., 1984 a,b). Furthermore, cassava has low photorespiration and elevated activities of the C_4 enzyme, phosphoenolpyruvate carboxylase, but lacks Kranz-anatomy typical of C_4 species (El-Sharkawy and Cock, 1987b, 1990). These physiological and biochemical characteristics could be advantageous to cassava photosynthesis, particularly when the crop has to endure a long period of drought coupled with dry-hot air in the regions where it is grown (El-Sharkawy and Cock, 1984; El-Sharkawy et al., 1992).

Across the set of 15 cassava cultivars, seasonal average net photosynthesis in upper canopy leaves was greater than 30 μmol CO_2 $m^{-2}s^{-1}$ (Table 1). Average dry root yield and total harvestable biomass were 17 and 23 t ha^{-1}, respectively. The relatively high seasonal photosynthesis and the yield and biomass production under rainfed conditions with a prolonged water deficit commencing early in the growth cycle (from June to September, 1990, the total rainfall was 267 mm while pan evaporation was 551 mm) illustrate the capacity of cassava to withstand drought. Under water stress, cassava restricts its leaf canopy, partially closes its stomata, and extracts soil water slowly (El-Sharkawy et al., 1992). These mechanisms allow the crop to maximize water use efficiency over a long period while maintaining good yield.

Seasonal average photosynthetic rate was significantly correlated with root yield (r = 0.56, $P < 0.05$) and with total biomass (r = 0.64, $P < 0.01$). These findings again confirm previous reports (El-Sharkawy and Cock, 1990; El-Sharkawy et al., 1990), showing direct relation between photosynthesis, as measured in the field under stressful environmental conditions, and productivity. Similar findings were recently reported in irrigated cassava (Ramanujam, 1990). The positive association of photosynthesis with yield suggests that selection for high photosynthesis in

parental materials may lead to higher yield when combined with other yield determinants such as high leaf area index (LAI), strong storage root sink and high partitioning ratio of assimilates toward roots (i.e. high harvest index).

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Table 1. Leaf net photosynthesis, yield and biomass of field-grown cassava under rainfed conditions at Santander de Quilichao (1990/1991).

Cultivar	Seasonal average net photosynthesis	Dry root yield	Dry total biomass [1]
	(μ mol CO_2 $m^{-2}s^{-1}$)	(t ha^{-1})	
CG 996-6	34	18	24
M Bra 191	36	17	24
CM 4864-1	34	19	26
CM 4145-4	32	17	22
CM 3456-3	32	18	23
CM 507-37	29	19	24
CM 4716-1	32	16	25
M Col 1684	31	17	22
CM 4575-1	33	18	22
CM 4617-1	31	17	23
CM 523-7	30	18	24
M Col 1468	30	15	22
CM 4701-1	31	18	24
CM 4711-2	31	17	25
CG 927-12	26	12	16
Mean of all cultivars	32	17	23
LSD (0.05)	1.8	1.7	2.4

[1] Excluding fibrous roots and fallen leaves.

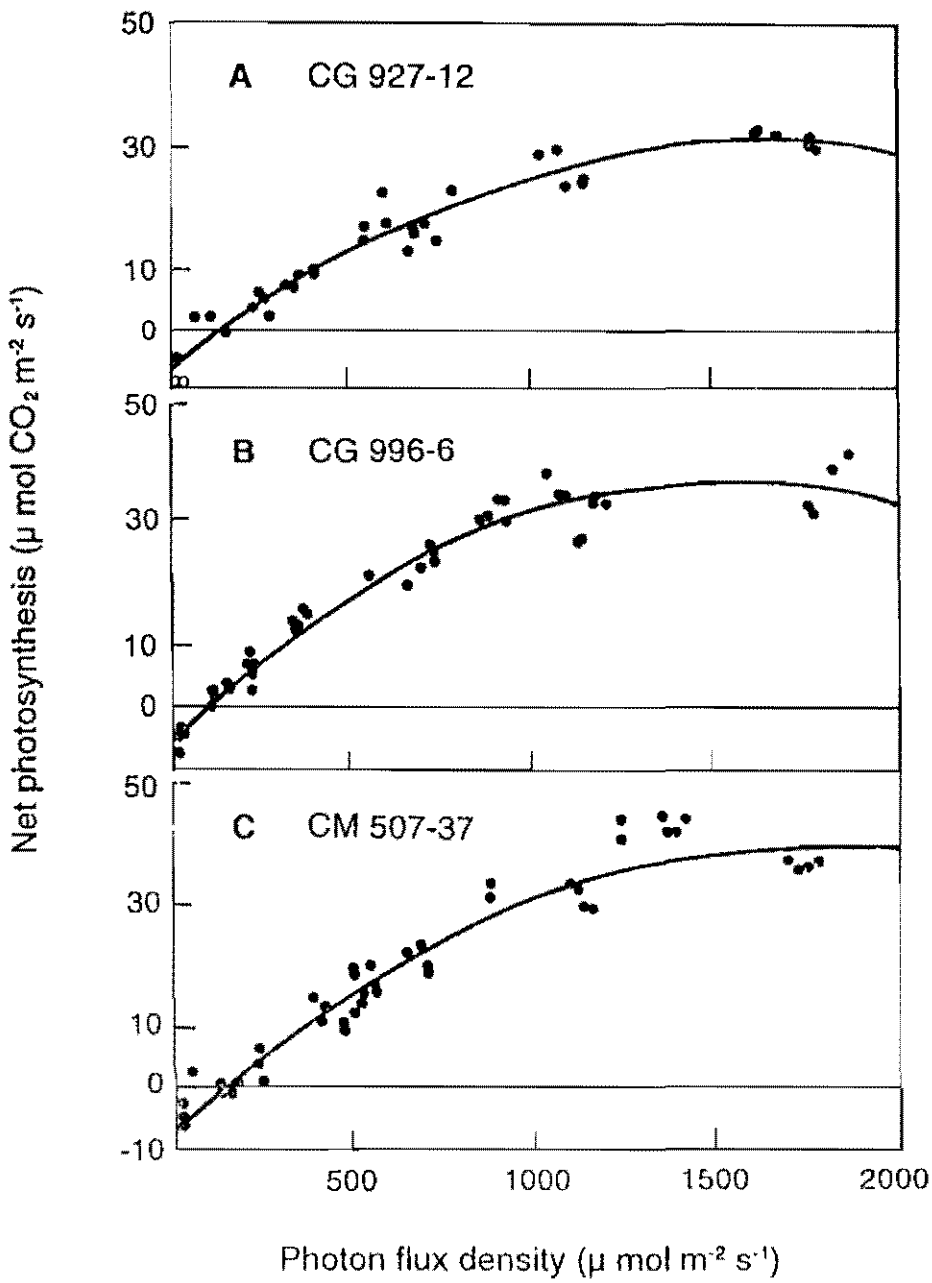

Figure 3. Responses in cassava of net photosynthesis to photosynthetic photon flux density in upper canopy leaves of field-grown cassava during the rainy season. (A) cv. CG 927-12; (B) CG 996-6; (C) CM 507-37.

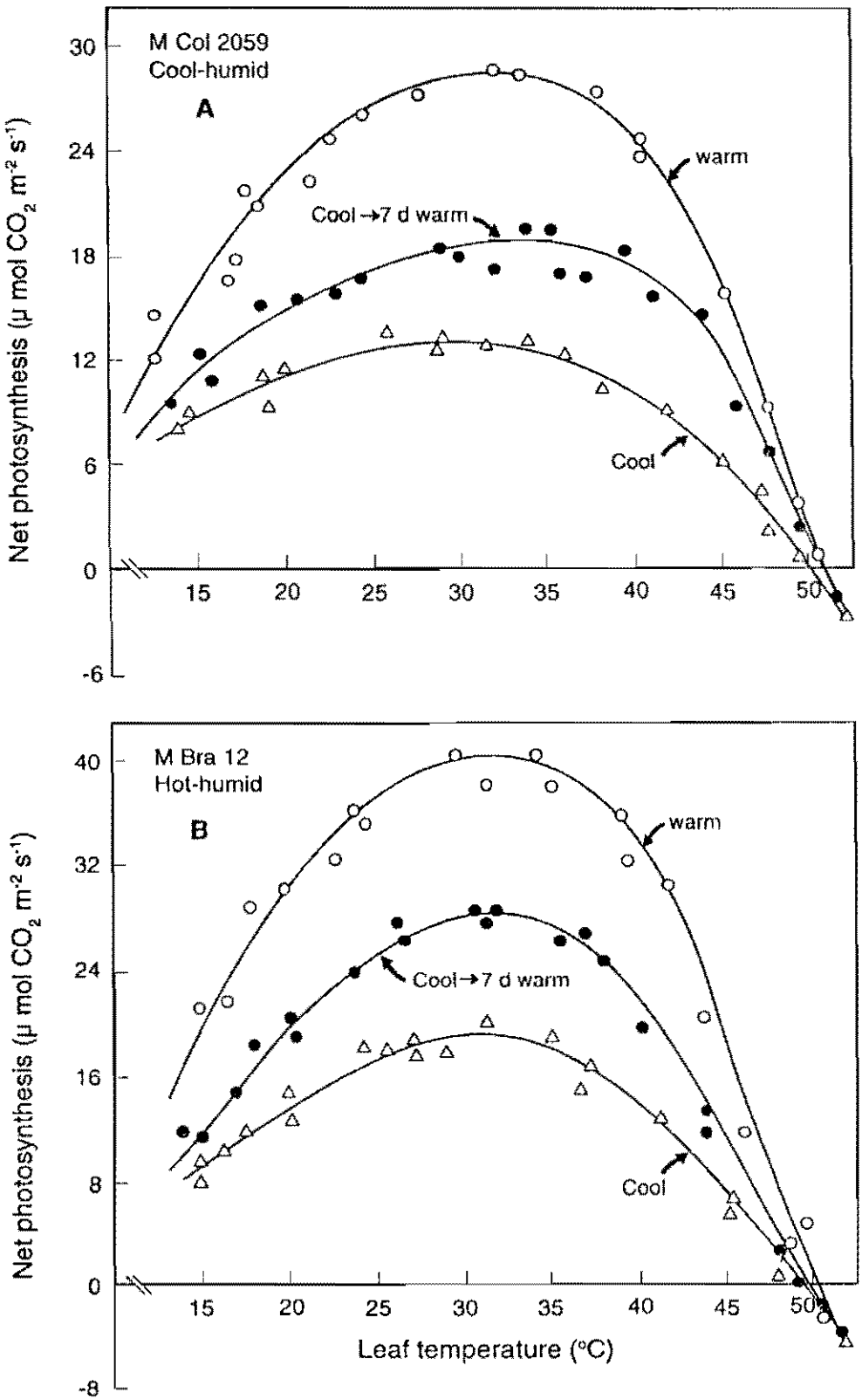

Figure 1. Responses in cassava of net photosynthesis to leaf temperature. (A) cv. M Col 2059, habitat: cool-humid; (B) cv. M Bra 12, habitat: hot-humid; ( ) leaves developed in cool climate; (o) leaves developed in cool climate and then acclimated for 1 wk in warm climates. (o) newly developed leaves in warm climate.

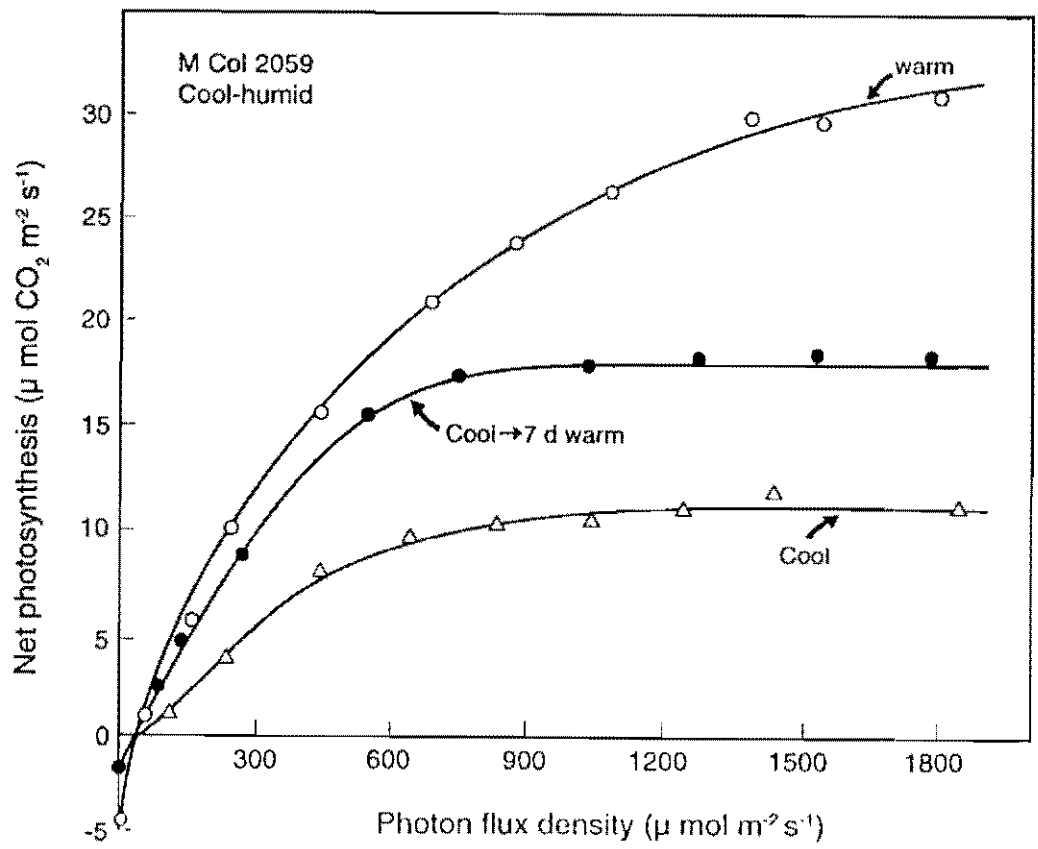

Figure 2. Responses in cassava of net photosynthesis to photosynthetic photon flux density in cv. M Col 2059. ( ) leaves developed in cool climate; (o) leaves developed in cool climate and then acclimated for 1 wk in warm climate; (o) newly developed leaves in warm climate.

NOVEL PHOTOSYNTHETIC CHARACTERISTICS OF CASSAVA, *Manihot esculenta* CRANTZ, A REPUTED C_3-C_4 INTERMEDIATE CROP SPECIES

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The biochemical pathway of CO_2 fixation in cassava leaves was reported initially as C_3 in work from Canada. But cassava is a tropical root crop and later work from Colombia in South America reported it was a C_3/C_4 intermediate photosynthesis plant. More recently it was again reported to be a C_3-type plant from Washington State. It has been claimed that these differences are because cassava is a tropical plant. Because of its importance as a productive crop on low fertility soils, in subsistence-type agriculture, we have investigated its leaf carbon metabolism further by working with plants grown both in Athens, GA, and Cali, Colombia. Our data show that whole leaf photosynthesis values can be quite high in air, indeed comparable to C_4 plants. But other gas exchange data such as leaf CO_2 compensation values and several photorespiration measurements, show C_3 values. The products of $^{14}CO_2$ leaf labeling experiments and their turnover in $^{12}CO_2$ air show C_3 patterns with no hing of C_4 organic acids. However, cassava exhibits a rapid and high labeling of sucrose which is novel for C_3 photosynthesis and its exhibits some high values for certain C_3 carbon cycle enzymes. Thus, cassava leaf photosynthesis, in both Athens and the tropics, is C_3; however, the leaves exhibit vigorous photosynthesis and have a very active sugar metabolism which likely contributes to the high productivity of the cassava crop in subsistence tropical agriculture.

INTRODUCTION

The biochemical pathway of photosynthetic CO_2 assimilation in cassava leaves has been in dispute. In early work, cassava was classified as a C_3 plant based primarily on leaf CO_2 exchange traits such as a CO_2 compensation point (Γ) above 45 μl CO_2 l^{-1}, low rates of leaf photosynthesis (PS), and high leaf water use efficiency values (1,2). Even so certain cassava cultivars exhibited a non-saturating response of leaf PS to increasing irradiances up to 2,000 uE $m^{-2}s^{-1}$ (2,3), high rates of individual leaf PS (4,5), a saturation of leaf PS near 500 μl CO_2 l^{-1} (1), or distinct green leaf bundle sheath cells (4). These latter traits are reminiscent of C_4 PS plants (6).

However, beginning in 1987, in a series of studies it was claimed that the PS biochemistry was variable in the cassava germplasm and that cassava is a C_3-C_4 intermediate PS plant with a partial C_4 biochemistry (7,8,9). These conclusions were based on a calculated leaf Γ value near 25 μl CO_2 l^{-1}; some of the anomalous traits just cited as reminiscent of C_4 plants; finding of 40 to 60% of the ^{14}C in C_4- organic acids after 5 to 10 s exposure to $^{14}CO_2$; and the detection of leaf PEP carboxylase activity between 15 to 40% of a C_4 plant, corn (7,8,9). But quite contradictory work

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was reported from Washington State with growth chamber and greenhouse grown cassava cultivars that concluded cassava was a C_3 plant (10). With cassava these workers found less than 3% $^{14}CO_2$ fixation into C_4-organic acids, without turnover; 85%, labeling of PGA and sugar-P in 3 s with subsequent turnover; a Γ value between 55 and 62 μl CO_2 l^{-1}; no leaf PEP case activity greater than in other C_3 plants; and C_4- acid decarboxylases were hardly detectable in cassava leaves.

Considering these studies, as a whole, the question of whether or not cassava has a partial C_4 cycle was in dispute. We have studied cassava, both in Georgia and at CIAT, over several years. From this work we will conclude i) that there is no evidence for the PS fixation of CO_2 into C_4- acids nor for the required C_4- acid turnover, ii) that cassava leaves have a novel sucrose metabolism, and iii) we have discovered new leaf morphological arrangements that may be related to efficient PS and drought tolerance of the cassava plant.

MATERIALS AND METHODS

The cassava accessions were obtained from CIAT as sterile plantlets, multiplied in the greenhouse each winter, and then field-grown each summer season. The PS experiments in Athens all were conducted with vigorously growing plants outdoors. In the late summer of 1991 Mr. Sun also conducted confirmatory studies at CIAT. Leaf gas exchange studies employed standard IR gas analysis techniques (11). The reaction assay mixtures for enzyme assays followed published procedures (12,13), as did assays for leaf sugars (13,14), $^{14}CO_2$ pulse-$^{12}CO_2$ chase PS studies (15), and leaf microscopy (12). Unless noted otherwise, we studied the intact first fully-expanded leaf (6th or 7th from branch tips) usually outdoors in full sunlight.

RESULTS

To determine the C_4 PS nature of cassava our first objective was to measure the initial levels of C_4-organic acids formed by cassava leaves and to follow the speed of C_4- acid turnover. But to be certain about these studies we also wanted to work with healthy vigorous cassava comparable to tropically grown plants. That work involved leaf photosynthesis studies as illustrated in Fig. 1. The intact cassava leaf has a C_4-type non-saturating highly active leaf PS as irradiances increased up to near 2,000 μE m^{-2} s^{-1} (Fig. 1A, 1,2,7). And unlike most C_3 plants (6), cassava leaf photosynthesis began to saturate slightly above air levels of CO_2 (Fig. 1B, 1). These leaf PS traits are more C_4-like than C_3- like (6), which lends some credence to the theory of cassava being a C_3-C_4 intermediate PS species (4,5). However we measured the leaf Γ values on numerous cassava cultivars and only found values between 49 and 61 μl CO_2 l^{-1} and could not find any values near the 25 reported by CIAT workers (4,5). Also we measured the O_2 inhibition of leaf PS, comparing 2% O_2 versus 21% at 340 μl CO_2 l^{-1}, and found 29 to 35% inhibition with all cultivars. Each of these values are typical of C_3 PS leaves.

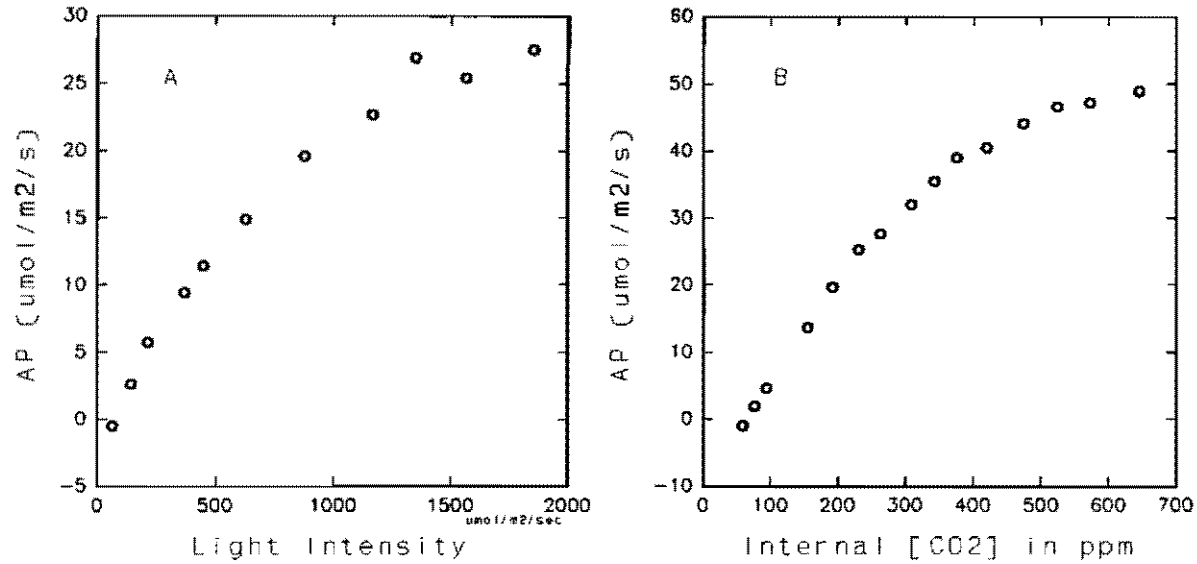

Figure 1 The influence of irradiance intensity (A) and changing internal CO_2 levels (B) on intact cassava (MCOL22) leaf photosynthesis.

These results indeed show that cassava leaves exhibit a unique set of gas exchange traits and that made us search for other unusual traits. But when we examined the pathway of PS CO_2 fixation, we could not find any evidence for a substantial early labeling of C_4-acids nor evidence of a rapid C_4-acid turnover during PS (Fig. 2A). The experimental results in Fig. 2A have been repeated with 4 cv's and during 2 growing seasons with similar data sets. Consequently we conclude there is no evidence for a C_4-type PS in intact cassava leaves.

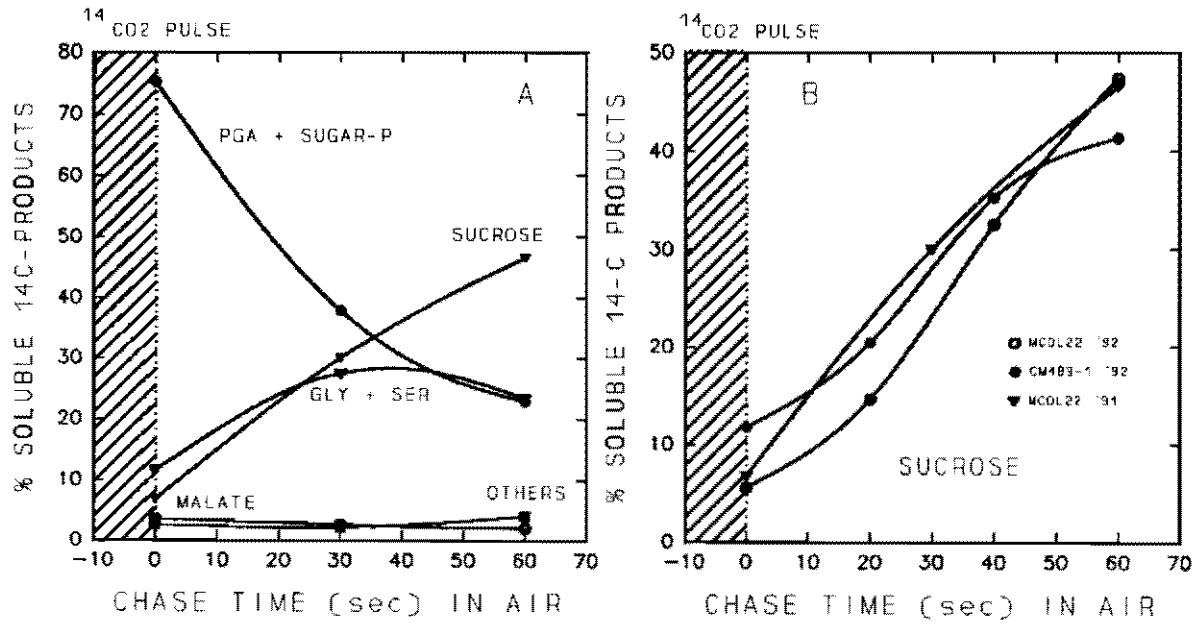

Figure 2. Tissue course of sucrose labeling in cassava.

However we found a remarkable and rapid ^{14}C-labeling of sucrose during cassava leaf PS. Fig. 2 presents time courses of sucrose labeling and the labeling is essentially linear. Studies over 2 years and cv's are plotted in Fig. 2B. To our knowledge, this is the most rapid sustained labeling of sucrose ever reported. Cassava PS is uniquely adept at synthesizing sucrose (Fig. 2) and we also have observed for several years that cassava can even excrete sucrose from its leaves and petioles (data not shown)! These unusual traits have not been observed in other plants. The cassava leaf exudate is comprised of sucrose plus about an equal amount of glucose and fructose (unpublished).

Then as we were studying the cassava leaf we examined it's morphology and discovered another novel trait of cassava. The lower leaf mesophyll surface has a network of papillae extending from certain individual epidermal cells that form a "fence-like structure" around the guard cells. Fig. 3 illustrates the papillose structure in a leaf cross section and in a direct view of the abaxial leaf mesophyll surface. Papillae are not prominent on vein surfaces.

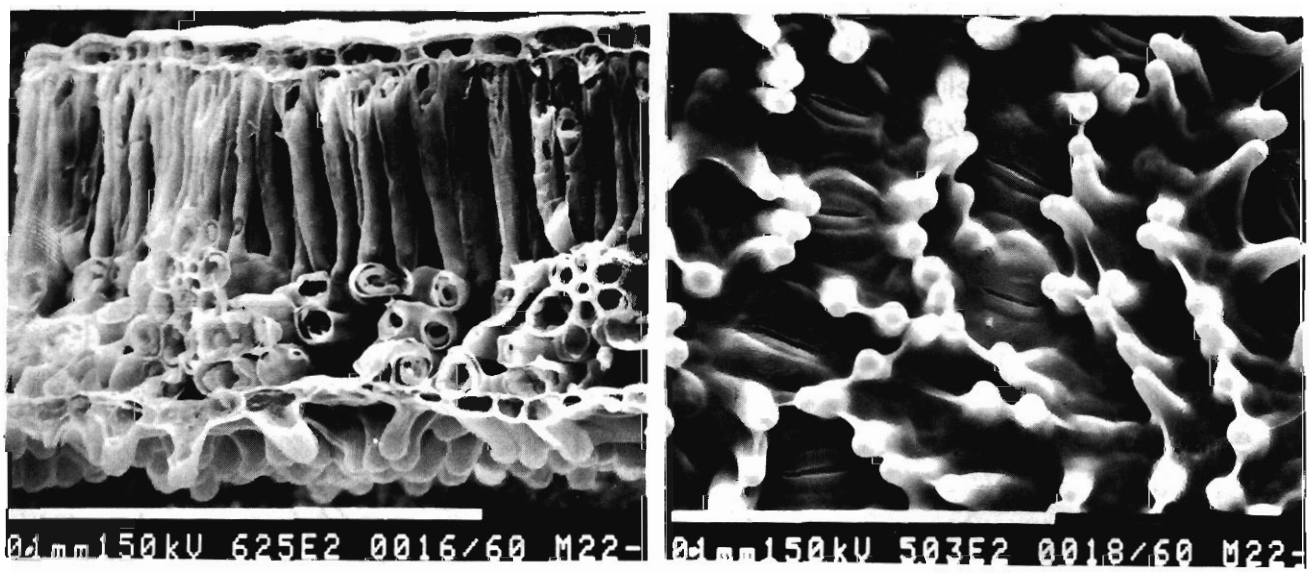

Fig. 3 Scanning electron micrographs of the cassava leaf in cross section (lèft) and papillae on the lower leaf surface over mesophyll cells (right).

The upper surface of the cassava leaf is relatively smooth with a few stomata (9) and trichomes. Our hypothesis is that the papillose structures influence the microenvironment of the stomata to help prevent desiccation and this allows the cassava plant to withstand extreme moisture deficient conditions.

CONCLUSIONS

From this work we conclude that the cassava leaf biochemistry follows a C_3 pathway of CO_2 assimilation; a novel biochemical pathway exists for the rapid synthesis of sucrose; the cassava leaf is uniquely adapted morphologically for maintaining its water; and these traits combine in the cassava plant to facilitate it's active photosynthesis and to support its ability to survive and grow in low moisture environments.

ACKNOWLEDGMENTS

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CLONING AND SEQUENCE ANALYSIS OF PEP-CARBOXYLASE FROM CASSAVA

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Cassava (*Manihot esculenta* Crantz) is very drought tolerant and shows high levels of photosynthesis at high temperatures. This makes cassava an important source of carbohydrates for several arid regions, especially in Africa. Some varieties perform better than others. Physiological performance, which covers a wide range between C_3 and C_4 plants, could be increased through directed breeding. In order to do so, a better understanding of the underlying molecular mechanisms is needed, as to develop well defined breeding criteria and tests that could aid to that purpose.

Several enzymes are involved in the fixation of CO_2 in plants, phosphoenolpyruvate carboxylase playing a central role. We want to analyze the compartmentation of this and other photosynthetic enzymes between palisade and mesophyll cells by using *in situ* hybridization of the radiolabelled genes on histological sections. For this purpose the homologous gene must be isolated. We constructed a cassava genomic DNA library from the variety 996 CIAT in an EMBL 3 derived bacteriophage cloning vector. The library was screened with a maize ppc probe (obtained from T. Nelson, Yale), and after three rounds of purification, a putative cassava ppc clone of 10 kb was obtained.

We are in the process of mapping and sequencing our clone. The next steps will include carrying out Northern analysis to assess levels of expression in different varieties, carrying out relatedness studies with other sequence characterized ppc genes, and the establishment of *in situ* hybridization techniques.

INTRODUCTION

Cassava accumulates starch in the storage roots even under harsh climatic conditions of high temperatures and drought stress, making cassava a unique crop for marginal arid zones. Biomass and root-yield of cassava are correlated with photosynthetic rate (El-Sharkawy et al., 1990). Cultivated species cover a wide range of photosynthetic activities, making it necessary to develop diagnostic tools to evaluate this trait for breeding purposes. To this end we have to understand the underlying mechanisms at the molecular level.

Several enzymatic parameters indicate that cassava has a peculiar, highly efficient photosynthesis (allowing us to classify cassava as a sort of C3-C4 intermediate species). These include the activities of PEP carboxylase (PEPC), malic enzyme (ME) and the ratio PEPC/RuBPC. Cassava shows a high photosynthetic rate and a low compensation point for CO_2, low photorespiration and high water use efficiency (WUE). The appearance of C4 acids is not conclusive yet and is a matter of controversy.

The C4 syndrome is found in around 20 different families, where it seems to have evolved separately. Examples exist where C3, C4, and C3-C4 intermediates

can be found within one genus, e.g. Flaveria. This is also indicated by the different C4 mechanisms that have evolved. Three main mechanisms can be classified according to the C4 compound shuttled (malate or aspartate), and the decarboxylating enzyme in bundle sheath cells (NAD-ME, NADP-ME or PEP carboxykinase [CK]) (Langdale and Nelson, 1991). The compartmentation of these enzymes has not developed in parallel to the development of the C4 anatomy, as can be derived from the study of several intermediate species (Edwards and Ku, 1987; Nelson and Langdale, 1991). Thus, C3-C4 intermediates provide very useful objects for evolutionary studies.

El-Sharkawy (CIAT Cassava Program) has developed a working model for cassava based on leaf cell ultrastructure and physiological data, which includes some degree of enzyme compartmentation in the leaf tissue. Some of the observations on which the model is based, include the positioning and number of mitochondria, chloroplast dimorphism, and large gas vacuoles penetrating deep into the palisade cell-layer, which is composed of very long cells funneling into bundle sheath cells. This arrangement could serve as a novel and efficient mechanism of CO_2 recycling and concentration.

Recycling of CO_2 has been postulated as a means of improving photosynthesis rates (PR) at high temperatures by Schuster and Monson (1990). Without evolving new biochemical pathways or altering the compartmentation patterns of photosynthetic reactions, the net PR of C3 plants could be increased by increasing intercellular CO_2 through increased stomatal conductance. This would lead to a higher PR per unit of RuBPC (higher photosynthetic nitrogen-use efficiency [NUE]), but at the cost of higher transpiration (lower WUE). The alternative would be the increase of RuBPC concentration, which would result in lower NUE and WUE, as stomatal conductance would have to be raised in order to deliver higher CO_2 concentrations for the higher enzyme concentration. The evolution of a C3-C4 mechanism involving the recycling of CO_2 has the potential to increase the rate of photosynthesis at warm leaf temperatures, without incurring the costs described above.

In situ hybridization techniques using antisense RNA on histological sections allow the analysis of the subcellular expression pattern of specific genes. This technique should enable us to shed light on the mechanisms involved in CO_2 assimilation in cassava by demonstrating the cellular compartmentation of the enzymes involved.

In situ hybridization is done with homologous probes, as heterologous probes are prone to result in high background noise due to the necessarily lower stringency hybridization conditions. To the present day, not many genes involved in CO_2 fixation from different plant species have been cloned. ME has been isolated from *Flaveria trinervia* and maize; MDH from watermelon, maize and sorghum; PEPC from F. trinervia, maize and tobacco; RuBPC from pea, Petunia, *Pinus thunbergii*, potato, tobacco, sorghum, spinach and maize. A homology of around 70% can be found in

some regions of the PEPC genes of maize and tobacco, a monocot and a dicot species, respectively. This homology would not be enough for in situ hybridization studies, but would allow the fishing of the corresponding genes from a cassava DNA library.

We have received gene probes for PEPC, NADP-ME, NADP-MDH and RuBPC from maize from Tim Nelson (Yale). The PEPC probe was used to fish out two clones from a lambda GEM11 genomic library, which contained around $2x10^5$ clones. The two clones went through three rounds of purification and have been partially sequenced. No homology has been found to PEPC from maize so far. We are in the process of cloning and sequencing additional clones for PEPC and will soon start doing the same for the other enzymes.

Southern blots of several cassava cultivars will be performed with the different maize probes to establish the degree of expected homology, and to get an estimate of the number of gene copies per genome. The blot shown in the Fig. 1 was done with the *ppc* probe on three differetn cassava cultivars, two of them showing high photosynthetic rates, as well as with *Zea mays* and *Phaseolus vulgaris* as controls. The two better cassava cultivars exhibit an additional band that could represent a second form of PEPC. This has to be confirmed utilizing a larger set of cultivars selected for this characteristic.

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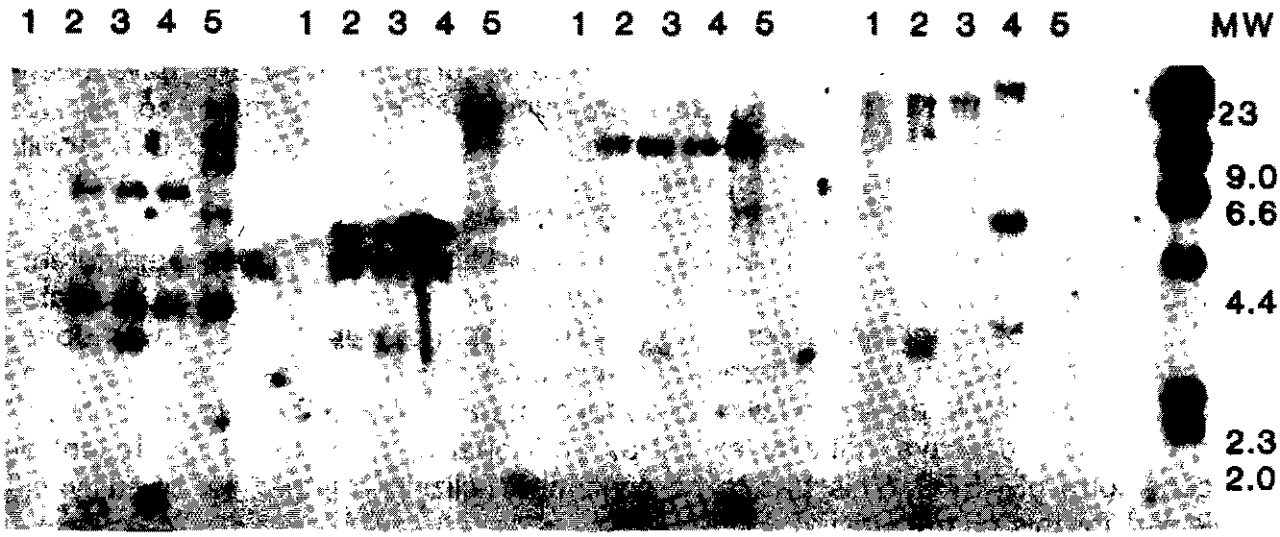

		Photosynthetic Activity
1. M-BRA 191		High
2. CG-996		High
3. CG-927		Low
4. Zea mays	C4	High
5. P. vulgaris	C3	Low

Fig. 1. Genomic Southern hybridization of three cassava genotypes with a ppc probe.

EVIDENCES FOR ENDOPHYTES PARASITING TRADITIONAL CLONES OF CASSAVA (Manihot esculenta Crantz)

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The existence of endophytes in cassava was suspected for the following reasons: (a) A wide range variation of root yield is frequent among disease-symptomless plants growing on the same plot; (b) root yield of low-yielding, virus-free plants of traditional clones can be increased by meristem culture; (c) the performance (i.e. root yield) of meristem-culture derived plants decreases sharply and uniformly under field conditions; and (d) the long growing cycle of cassava and its vegetative propagation allow infection and dissemination of these parasites. The existence of endophytes affecting cassava was demonstrated by the following: (a) Several fungal species were isolated from internal tissues of the epidermis, colenchyma and parenchyma of stems of symptomless low-yielding plants of various clones. Most of these species have been reported as endophytes in the literature; (b) inoculation on plantlets and callus tissues did not induce visible symptoms, but inoculated fungal species were reisolated from internal tissues near the inoculated points 30 days afterwards; (c) total root dry weights (t/ha) of plots planted with stakes taken from commercial fields and treated with a systemic fungicide were higher than for similar plots treated with a protectant fungicide or untreated controls; and (d) histological studies observing fungal invasion into the host tissues have shown hyphal growth in colenchyma and parenchyma tissues of affected stems. Additionally, the existence of beneficial endophytes is suspected; this could be of importance to increase biomass production or induce plant protection against detrimental parasites.

INTRODUCTION

Endophytic fungi are symbionts occurring in living plant tissues. The relation to their host may be mutualistic as in mycarrhizas and Acremonium endophytes of forage grasses, antagonistic (pathogenic) or neutral (Cooke, 1977). Most endophytes seem to be neutral facultative symbionts which grow out of symptomless plant organs after surface sterilization.

Endophytic fungi infect root, stem and leaf tissue internally without inducing visible symptom expressions (Petrini and Carroll, 1981) or only causing a few host symptoms (Riesen and Close, 1987) independent of the host-endophyte association (White and Cole, 1986). They complete part or the whole life cycle in their respective host (Latch et al, 1985; Siegel et al, 1985).

Endophytic fungi have been found in all plant families thus far investigated, affecting many species in different geographic regions of the world (Carroll et al, 1977; Petrini and Carroll, 1981). However, no report on the existance of endophytic fungi parasiting Manihot spp. is known excepting mycarrhiza species in Manihot esculenta. Because the effect of endophytes in crop species may be detrimental, beneficial or neutral this investigation was undertaken to prove their existance and test their possible effect in affected clones.

The existence of endophytes in cassava was suspected for the following reasons: a) a wide range variation in root yield is frequent among disease-symptomless plants growing in the same plot; b) root yield of low-yielding, virus-free plants of traditional clones can be increased by meristem culture (CIAT Annual Report, 1976); c) the performance (i.e. root yield) of meristem culture-derived plants decreases sharply and uniformly under field conditions (CIAT Annual Report, 1988); and d) the long growing cycle of cassava and its vegetative propagation allow infection and dissemination of these parasites.

FINDINGS

1. Several fungal species were isolated from internal tissues of the epidermis, colenchyma and parenchyma of symptomless low-yielding plants of various clones. The fungal species isolated and their frequency were: Septoria nodurum, 52.1; Fusarium oxysporum, 7.2; Colletotrichum gloeosporioides, 5.8; C. graminicola, 4.3; Alternaria termissima, 2.9; Trichoderma sp., 2.9; Botrytis sp., 1.4; Torula sp., 1.4; Nigrospora sp., 1.4 and others 20.4. Most of these species have been reported as endophytes in literature.

2. Inoculations on plantlets and callus tissues by spraying, puncturing or immersion in a fungal suspension of some of the isolated fungi did not induce visible symptoms, but inoculated fungal species were reisolated from internal tissues near the inoculated points 30 days afterward.

3. Total root dry weight (t/ha) of plots planted with stakes taken from commercial fields and treated with a systemic fungicide were higher than for similar plots treated with a protectant fungicide or untreated controls (Table 1). However, differences were recorded among plots with plants derived from rooted shoots; this may be due to the existence of a higher probability of endophyte infections of stakes than shoots.

4. Histological studies observing fungal invasion into the host tissues have shown hyphal growth in colenchyma and parechyma tissues of affected stems.

EFFECT

The effect of nine of the isolated endophytes was studied on three cassava clones (M Col 2215, M Bra 191 and M Col 1468) inoculated by spray, immersion and puncturing methods. Results were as follows (Fig. 1):

1. Both detrimental and beneficial endophytes affect cassava, but most of the isolated species were detrimental.

2. The detrimental/beneficial effect of some endophytes depended upon the inoculation method: on M Col 2215, Curvularia sp. was detrimental when spray-inoculated but beneficial when inoculated by immersion or puncturing.

3. Some fungal species behaved as endophytes in a given plant tissue but as pathogens in others: Rhizoctonia sp. did not induce symptoms in leaf and stem tissues, but induced necrosis when the roots were mechanically wounded, similar to any root pathogen.

4. There were varietal differences in relation to the behavior of the endophytes on cassava, which could be of importance for genotype selection.

DISCUSSION

These findings stress the importance of selecting planting material from high-yielding plants of commercial plots and the need for genotype evaluations through several growing cycles under field conditions in order to eliminate susceptible clones showing low root yield stability. On the other hand, the existence of detrimental endophytes in cassava may partly explain the gradual degradation of cassava clones over continuous growing cycles. Further research is needed to elucidate other interactions between cassava endophytes and genotypes of the crop, as well as epidemiological features related to this group of parasites before control/prevention systems can be devised. Special attention will be given to the beneficial endophytes in order to increase biomass production or induce plant protection against detrimental parasites.

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Table 1: Root dry weight (t/ha) of a plot planted with stakes taken from commercial fields and with plants derived from rooted shoots; both were treated with systematic or protectant fungicides every 2 weeks, 3 months after planting and for a 6-month period.

Clone	Source of Planting Material	Treatment		
		Benomyl (systemic, 1500 ppm)	Chlorothalonil (protectant, 1500 ppm)	Control (Distilled H_2O)
M Bra 191	Rooted shoots	24.4[1]	19.7	17.1
	Stakes	25.5	15.0	14.7
M Col 1468	Rooted shoots	20.8	7.6	6.7
	Stakes	22.1	14.0	11.8
M Col 2215	Rooted shoots	21.8	15.6	12.4
	Stakes	17.7	12.8	13.2

[1] Average data taken from 9 pl/plot, 3 reps; plants harvested at 10 months.

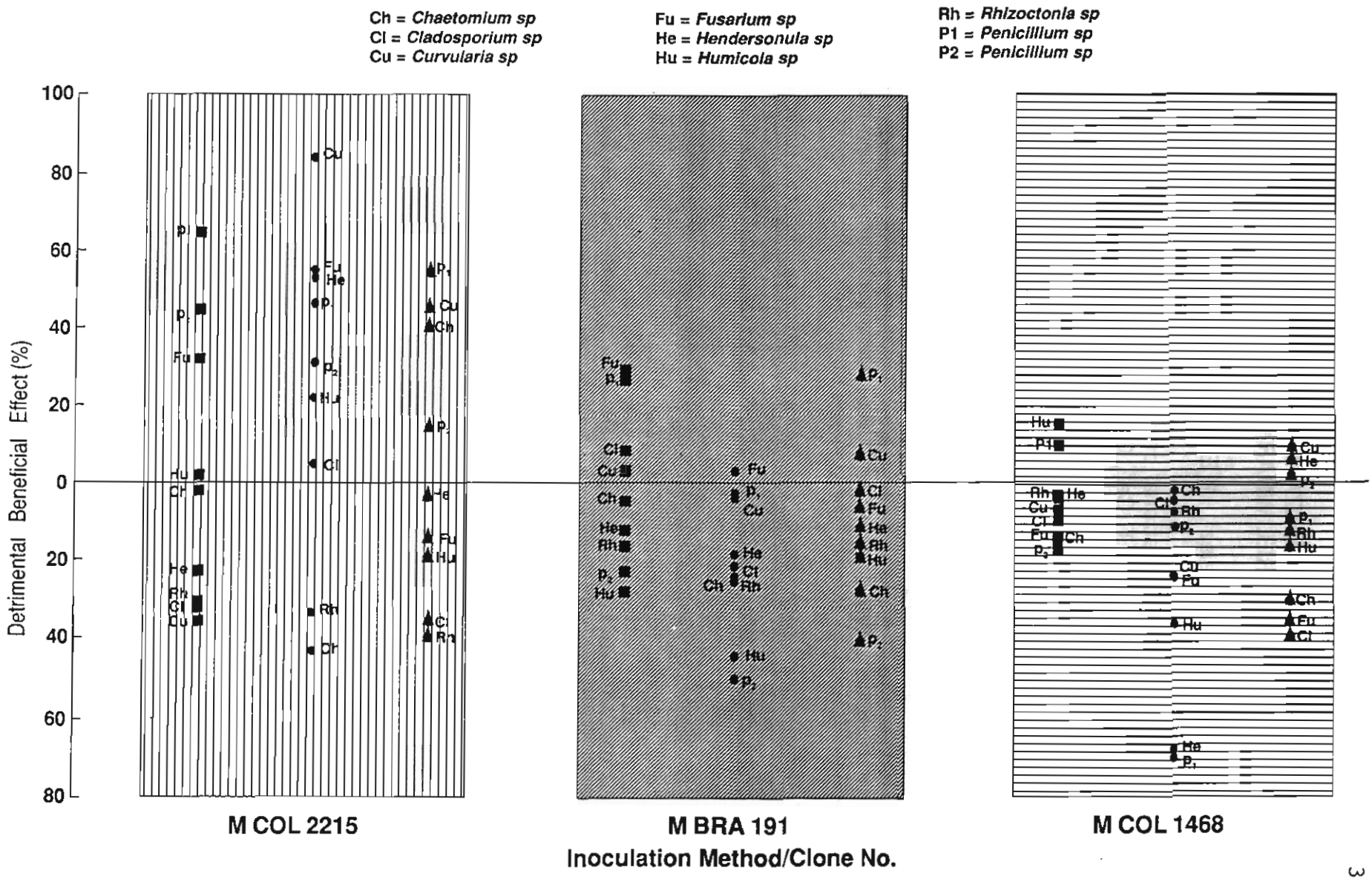

Figure 1 Effect of 9 cassava endophytes(inoculated by ■=spray, ●=immersion, and ▲=puncturing) on 3 cassava clones in relation to the percent of root weight of uninoculated controls.

BIOCHEMICAL CHARACTERIZATION OF PEPC FROM CASSAVA: A PRELIMINARY REPORT

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We want to understand the physiological mechanisms underlying the high photosynthetic rates of cassava under drought stress and high temperatures. Histological analysis of leaf cross sections with cassava PEPC specific antiserum to establish the enzymic compartmentalization pattern is our next goal.

Phosphoenolpyruvate carboxylase (PEPC) from cassava has been purified to >95% purity by liquid chromatography (fractionated ammonium sulphate precipitation, desalting by Sephadex G-25, DEAE Sepharose ion exchange, and gel filtration through Sephacryl S-300 HR). One peak of activity was eluted from DEAE Sepharose by salt gradient at 0.125 M ammonium sulphate. Gel filtration yielded two peaks of 350 and 400 kDa, respectively. Specific activity of the main peak was 5.5 units/mg protein.

PEPC activities from maize, beans, and cassava leaves were compared using a spectrophotometric assay and Fast Violet detection on polyacrylamide gels. PEPC relative content and activity in cassava have intermediate values between maize and beans.

A maize PEPC specific antiserum cross-reacts with cassava PEPC, indicating homologous antigenic determinants. This has also been shown at the DNA level in hybridization studies with a maize ppc probe and total, enzyme digested cassava genomic DNA. The production of a specific antiserum will enable the conduction of histological analysis of PEPC within photosynthetic tissues using immunofluorescence techniques.

INTRODUCTION

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A maize PEPC specific antiserum cross-reacts with cassava PEPC, indicating homolgous antigenic determinants. This has also been shown at the DNA level in hybridization studies with a maize ppc probe and total, enzyme digested cassava genomic DNA. The production of a specific antiserum will enable the conduction of histological analysis of PEPC within photosynthetic tissues using immunofluorescence techniques.

METHODOLOGY AND RESULTS

For the same reasons explained in the abstract on the isolation of the ppc gene by Tenjo and Mayer, the purification of the enzyme phosphoenolpyruvate carboxylase (PEPC) from cassava was also pursued. PEPC plays the central role in the primary fixation of CO_2 in C4 plants. The purification of the enzyme will allow the production of antibodies for quantification and subcellular localization of the enzyme using immunofluorescence techniques on histological sections, analog to the in situ hybridization with antisense RNA.

PEPC from cassava (*Manihot esculenta* Crantz) was purified to >95% purity by liquid chromatography (fractionated ammonium sulphate precipitation, desalting by Sephadex G-25, DEAE Sepharose anion exchange chromatography, and gel filtration through Sephacryl S-300 HR). One peak of activity was eluted from DEAE Sepharose by
salt gradient at 0.125 M ammonium sulphate. Gel filtration yielded two peaks of 350 and 400 kDa, respectively. Specific activity of the main peak was 5.5 units/mg protein.

The K_m of PEPC from cassava (0.18 mM) lies lower than that of maize (1.5 mM), indicating higher substrate affinity. If this can be substantiated, it could explain in part the high efficiency of CO_2 assimilation in cassava.

Phosphoenolpyruvate carboxylase (PEPC) is a key enzyme of primary photosynthetic CO_2 fixation by C4 and CAM plants, it is also present in C3 plants, but at much lower levels. Levels of PEPC in leaves of C4 plants are nearly twenty times higher than in C3 plants on a chlorophyll basis. In maize, PEPC constitutes 10-15% of leaf total soluble proteins and is mainly localized in the cytosol of mesophyll cells. PEPC activities from maize, beans, and cassava leaves were compared using a spectrophotometric assay and Fast Violet detection on polyacrylamide gels. PEPC relative content and activity in cassava have intermediate values between maize and beans under stress conditions which drop to normal C3 values under non-stress conditions (Table 1).

A maize PEPC specific antiserum cross-reacts with cassava PEPC, indicating homologous antigenic determinants. The production of a specific antiserum is necessary for the conduction of the immunofluorescence experiments on leaf sections. For the production of PEPC antiserum, the protein stemming from the last DEAE Sepharose purification step will be separated by PAGE, the band containing PEPC will be cut out, and the finely powdered gel will be resuspended in buffer plus Freund's Adjuvant for the production of rabbit antiserum.

Purification of PEPC

PEPC was isolated using a combination of fractionated ammonium sulphate precipitation and anion exchange chromatography on DEAE Sepharose CL-6B,

followed by gel filtration chromatography on Sephacryl S-300 HR. PEPC activity precipitated between 40 and 60% ammonium sulphate saturation. The desalted fraction was loaded onto the DEAE column and eluted with an ammonium sulphate gradient (0.05-0.4 M). The peak fraction was concentrated by ammonium sulphate precipitation and passed through the Sephacryl column. Tris-SO_4 buffers were used throughout, as chloride is deleterious to the enzymatic activity of PEPC.

Enzyme assays.

PEPC. The oxidation of NADH in a coupled enzymatic reaction with MDH was quantitated photometrically at 340 nm. In this reaction PEP is converted to oxaloacetate and further to malate.

RUBISCO. The conversion of phosphocreatine to phosphoglycerate and diposphoglycerate in a coupled enzyme assay utilizing PGDH and PGK is quantitated as with PEPC.

NAD-ME. The reduction of NAD in the conversion of malate to pyruvate and CO_2 is quantified photometrically.

Immunological and electrophoretical analysis. Protein preparations were assayed using the double immunodiffusion technique on agarose gels buffered with 0.05 M barbital pH 7.6. The precipitin reaction was stained with Coomassie Blue R-250 in methanol/acetic acid. Proteins were also analyzed by Western blot of non-denaturing gels. Primary antibodies were detected with anti-rabbit-IgG coupled to peroxidase. PEPC activity was also detected in situ on gels by Fast Violet stain (detects oxaloacetate).

Table 1.

Activities of PEPC, RuBPC and NAD–ME in Cassava Leaf Extracts

VARIETY	PEPC			
	μmol / gFW min	μmol / mg CHL min	μmol / mg PROT min	PEPC / RuBPC
CM 523–7	5.58±0.53	1.57±0.10	0.068±0.007	0.43
CM 507–37	2.96±0.16	1.91±0.10	0.049±0.003	0.28
MCOL 1684	4.28±0.27	2.90±0.19	0.058±0.003	0.42
MCOL 1468	4.33±0.39	3.07±0.27	0.052±0.005	0.36
C4				1.7–5.0
C3				0.05–0.10
C3–C4				0.25–0.50

	RuBPC		
CM 523–7	12.84±2.20	3.62±0.62	0.156±0.026
CM 507–37	10.60±1.02	6.84±0.66	0.174±0.017
MCOL 1684	10.22±1.74	6.96±1.18	0.138±0.023
MCOL 1468	12.18±1.88	8.16±0.71	0.146±0.022

	NAD–ME		
CM 523–7	1.94±0.29	0.55±0.08	0.024±0.004
CM 507–37	2.01±0.16	1.30±0.10	0.033±0.003
MCOL 1684	2.27±0.15	1.54±0.10	0.031±0.002
MCOL 1468	2.10±0.17	1.48±0.12	0.025±0.002
C3		0.1–0.3	
C4		5.0–9.0	
Cassava/C3		5.0–15.0	
Cassava/C4		0.2–0.3	

BIOLOGICAL CONTROL OF CASSAVA DISEASES USING FLUORESCENT PSEUDOMONADS

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Recent investigations on cassava and its relationships with *Pseudomonas fluorescens* and *P. putida* have shown (a) isolates with none or very mild inhibition of pathogenic bacteria and fungi; (b) isolates that strongly inhibit pathogenic bacteria, with none or very mild inhibition of fungi; (c) isolates causing very strong inhibition of fungi, with none or very mild inhibition of bacteria; and (d) isolates inducing strong inhibition of both fungi and bacteria. There were more isolates able to inhibit pathogenic bacteria than fungi and *P. fluorescens* showed broader *in vitro* inhibition of the bacterial and fungal species tested than *P. putida*. There was also found the following in relation to the ability of these two species to promote root system growth of plantlets as related to pathogen inhibition *in vitro*: (a) Isolates that did not inhibit cassava pathogens *in vitro* or increased root growth of cassava plantlets; (b) strains showing a high inhibitory effect *in vitro* of cassava pathogens, but did not promote an increase in foliage or root system inoculated clones; (c) strains that did not inhibit cassava pathogens, but increased both the foliage and root systems of inoculated clones; and (d) strains that induced a strong inhibitory effect on cassava pathogens and a significant increase in the aerial parts and roots of inoculated plantlets. As a result of the aforementioned the following practical applications have been envisaged at various steps in systems of cassava aimed at increasing production: (a) Biological control of foliar pathogens; (b) biological control of preharvest root rots; (c) biological control of postharvest root rots: and (d) using strains as root promotors, i.e. to speed up and increase the root system of cassava plantlets in rapid multiplication systems. The effectiveness of the above depends upon the selection of appropriate strains or strain improvement by genetic manipulation.

INTRODUCTION

The biocontrol of diseases of different crops has been reported as a feasible measure (Baker and Cook,1974). Investigations on the subject are increasing and are being carefully taken into consideration in many plant pathology programs around the world.

There are groups of beneficial microorganisms which have shown to be effective for the control of plant pathogens in different crops. Among these, beneficial bacteria belonging to fluorescent pseudomonads (Pseudomonas putida and P. fluorescens) are the most promising for use in biocontrol programs due to their nutritional diversity, ability to grow under a wide range of environmental conditions, and ability to colonize the rhizosphere of many plant species (Kloepper et al., 1980).

They can be easily isolated from soil or the plant rhizosphere of many crop species by using King's B (KB) medium incubated at 27°C for 24-36h. Bacterial isolates showing fluorescence on KB under ultraviolet light can be purified from single colonies after serial dilutions seeded on KB medium (Hernandez et al, 1986).

SURVIVAL OF BACTERIAL POPULATIONS

High bacterial populations were found in the rhizosphere of cassava plants or rooted shoot tips two months after inoculation. There were variations in bacterial populations among clones and between strains; but a clone X strain interaction was not found (Rosas, 1986), which indicates lack of host specificity with the strains tested. It has been reported that survival of fluorescent bacteria is probably dependent upon nutrient availability, space and cellular migration to new sites, or a combination of these factors (Brown, 1974; Burr et al, 1978). Similarly, quality of exudates (e.g. production and quantity of specific amino acids or toxic compounds) may influence the survival of these bacterial species in the rhizosphere.

EFFECT ON CASSAVA PLANTLETS UNDER GLASSHOUSE CONDITIONS

Isolates of beneficial fluorescent pseudomonads have been characterized according to their: (I) inhibitory effect, in vitro on both bacterial and/or fungal pathogens; and (II) according to their ability to promote root system growth of plantlets as related to pathogen inhibition in vitro. With method I four groups were identified: (a) isolates with no or very mild inhibition of pathogenic bacteria and fungi; (b) isolates that strongly inhibit pathogenic bacteria, but no or very mild inhibition of fungi; (c) isolates causing very strong inhibition of fungi, but no or very mild inhibition of bacteria; and (d) isolates inducing strong inhibition of both fungi and bacteria (CIAT, 1986). Generally, there were more isolates able to inhibit pathogenic bacteria than fungi. Among all isolates collected, those of P. fluorescens showed broader in vitro inhibition of the bacterial and fungal species tested than P. putida (CIAT, 1985).

Isolates characterized by method II were also classified into four groups: (a) those that did not inhibit cassava pathogens in vitro, or increase root growth of cassava plantlets; (b) strains showing a high inhibitory effect in vitro of cassava pathogens (both fungi and bacteria; some strains of group d in method I), but did not promote an increase in foliage or root system of inoculated clones. As reported for other crop species (Leisinger and Margroff, 1979), secondary metabolites may have phytotoxic or antibiotic activity; (c) strains that did not inhibit cassava pathogens, but increased both the foliage and root system of inoculated clones. It is possible that antagonistic interactions with pathogens (nutrient competition or antibiotic effects) resulted in the exclusion of pathogens from the rhizosphere. Healthy roots promote better total plant growth; (d) strains of this group induced a strong inhibitory effect on cassava pathogens and a significant increase in the aerial parts and roots of inoculated plantlets (Fig. 1) (CIAT, 1986). It has been reported for other crop species that some strains of fluorescent pseudomonads produce growth regulators (Eklund, 1970).

PRACTICAL APPLICATIONS

1. Biocontrol of foliar pathogens

Strains of method I group b, which induced the highest in vitro inhibition of Xanthomonas campestris pv. manihotis (causal agent of cassava bacterial blight, CBB), were used to spray plots of susceptible, intermediate resistant and resistant clones planted in an area where CBB is epidemic. Results of these treatments are shown in Tables 1 and 2. Both the number of angular leaf spots/leaf and the number of blighted leaves/plant were significantly reduced by foliar applications of a strain of P. putida (Table 1). Yield of the susceptible clone (M Col 22) also increased 2.7 times (Table 2) but did not increase in either the intermediate resistant or resistant clones (CIAT, 1985; Lozano, 1987).

The control of other foliar pathogens of cassava by spray applications of specific strains of beneficial fluorescent pseudomonads has not been reported, but is possible. Practical and economic problems exist, such as base inoculum production, inoculum storage and preparation under aseptic conditions and costs for spray applications. Further research is required to test the practical feasibility of controlling foliar pathogens of cassava.

2. Biocontrol of preharvest root rots

When soils infested with Pythium spp. or Diplodia manihotis were drenched with a bacterial suspension of P. putida (method I, group d) before planting cassava plantlets, satisfactory control of root rot was obtained (Hernandez et al, 1986). Strains of P. fluorescens (belonging to method I, group c) were also able to protect cuttings against D. manihotis (Table 3 and 4)(CIAT, 1985; Lozano, 1987). The protective effect was evident when cuttings were treated with the bacterial suspension before or after fungal inoculations. Protection was related to sprouting of buds and fungal establishment, as well as invasion through the tissues of the cuttings (Table 3). This type of protection was also evident for three clones taken either from farmers' fields or from meristem-derived plants. The bacterial protection was nearly as efficient as that obtained with the best fungicidal treatment (Table 4) (CIAT, 1985).

Yield of fresh roots increased when plants were watered with a 10ml bacterial suspension of a beneficial strain of P. fluorescens (method II, group d). Yields tended to increase with an increased number of waterings (Table 5). However, level of increase also varied according to clone (CIAT, 1985), suggesting differential clonal responses to bacterial strains used or differences in susceptibility of the pathogens to the beneficial bacteria.

These results open the possibility of controlling root rots of cassava with beneficial bacteria, replacing fungicidal treatments of cuttings. However, more

extensive research on this subject is needed to define practical systems for biocontrol of root rots.

3. Biocontrol of postharvest root rots

In an experiment to test the potential of biocontrol of postharvest root rots, recently harvested roots were dip-treated in a bacterial suspension of P. fluorescens and P. putida (strains belonging to method II, group d) and stored for three weeks in sealed plastic bags under ambient conditions (Table 6). Strains of P. putida gave variable results, however, a strain of P. fluorescens gave consistently good root rot control during the first two weeks of storage and moderate control after three weeks (Table 6) (CIAT, 1985; Lozano, 1987).

The eventual aim of using a biological rather than a chemical means for controlling postharvest microbial deterioration in cassava is to avoid toxic risks arising from some chemicals. However, these still require considerable adaptive and applied research in order to develop a practical method for on-farm use.

4. Plant growth effects

Dip-treated cuttings (with strains from method II, groups c and d) planted in pots with sterile soils under glasshouse conditions showed a significant (at 5% level) increase in number and weight of roots in relation to distilled water-treated controls (CIAT, 1986; Rosas, 1986). Similarly, shoot tips rooted in a suspension of beneficial bacteria (strains belonging to method II, group c) showed the following significant effects (at 5% levels) in relation to controls: (i) faster root initiation (8 days vs. 10 days); (ii) higher number and longer roots; and (iii) increased root system weight (Hernandez and Lozano, unpublished data). The above results, especially those obtained with strains belonging to method II, groups c and d, strongly suggest the production of growth regulator(s) by strains of these bacterial species. Inoculations of plantlets (obtained by the shoot rooting system in water (Cock et al, 1976) , under sterile conditions in Leonard jars (Bradley et al, 1985) confirmed previous findings (Table 7). Further research on the purification of such growth regulator(s) is in progress (Rosas, 1986; CIAT, 1986). The above results suggest the possibility of using strains of both P. putida and P. fluorescens as root promotors, especially to accelerate and increase the root systems of cassava plantlets in rapid multiplication systems. Applications for accelerating the rooting and sprouting of cuttings in commercial plantations requires further research.

GENERAL CONCLUSIONS

Even though research on the use of beneficial fluorescent pseudomonads to control of cassava pathogens is recent and preliminary, results suggest it is feasible.

The use of strains of fluorescent pseudomonads effective in control of CBB needs more investigation on practical storage of beneficial strains and the distribution

and multiplication of inoculum. Effective strains of this beneficial bacteria are available, and the methodology for their identification is known. Bacterization of cuttings is feasible in special situations, such as in planting material production fields, to control pathogens infesting the cuttings, and to protect them against pathogens in infested soils. This technology may not be useful in traditional cassava production systems because the treatment requires technical work and aseptic handling during the production of the inoculum. The use of beneficial bacterial suspensions to treat cassava roots before their storage in order to control microbial deterioration needs further research on the identification of effective strains and the development of treatment systems, giving levels of control similar to those obtained with thiabendazole (CIAT, 1983; Wheatley et al, 1984). Similarly, the use of growth-stimulating strains of fluorescent pseudomonads to treat both cuttings or plantlets before planting, for the promotion of root system growth, appears to be feasible in the near future.

It can be seen from the foregoing that investigation on this topic will possibly open new fields that can be incorporated successfully in many cassava production systems. The integration of this control measure with others already known, especially applied to resistant clones, can undoubtedly lead to the obtainment of better stable yields.

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Table 1. Control of cassava bacterial blight by foliar applications* of Pseudomonas putida (strain F-44 and F-88) on two susceptible clones during 1984 and 1985.

Clone	Scoring System	Year of control/strain number					
		1984		1985			
		F-44		F-44		F-88	
		Sprayed	Controls	Sprayed	Controls	Sprayed	Controls
M Col 22	Angular leaf spots/leaf**	1.0a	7.0b	2.5a	5.7b	2.5a	5.7b
	Number of blighted leaves/plant***	1.0a	6.0b	1.5a	5.0b	1.0a	4.0b
M Col 72	Angular leaf spots/leaf	-		4.3a	9.5b	4.2a	8.7b
	Number of blighted leaves/plant	-		2.1a	6.06b	2.3a	7.0b

* Plant received six foliar applications of a 1 x 10^9 cells/ml suspension of the respective strain of P. putida at 15-day intervals. Control plants were sprayed with distilled water. Numbers followed by the same letter are not significantly different at a 0.05 level of the Duncan Multiple Range Test.

** Average number of leaf spots/leaf of 15 leaves/plot. Plots contained 36 plants with three replications.

*** Average number of blighted leaves/plant of 20 leaves/plot. Plots contained 36 plants with three replications.

Table 2. Yield (t/ha) of a CBB-susceptible)M Col 22), intermediate resistant (CM 523-7), and resistant (M Ven-77) clones after foliar applications of Pseudomonas putida (strain F-44).

Clones	Sprayed plots	Control plots
M Col 22	6.8a	2.5b
CM 523-7	14.7a	14.0a
M Ven 77	9.6a	9.1a

* Yield was recorded from three replicated plots of 30 plants each (12 harvested plants). Border plants were eliminated. Yields followed by the same letter(s) are not significantly different at the 0.05 level of the Duncan Multiple Range Test.

Table 5. Average yield of fresh roots of several clones planted in Carimagua, Media Luna and CIAT, in relation to treatments with Pseudomonas fluorescens (PF-88).

Location	Clone	Yield (ton/ha) in relation to bacterial treatments*			
		0	1	2	3
Carimagua	M Col 1914	12.1b**	14.5b	16.5a	17.6a
	M Col 1916	11.2b	14.5b	15.7a	17.7a
	M Pan 19	10.7a	10.1a	12.0a	12.3a
	M Ven 77	14.5b	14.5b	18.1a	21.6a
Media Luna	CM 342-170	9.0b	11.1ab	11.3ab	12.5a
	M Col 72	12.5b	16.0a	16.3a	18.3a
	Venezolana	10.3b	11.3b	12.3ab	13.3a
CIAT-Palmira	M Col 1468	38.1b	40.2b	44.5ab	49.3a
	M Col 72	42.1b	46.6ab	46.8ab	47.0a

* Bacterial treatments: 10 ml of a bacterial suspension (1.1×10^9 cells/ml) were poured at the base of each plant at: 1 = one month; 2 = one and two months; 3 = one two and three months after planting the cuttings.

** Data taken from three replicates of 30 plants each/location/clone and treatment. Border plants were eliminated. Yield data followed by the same letter(s), compared across bacteria treatments, are not significantly different at the 0.05 level of the Duncan Multiple Range Test.

Table 6. Effect of six strains of Pseudomonas putida (Pp.), two of P. fluorescens (Pf.) and one Bacillus (Bsp.) on postharvest microbial deterioration of cassava.

Bacterial strain number	Root deterioration (%) after storage*								
	CMC 401		HMC-1		CMC 40		M COL 22		
	1	2	1	2	1	2	1	2	3
Pp. f-56	0.6**	30.5	7.0	18.0					
Pp. f-44	8.8	10.0	7.3	23.0					
Bsp.	15.2	32.5	15.3	25.0					
Bsp. + Pp. f-56	6.6	23.5	7.8	28.5					
Bsp. + Pp. f-44	7.0	9.0	7.6	16.0					
Pf. c-5a					0.0	10.0	4.5	9.9	38
Pp. c-7a					20.4				
Pp. c-4b					7.8	14.9			
Pf. c-88					11.0	47.3			
Pp. c-5b							7.0	16.5	
Pp. c-7c							5.0	18.0	
Pp. c-7c							2.0	8.4	
Mertecc	8.6	13.5	4.8	15.0	1.8	4.0	2.0	8.4	5
Control	10.3	34.5	13.8	74.5	32.5	77.0	18.0	64.0	100

* Readings taken after 1, 2 and/or 3 weeks of storage.

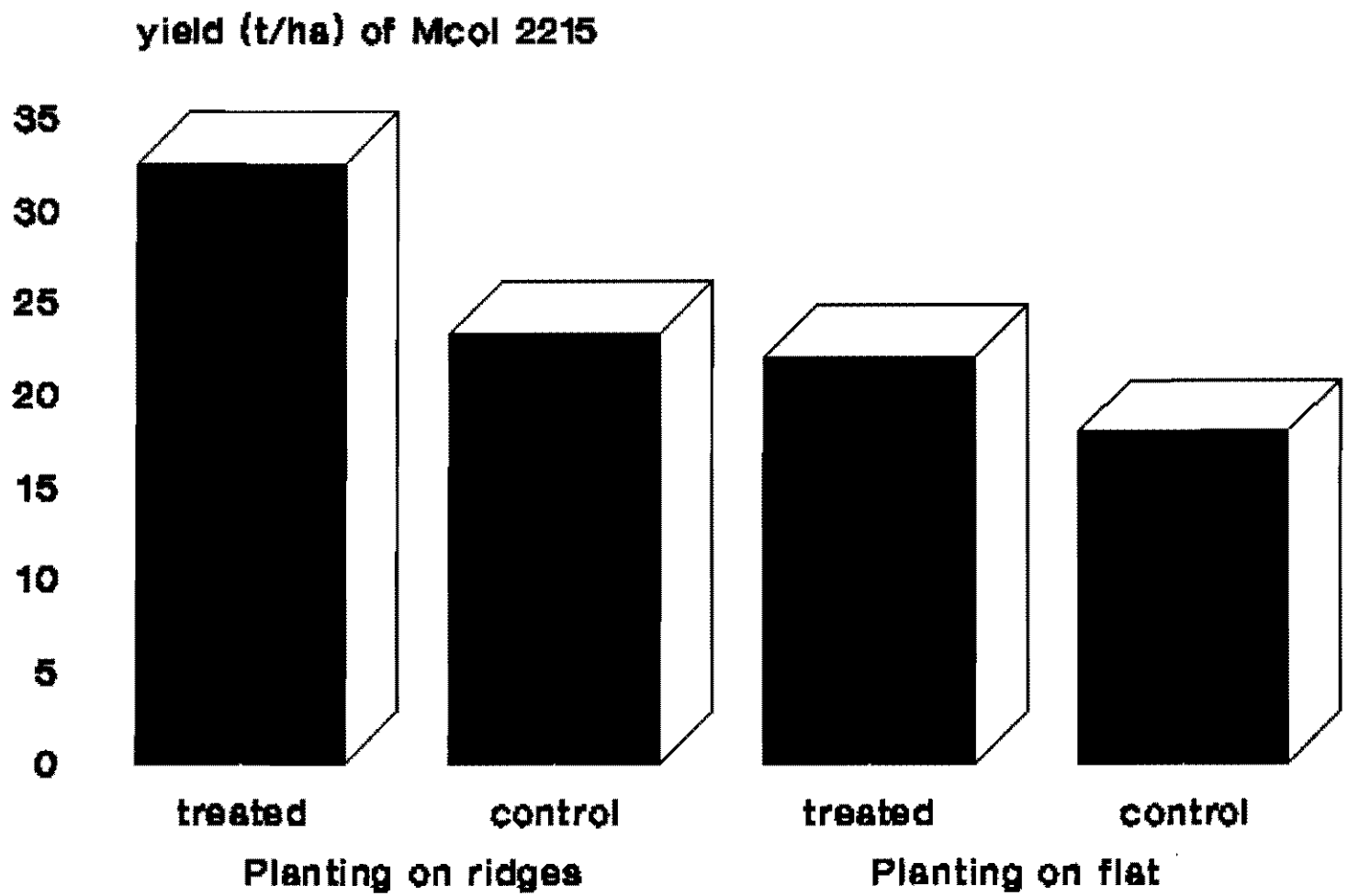
** Average score from 500 roots of approximately 0.3 Kg each. Score of 0 to 100 refers to percentage losses due to microbial deterioration (Wheatley, et al., 1984).

Table 7. Effect of P. fluorescens (strain 88) on the root system of cassava (Clone M Ven 77) two months after inoculation and incubation under sterile conditions.

Treatment	Root weight (g)	Increas (%)
P. fluorescens plus nitrogen	4.5*	164
Uninoculated control plus nitrogen	1.7	-

* Results of 10 plantlets which were immersed for 20 min in a bacterial suspension of 1 x 10^9 cfu/ml. Leonard jar contained sterile Morri's medium plus washed quartz.

FIG. 1. EFFECT OF Trichoderma harzianum TREATMENTS ON YIELD OF CASSAVA (MCOL 2215) PLANTED ON A PLOT NATURALLY INFESTED OF Fusarium solani, A ROOT ROT PATHOGEN



MICROSCOPIC STUDY OF THE ESTABLISHMENT OF *XANTHOMONAS CAMPESTRIS PV.MANIHOTIS* ON *IN VITRO* MICROCUTTINGS OF CASSAVA

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The establishment of 2 strains of *Xanthomonas campestris* pv.*manihotis* (i.e. strain CIAT1111M, non aggressive and strain ORST 17 aggressive) on *vitro* plants of cassava was monitored by scanning electron microscopy (SEM). The inoculum was deposited on leaf surfaces of *in vitro* plants with a fine brush. Immediately after inoculation the appearance and the distribution of bacteria were similar for both strains.

Within 6 days the aggressive strain increased rapidly, masses of bacteria were visible near stomata and lesions developed on leaves. On the other hand, the non-aggressive strain did not lead to visible alterations in leaf tissues. SEM revealed bacterial cells under a thin layer of unknown composition.

Using SEM examinations, the localization of bacterial cells and their spreading on the leaf surface were more accurately defined.

Our results suggest the relative importance of colonization of stomata in aerial bacterial infection. A relation was reported between strain aggressiveness and colonization of cassava leaves. *In vitro* culture provides a material suitable for studying different steps of host pathogen interactions.

INTRODUCTION

Xanthomonas campestris pv. manihotis causes the severe cassava bacterial blight disease characterized by angular leaf spots and blight, and a systemic infection of the stem leading to wilt. The epiphytic phase of X.c pv. *manihotis* has important implications in the epidemiology of the disease constituting one source of inoculum (DANIEL & BOHER, 1985). The objective of this work was to investigate the establishment of virulent and avirulent strains of *Xanthomonas campestris pv. manihotis* on cassava leaves. This study was monitored by scanning electron microscopy (SEM).

MATERIALS AND METHODS

Plant materials *In vitro* plants (cultivar IITA 30555) were used in the experiment. They were produced from meristem and multiplied by the micropopagation technique on Murashige & Skoog medium. Two strains of *Xanthomonas campestris pv. manihotis* were used in the present study. The virulent strain ORST17 was isolated from cassava leaves in the Congo.

The strain ORST4-CIATIIIIM has lost its virulence and was isolated in Colombia in 1974.

The bacteria were cultured on LPG medium for 2 days, washed twice with sterile distilled water and centrifuged at 9000g for 10mn prior to inoculation. The bacterial inoculum was adjusted to 10l cfu per milliliter in sterile water. The inoculum was deposited on leaf surfaces of in vitro plants with a fine brush.

Scanning electron microscopy.

Inoculated leaves were sampled 15mn, 2 and 6 days after inoculation and prepared for scanning electron microscopy as previous methods described (VERDIER, 1988, VERDIER et *al,* 1990). Two methods were used to process samples: examination in fresh state with a cryoscan device and after glutaraldehyde-OsO4 fixation followed by critical point drying.The specimens were coated with gold and examined with a JEOL JSM 35 scanning electron microscope (SEM).

RESULTS

Observations of healthy cassava leaves.

Both methods (cryoscan and critical point dried) maintained structural organization of samples but "cryoscan" gave better preservation of plant tissues than critical plant drying. the reverse was observed for bacterial cells. Comparing both methods, thus helped to avoid major misinterpretations.

Epidermic cells at the upper face are smooth and convex. Stomata are ranged throughout the principal and secondary veins. At the lower epidermis face there were a high number of stomata surrounded by digited cells (Fig 1, 2, 3).

Morphology and distribution of bacteria cells.

The bacteria can be easily observed (1 to 2μm length). Small aggregations or single cells appear on the leaf surface (Fig 4, 5). Soon after the inoculation the distribution and establishment of the bacteria is same for both the virulent (Fig 6, 7) and avirulent strain (Fig 8, 9). No marked differences in bacterial numbers were noted between the virulent and avirulent strain within two days after inoculation. Six days after the inoculation, masses of bacteria (ORST17) were visible near the stomata which resulted in the multiplication of the virulent strain on leaf surfaces (Fig 10, 11, 12). Bacteria seemed to burst out of the stomata (fig 10). Bacterial masses emerging from the stomatal cavities were enmeshed in strands of unknown composition, possibly polysaccharide slime (Fig 12). The tissues reveal lesions necrosis of parenchymatous cells, fading of stomata cells and peeling off of the cuticle. On the other hand, the non-aggressive strain did not lead to visible alterations in leaf tissues. SEM revealed bacterial cells trapped under a thin layer of

unknown composition (Fig 13, 14). The avirulent strain multiplied slightly or not at all, their numbers were considerably lower than the virulent strain.

DISCUSSION

Using SEM examinations, the localization of bacterial cells and their spreading on the leaf surface were more accuratly defined. Our results confirm the relative importance of colonization of stomata, and their surroundings, in aerial bacterial infection (MILES et *al*, 1977, BASHAN et *al*, 1981, HUANG, 1986). A correlation was reported between strain virulence and colonization of cassava leaves. In an incompatible host-bacterial system, bacterial multiplication was considerably reduced. The production of exudate by the host immobilized the avirulent bacterial cells. A similar phenomenon was demonstrated with other host-bacteria system (MEW et *al*, 1984). *In vitro* culture provides a material suitable for studying different steps of host pathogen interactions.

Adopting the inoculation method on vitroplants as proposed here, we were able to reproduce the typical symptoms of the disease (angular leaf spot, wilting). This opens the possibility of developing a simplified screening system to identify cultivars resistant to bacterial blight.

Moreover this simplified system allowed the distinction of pathogenic or non pathogenic strains of *Xanthomonas campestris pv. manihotis*.

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Figures 1-14. Scanning electromicroscopic of meristem culture-derived cassava leaves innoculated with *Xanthomonas campestris* pv. *manihotis*.

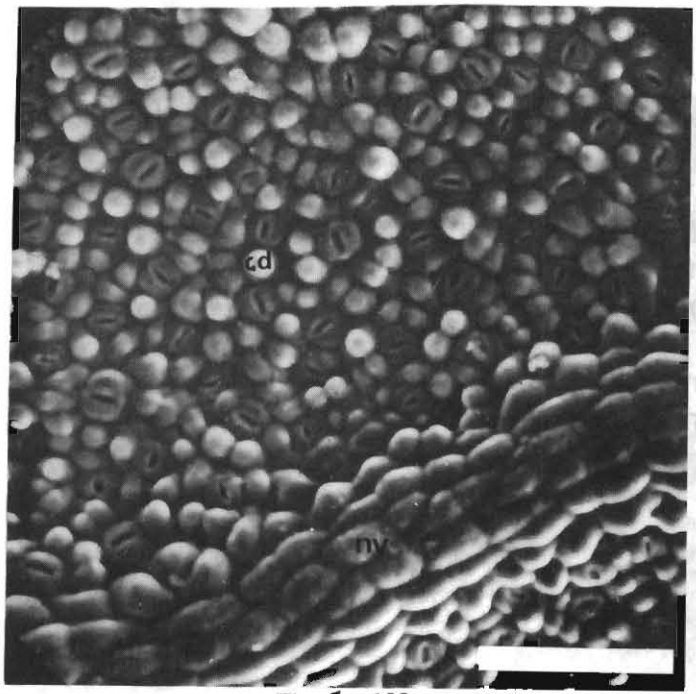

Fig. 1 100 µm

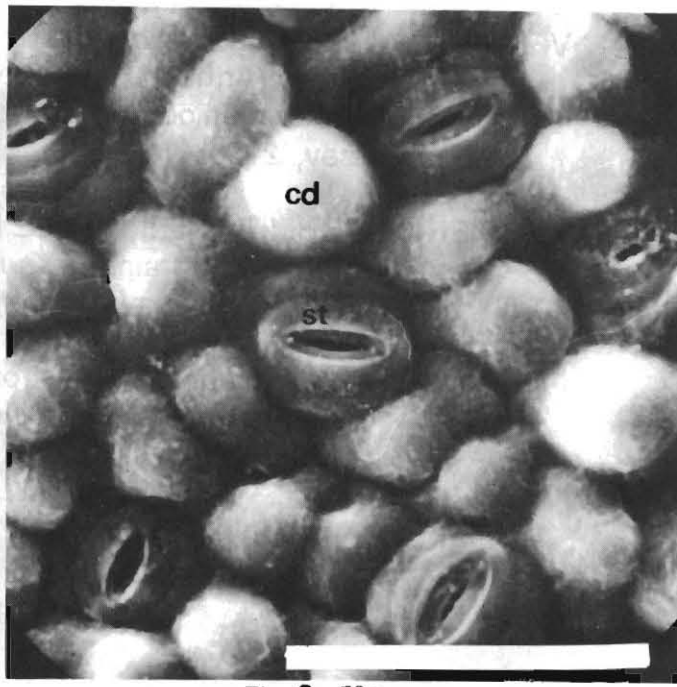

Fig. 2 50 µm

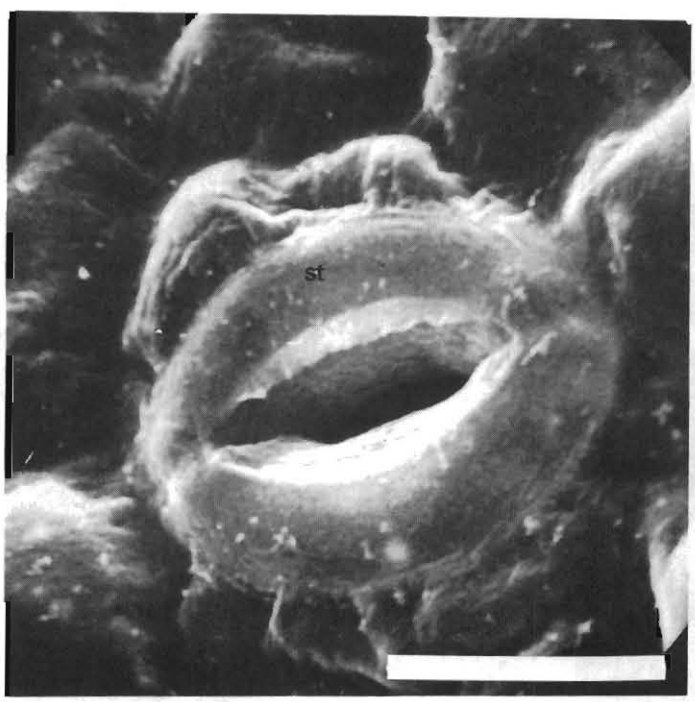

Fig. 3 10 µm

Fig. 4 5 µm

Figures 1 - 14. Scanning electron microscopy of meristem culture-derived cassava leaves inoculated with *Xanthomanas campestris* pv. *manihotis.*

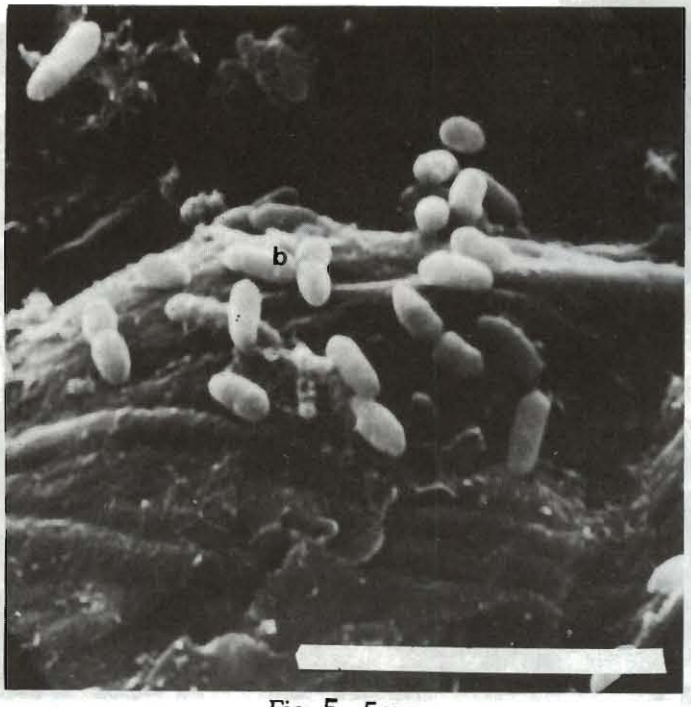

Fig. 5 5 μm

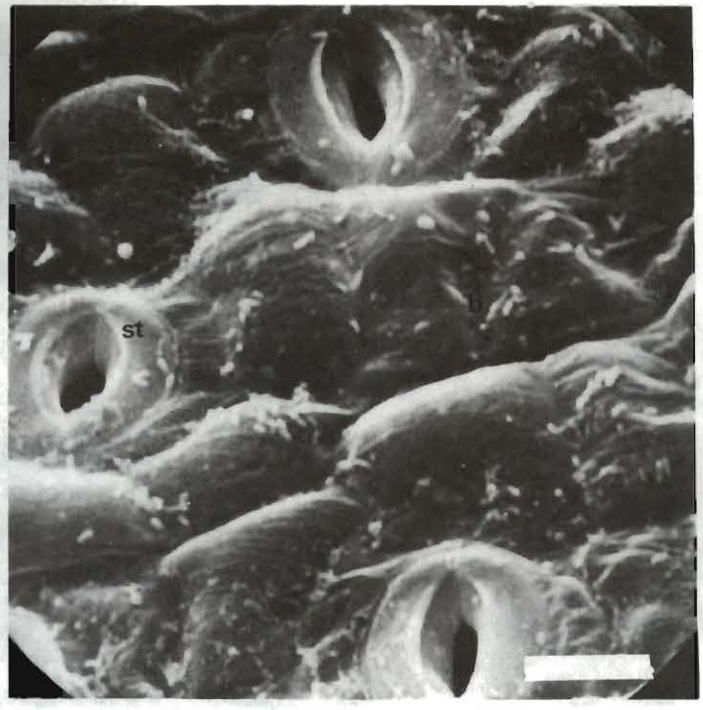

Fig. 6 10 μm

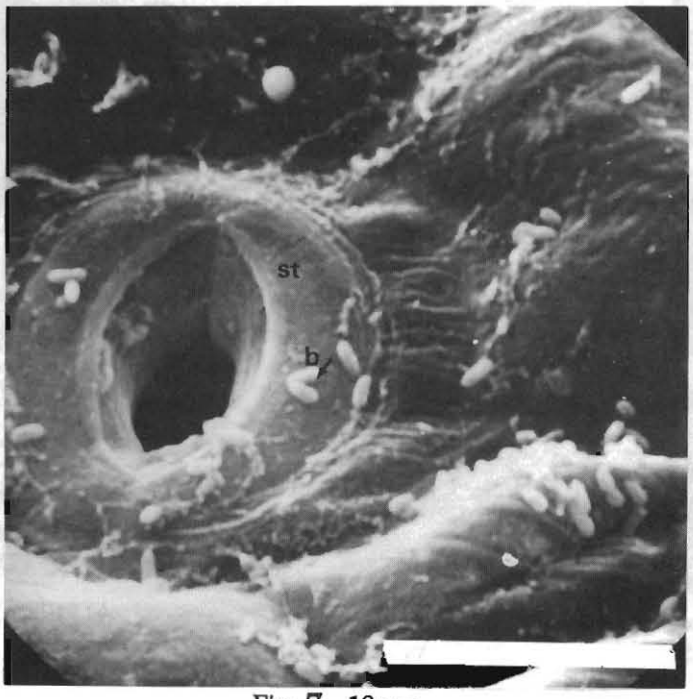

Fig. 7 10 μm

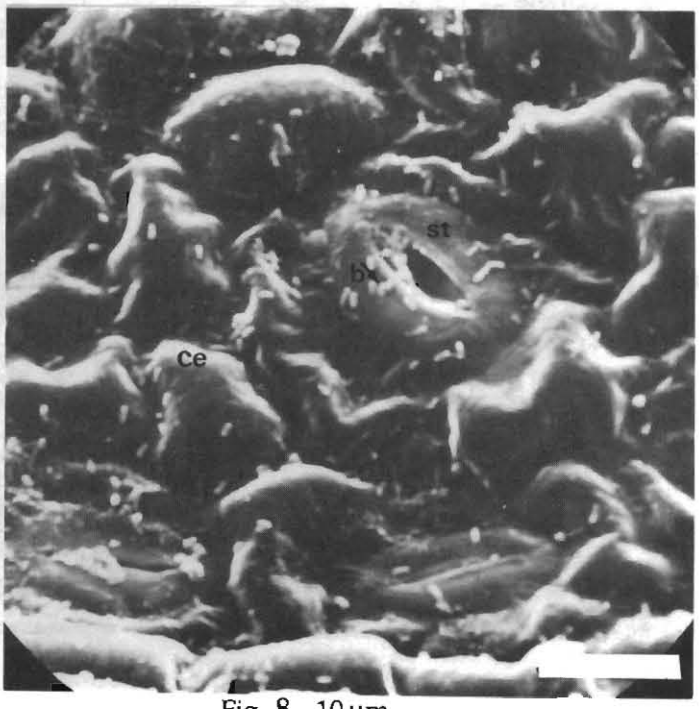

Fig. 8 10 μm

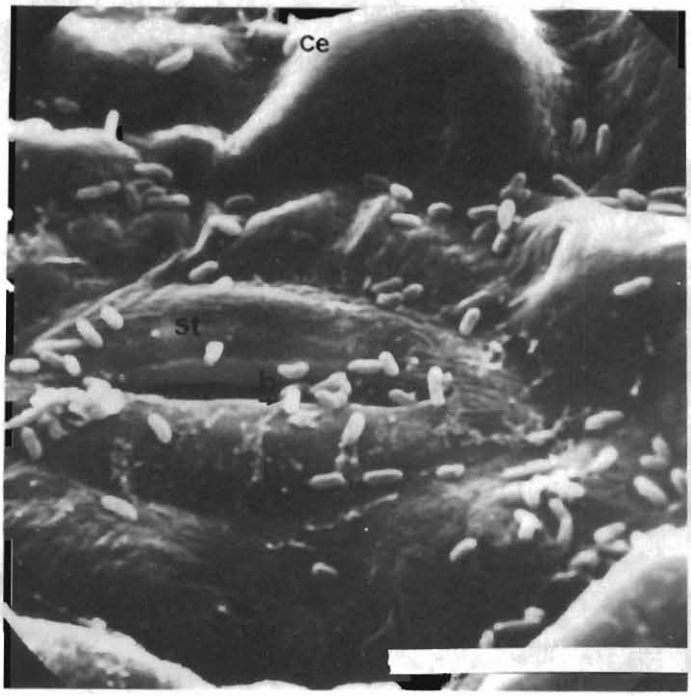

Fig 9 10 µm

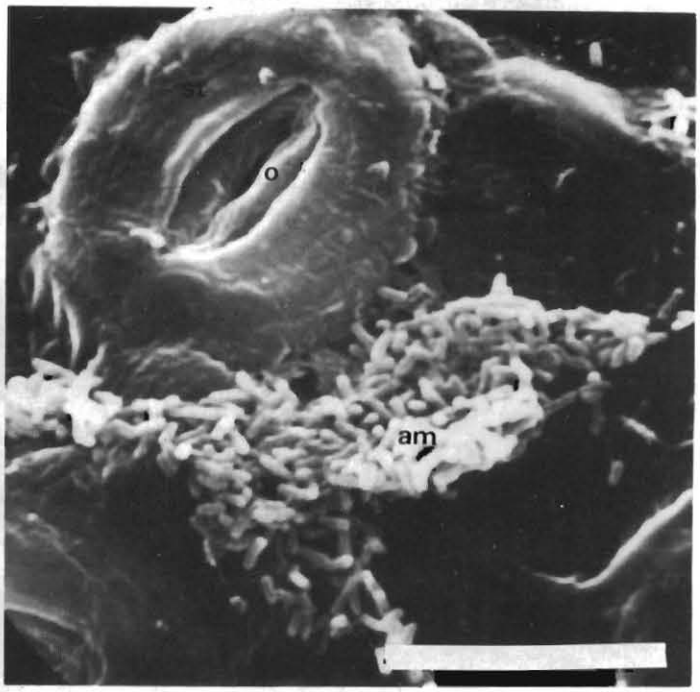

Fig. 10 10 µm

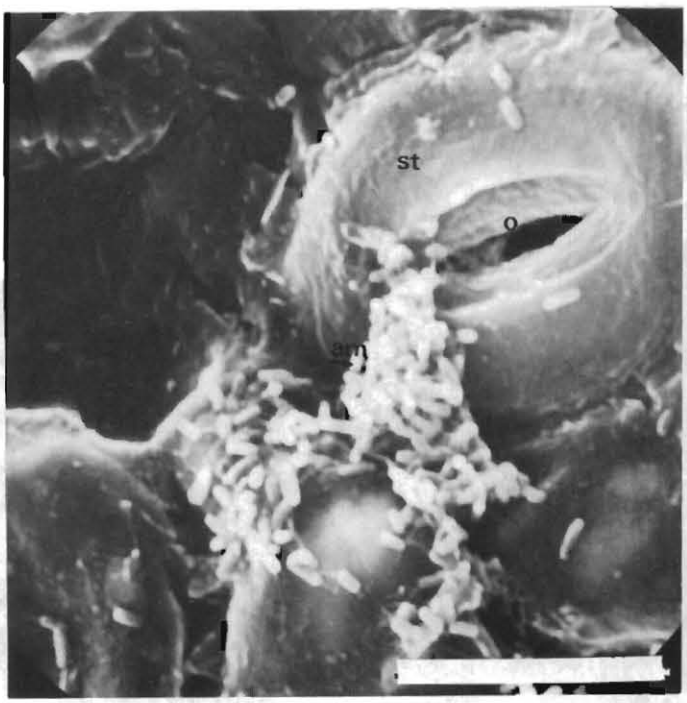

Fig. 11 10 µm

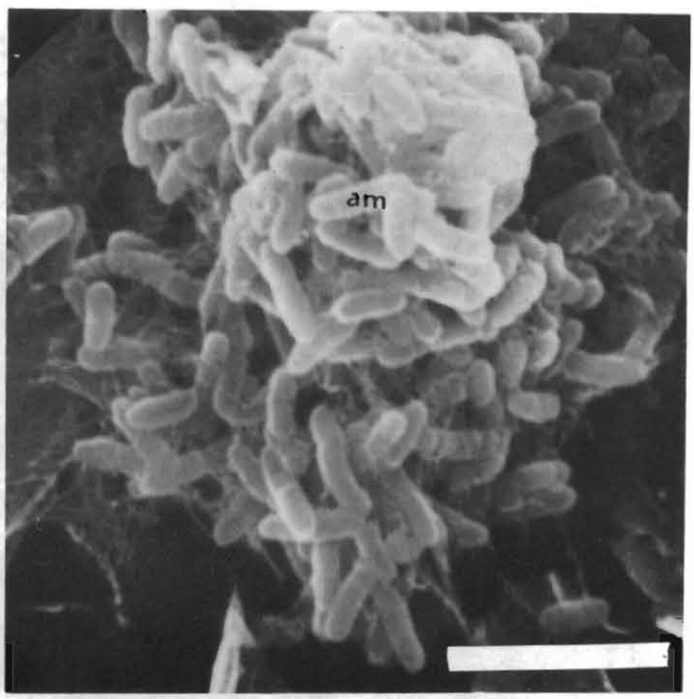

Fig. 12 5 µm

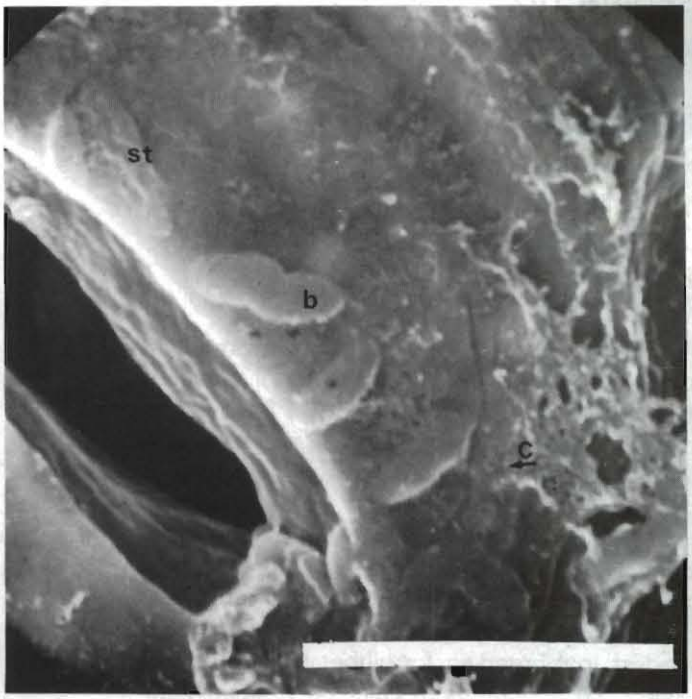

Fig. 13 5 µm

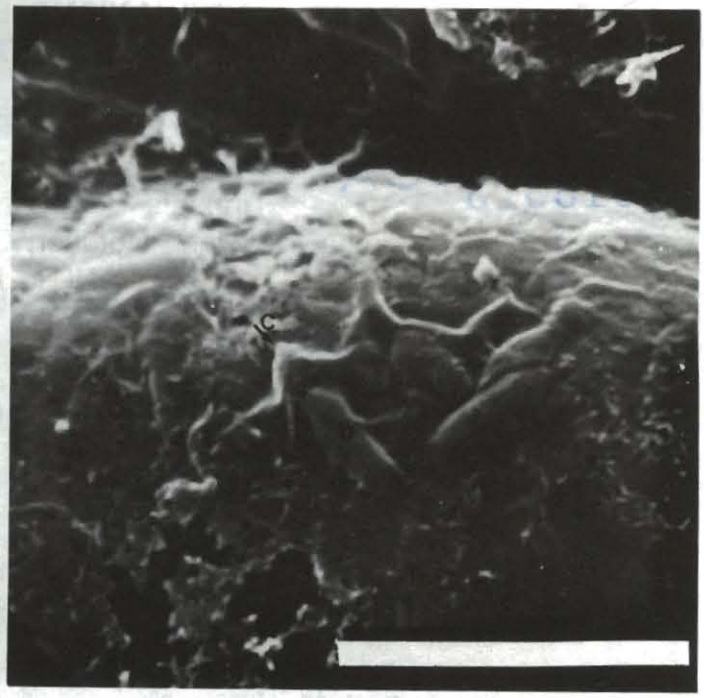

Fig. 14 5 µm

Abbreviations used in Figures :

b : bacteria
st : stoma
cd : digited cells
ce : epidermic cell
am: bacterial mass

CAUSAL MECHANISMS BEHIND HUMAN DISEASES INDUCED BY CYANIDE EXPOSURE FROM CASSAVA

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New chemical methods for determination of cyanogen substances in cassava products and of cyanide exposure in humans were combined with modern methods for elucidation of the socio-economic determinants of toxic effects from cassava. Studies in Mozambique, Tanzania and Zaire revealed that food security problems that induce short-cuts in established processing methods are the underlying cause of cyanide exposure from cassava that can cause acute intoxications and aggravation of goitre. Recent studies in Africa also show a causal role of cyanide exposure from cassava in a new epidemic paralytic disease named konzo but not in diabetes as earlier claimed. All toxic effects from cassava can be avoided by sufficient processing. Breeding for lower levels of cyanogenic glucosides is being regarded as the only way that biotechnology can prevent toxic effects. Our new findings suggest that breeding for higher producivity as well as improved "processability" and pest tolerance may be more efficient, especially as a positive effect of the cyanogenic glucosides on yields cannot be excluded in the areas with marginal land where severe consequences of cassava toxicity occur.

INTRODUCTION

The implications of cassava toxicity have been a matter of conflicting views. Opinions have varied from regarding cyanogenesis as a peculiarity with little practical importance to viewing cassava toxicity as a major public health hazard. This paper summarizes the recent advances that provide an intermediate opinion. On one hand it is clear that cyanogenic glucosides in cassava under special circumstances may cause toxic effects in humans that occasionally may be severe. On the other hand these effects are very rare in relation to the wide use of bitter cassava roots with high glucoside levels as staple food. The main reason for this apparent contradiction is that cyanide (CN)-yielding substances in cassava can easily be reduced to negligible levels by effective processing. One such method is to grate fresh roots and ferment the mash under pressure followed by rapid roasting to obtain a product known as gari in West Africa (Vasconcelos 1990). This method was developed in Amazonas thousands of years ago. Community based trial-and-error have also provided other effective cassava processing methods (Hahn 1989). Strict adherence to such methods have enabled populations in the tropics to use roots from bitter cultivars as staple food without adverse effects.

Toxic effects of cassava following consumption of insufficiently processed products constitute a significant public health problem only when bitter cassava cultivars are of critical importance for food security in poor populations. The recently reported outbreaks in Africa of acute intoxications and paralysis induced by CN

exposure from cassava occurred when bitter cassava cultivars were the sole staple food available for the affected populations.

Our studies in Africa have revealed a fairly constant chain of events behind the human diseases induced by CN exposure from cassava. The chain starts with a gradual ecological collapse of shifting cultivation that over decades results in a farming system dominated by high yielding bitter cassava cultivars. General food shortage (Ministry of Health Mozambique 1984) combined with intensive cassava sales that compete with household needs (Tylleskär et al. 1991) lead to short-cuts in established cassava processing. In these situations, the diet of the cassava farmer and her family will, due to poverty and heavy workload, consist almost exclusively of cassava flour with high amounts of remaining cyanogens. The resulting high CN intake may induce acute intoxications, the paralytic disease konzo and aggravate goitre. The least understood part in this chain of events is the biological role of cyanogenesis for root and leaf yields in cassava dominated farming systems on marginal land.

CYANOGEN REMOVAL DURING PROCESSING

To understand the characteristics of cassava toxicity, it must first be realized that cassava roots and leaves do not contain cyanide (CN). They contain varying amounts of cyanogenic glucosides, mainly linamarin, that can yield the corresponding cyanohydrins and hence hydrogen cyanide (HCN) (Cooke 1981). These substances, collectively known as cyanogens, can all be reduced to negligible levels through effective processing. Insufficiently processed cassava products contain varying proportions of cyanogens. During processing different factors influence the break down of glucosides to cyanohydrins and cyanohydrins to HCN, respectively. The first reaction depends on disintegration of plant cells to liberate the endogenous enzyme linamarase. The second reaction is, in an unclear manner, facilitated by alkaline pH, drying, heating, and enzymatic action. The HCN finally yielded does not remain long in the products. It either dissolves in water, evaporates into the air, or disappears by reaction with carbohydrates to form secondary cyanohydrins (Banea 1992).

If cassava root pieces are rapidly dried, high glucoside levels will remain in the flour due to insufficient time for enzymatic breakdown (Mlingi 1992). If root cells are disintegrated mechanically or by fermentation before drying the glucosides will rapidly disappear. The cyanohydrins yielded are fairly stable if lactic acid fermentation has resulted in a pH below 6. If acid root products are consumed without allowing for sufficient drying or heating, high amounts of cyanohydrins will be ingested (Tylleskär 1992).

The fate of glucosides, cyanohydrins and hydrogen cyanide differ during digestion in the gut and metabolism in the body. Considering that the amounts of HCN found in cassava products are always low, most of the CN exposure from the consumption of insufficiently-processed roots must be derived from the glucosides or cyanohydrins. Our studies indicate that remaining cyanohydrins are the major

source of dietary CN exposure. Cyanohydrins seem to withstand short boiling; however, in the alkaline environment of the gut they can rapidly and completely break down to CN resulting in dietary cyanide exposure (Tylleskär 1992). Rat studies have shown that cyanogenic glucosides may break down to CN in the gut if suitable glycosidases are provided by the microflora, but they can also be absorbed and excreted intact in the urine. We found that a substantial part of ingested glucosides may be absorbed and excreted intact in the urine also in humans. Findings in Tanzania even suggest that flour from sun-dried roots having considerable amounts of remaining glucosides but no cyanohydrins can be consumed without any CN exposure (Mlingi 1992). The factors controlling the breakdown of glucosides and cyanohydrins in cassava products and the kinetics in humans of these substances must be elucidated in detail if requirements for cassava processing and safe cyanogen limits for cassava products shall be based on sound scientific grounds.

CYANIDE DETOXICATION IN HUMANS

A cyanide dose of 50-100 mg (2-4 mmol) may be lethal to an adult within minutes by an inhibitory effect on cell respiration. On the other hand CN is a natural toxin that has been with us through evolution and humans have inherited two effective defense lines protecting us against low dose rates. First, about 10 mg (0.4 mmol) CN can be temporally "neutralized" by a reversible reaction with the methemoglobin fraction in the red blood cells (Lundquist 1985). Second, the cells in most tissues contain the enzyme rhodanese that, by a reaction with sulphur originating from dietary sulphur amino acids, converts the main part of a CN dose to the less toxic thiocyanate (SCN) that is slowly excreted in the urine. The conversion rate of CN to SCN in well-nourished adults is about 50 mg (2 mmol) CN/24 hours (Schulz 1984). The rate limiting step is sulphur availability. Injection of the antidote thiosulphate acts by increasing the conversion rate several times. Protein malnutrition, especially low intake of proteins rich in sulphur amino acids, will decrease the CN conversion rate. However, even subjects with very low intake of sulphur are able to form at least 0.5 mmol of SCN/24 hours (Tylleskär 1992) and no evidence suggests adverse effects of CN exposure at dose rates below 5 mg (0,2 mmol) CN/ 24 hours.

SAFE CYANOGEN LEVELS IN CASSAVA

In Codex Alimentarius (1988), the safe limit for cyanogens in cassava products is set at 10 mg (0,4 mmol) CN equivalents/kg dry weight. Subjects with cassava as the main staple and estimated daily intake of 0,5 kg flour will not be exposed to more than 0,2 mmol CN per 24 hours. Since the detoxication rate is ten times higher in well-nourished subjects, the limit set in the FAO/WHO document leaves a safety margin even in malnourished subjects with decreased detoxication rate.

The levels of cyanogenic glucosides in fresh roots from bitter cultivars may reach 1500 mg CN equivalents/kg dry weight (O'Brien 1992), which is 150 times greater than the safe limit. If 0.5 kg dry weight of such roots are consumed

unprocessed daily the potential CN intake is 30 mmol/24 hours which is 15 times the lethal dose rate. The upper limit for cyanogenic glucosides in so- called "low CN varieties" has for breeding purposes been set at 10 mg/100g fresh weight (Hahn 1985), i.e. 300 mg or 12 mmol/kg dry weight. The level of cyanogenic glucosides in fresh roots of these improved varieties is 30 times higher than the level considered safe for human consumption. These discrepancies show just how crucial processing is for prevention of toxic effects from cassava. Some procedures can reduce the CN yielding capacity to less than one percent of initial values (Hahn 1989). When "safe" levels are set for breeding purposes, the processing assumed to be undertaken before consumption must be specified. The elaborate traditional processing methods and modern mechanical processing procedures seem to include a considerable safety margin and can therefore supply safe products even from roots with the highest cyanogen levels. On the other hand, if roots from so called "low CN varieties" are used for preparation procedures intended for sweet cassava cultivars with very low glucoside levels, a high dietary CN exposure may result.

ACUTE CASSAVA POISONING

The fact that consumption of insufficiently-processed bitter cassava roots may cause acute poisoning is well known by all populations growing such cultivars, though such intoxications are poorly documented in scientific literature. A few case reports and a greater amount of anecdotic information do, however, describe a fairly consistent clinical picture. The symptoms start a few hours after consumption of the insufficiently processed cassava product. These unspecific symptoms include nausea, vomiting, dizziness, weakness and sometimes collapse that occasionally leads to death. Typically several subjects from one family are affected to varying degrees and all symptoms clear within 12-24 hours leaving no sequel (Mlingi 1992). Acute cassava poisoning has been assumed to be caused by CN, since the symptoms correspond to those of CN intoxication from other sources. The time lag between meal and onset of symptoms is compatible with the hypothesis that cyanide originates from ingested cyanohydrins that break down to cyanide in the alkaline environment of the gut. The low mortality in several outbreaks of acute cassava poisoning suggest that the cyanogenic glucoside *per se* or some other less toxic substance may be responsible for many of the symptoms.

To our knowledge the only documentation of high blood CN levels in cases of cassava poisoning is the report by Akintonwa and Tunwashe (1992) of subjects from Lagos that died from acute poisoning after gari consumption. They had blood CN ranging from 34 to 54 μmol/l which is higher than the normal range (<1 μmol/l), still below what is considered as lethal level (>100 μmol/l). Unfortunately, this important report lack information on the type and source of the gari consumed. There are, however, reasons to believe that the intoxications were caused by CN exposure from gari since a number of acute poisonings from consumption of gari has been reported from Nigerian cities during the last few years. These poisonings occurred when both fermentation and drying time were reduced in urban gari processing (Sanni M.O.,

pers. com.). Remaining cyanohydrins are the most probable source of dietary CN in such gari products and improved drying may be a sufficient prevention.

Outbreaks of acute poisoning from cassava have otherwise mainly been reported when toxic cultivars were critically important for food security in poor rural populations. Most commonly acute poisonings occur when a population for the first time consume bitter cultivars without having acquired proper knowledge of processing, including when populations used to sweet cultivars start to consume bitter cultivars without changing to the processing required. Outbreaks of acute poisoning also occur when drought increases glucoside levels and when short-cuts in processing are done due to food shortage or intensive sales.

IODINE DEFICIENCY DISORDERS

A low dietary intake of iodine due to low iodine content in the soil causes goitre, cretinism and other health problems, jointly called iodine deficiency disorders (IDD). Globally IDD still remains a major nutritional problems although it can be easily controlled by various forms of iodine supplementation. Daily dietary exposure of CN from insufficiently processed cassava may aggravate iodine deficiency disorders as the detoxification product SCN competitively interferes with iodine metabolism. Populations with high SCN loads from insufficiently processed cassava do not develop goitre if iodine intake is adequate (Cliff 1986). The goitrogenic effect of SCN will only be of importance in populations with marginal or low iodine intakes and iodine supplementation can overcome the goitrogenic effect in these situations (Delange 1983). The goitrogenic effect from cassava is of limited importance for the global occurrence of IDD. There are, however, good reasons to include promotion of effective cassava processing as part of IDD control programmes in cassava eating populations, since regular iodine supplementation may not reach those most in need.

TROPICAL ATAXIC NEUROPATHY AND KONZO

The neurological diseases tropical ataxic neuropathy (TAN) and konzo have both been attributed to CN exposure from insufficiently processed cassava. TAN is a slowly progressive paralysis affecting rural populations in Nigeria (Osuntokun 1981). The diagnosis requires two of the following symptoms: degeneration of the spinal cord causing balance disturbance (ataxia), damage to the optic nerves, deafness or symmetrical damage to peripheral nerves. Konzo is a form of spastic paralysis affecting both legs with an abrupt onset and a non-progressive course that recently was identified as a distinct disease entity (Howlett 1990, Tylleskär 1993). It was named konzo after the local designation among the population in Zaire where it was first reported. In the last decade epidemics of konzo occurred in Mozambique (Ministry of Health 1984, Cliff 1985, Essers 1991), Tanzania (Howlett 1990, 1992, Mlingi 1991) and Zaire (Tylleskär 1991, 1992, Banea 1992). In 1992 we also found konzo in the Central African Republic. Several epidemiological studies implicate several weeks of high dietary CN exposure due to exclusive consumption of insufficiently-processed bitter cassava roots as the main cause. All konzo-affected

areas are characterized by a severe agro-ecological crisis and cultivation of marginal land with high-yielding bitter cassava. Konzo epidemics have only been reported from these poor communities of Africa. The outbreaks coincided with food shortage and/or intensive sale of cassava, shortcuts in processing, high CN exposure and low intake of sulphur for CN detoxification. The etiological role of CN in konzo is not confirmed beyond all doubt, but the evidence is strong enough to urge for preventive action.

Konzo and TAN are both attributed to dietary CN exposure from cassava but differ in most other aspects. In contrast to konzo, TAN is a progressive disorder with slow onset that mainly affects older adults. Konzo is a disease of upper motoneurons, whereas TAN is caused by damage of other neurons in the spinal cord. That different diseases are induced by the same toxin may be explained by different rates of exposure.

OTHER DISEASES

CN exposure from cassava has also been implicated as an etiological factor in malnutrition related diabetes. However, recent studies have failed to confirm such a relationship between this disorder and CN exposure from cassava (Swai 1992).

There is good reason to believe that dietary CN intake substantially contributes to protein malnutrition and in the stunting of children in populations consuming insufficiently processed cassava. The reason is that up to a quarter of the limited intake of the essential sulphur amino acids in their diet is used for CN to SCN conversion instead of protein synthesis. This is supported by unpublished findings of severely reduced height for age combined with almost normal weight for height in children below 5 years of age in populations with verified high dietary CN intake from cassava. The practical importance is that protein malnutrition in children on cassava-dominated diets that has been attributed to the low protein content of cassava roots may be partly due to insufficient processing.

THE ROLE OF CYANOGENESIS IN CASSAVA

Small scale cassava farmers are heterogeneous. Thai farmers producing for export, Colombian farmers producing domestic animal feed and African farmers in a diversified agro-economy producing for urban markets should be well differentiated from the poor populations producing cassava for their survival in collapsing shifting cultivation systems (Rosling 1988, Romanoff 1992). Cassava toxicity is mainly a problem for the poorest group of small scale cassava farmers in the same way as short shelf-life of sweet cultivars mainly is a problem for other groups and different research will thus benefit different groups of small scale farmers.

Each scientific discipline tends to propose preventive strategies for cassava toxicity that fit their area of research rather then the setting in which it should be applied. Promotion of other crops has been proposed by medical science. Breeding

for low cyanide varieties has been the main option for agricultural science, whereas food science has argued for better processing and economists for improved marketing. The human diseases we have studied appear to be best prevented by improved processing but other measures may be better in other situations. Ongoing studies indicate that a dry cassava flour obtained by mechanical milling does not cause cyanide exposure, even if pre-milling fermentation of bitter roots has been shortened. A focus on post-harvest technology appears to be the best short-term option for prevention of cassava toxicity in poor populations growing cassava for survival.

Apparently most communities growing cassava as the major crop in shifting cultivation on poor soil prefer to grow bitter cultivars as staple. An example is the west-to-east drift of bitter cassava cultivars that has occurred during the last decades from Central Africa to drought-threatened cassava growing areas with poor soil in Mozambique (Essers, 1992), Tanzania (Howlett 1990) as well as Malawi and Uganda (Essers, pers. com.). The high yields of bitter cultivars observed by farmers in these areas have not been documented by agricultural investigations nor are the biological reason for the preference for bitter cultivars understood. Cyanogenesis may protect the crop against pests and predators but may also act as a marker of an associated preferred characteristic.

The importance of bitter cassava cultivars for food security in savannah and rain forest areas with poor soils (Romanoff 1992, Rosling 1988) leads us to propose the following long term research priority to the international cassava research community: "*Elucidation of the biology of cassava cyanogenesis in farming systems on marginal soils to enable optimal use of cyanogenesis for food security*".

The methodological approach we use for studies of cassava cyanogenesis may be labeled "molecular anthropology". We use explorative interview and observational methods in combination with verification through community-based food chemistry experiments and epidemiological studies with biomarkers for cyanide exposure. The participant observations, focus group and unstructured individual interviews as well as use of key informants are beneficial for two reasons. First, because structured questionnaires will always miss many aspects of the rapid change in agricultural, commercial, and processing practices of cassava in collapsing shifting cultivation systems (Tylleskär 1991, Banea 1992). Second, because the considerable local knowledge among cassava-growing women is not accessible through structured interviews. The same methods are useful in agricultural research in populations in marginal life conditions (Fresco 1986).

Needs and knowledge of small scale cassava farmers in areas with severe agro-ecological problems can be identified by these modern survey methods. Thereafter the findings can be verified in differently designed studies and hence guide biotechnology at the molecular level. Multidisciplinary research on the role of cassava cyanogenesis can become more than wishful thinking. Explorative surveys should be done by several types of biological scientists in direct communication with

the poorest group of cassava farmers. The reason for this is that biological knowledge is essential for doing relevant biological observations. As Louis Pasteur noted in 1854: "*In observational studies, chance favours the prepared mind*".

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DIFFERENTIAL BIOSYNTHESIS AND TRANSPORT OF LINAMARIN IN HIGH AND LOW CYANIDE CULTIVARS OF CASSAVA (MANIHOT ESCULENTA)

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Cassava varieties can be subdivided into low and high cyanide groups, based upon the linamarin content in the roots. It is generally assumed that differential rates of linamarin synthesis in leaves account for the varietal differences in root linamarin content, however steady-state concentration in leaves of low and high-cyanide cultivars are identical. In order to account for the differential accumulation of linamarin in roots, ^{14}C-valine, the precursor to linamarin, was fed to leaf blades of low (HMC-1) and high (CM 966-6) cyanide cultivars of cassava at progressive days after seed germination. In both varieties, linamarin biosynthesis peaked when the plants were about 45 days old, and then declined. However, during the period when linamarin biosynthesis was most rapid, the high-cyanide variety produced four times as much linamarin as the low-cyanide variety. After 83 days, both cultivars maintained similarly low levels of ^{14}C-linamarin in their leaves. Levels of labelled linamarin in petioles, i.e. transported linamarin, showed similar trends as those seen in leaves. These data suggest that increased levels of cyanogenic glycosides in high-cyanide roots is due to both higher biosynthesis in leaves and transport to the roots in young cassava plants.

INTRODUCTION

The nutritional quality of cassava is compromised by the presence of the cyanogenic glycosides linamarin (95%) and lotaustralin (5%). Cassava cultivars are characterized as either high or low cyanide based upon the amount of cyanogenic glycosides present in the root (Nartey, 1968). Cyanogenic glycosides are synthesized in the leaves of the plant and transported to the roots (Koch et al., 1992; Makame et al., 1988). The mode of cyanogenic glycoside transport, either symplastically or apoplastically, may be inferred by determination of the chemical form of cyanogenic glycoside which is transported from the leaves. Transport through the apoplast would presumably require conversion of linamarin into a non-hydrolyzable glucoside due to the presence of the β-glucosidase, linamarase, in the cell wall (Mkpong et al., 1990). Symplastic transport, however, would presumably not require conversion of linamarin into a non-hydrolyzable form, e.g. linustatin. Our objectives were to determine the levels of cyanogenic glycoside produced in leaves of low- and high-cyanide plants, and to identify the form of cyanogenic glycoside which is transported from leaves to roots. For our purposes, leaves were labelled with ^{14}C-valine, the precursor of linamarin, and the identity and quantity of labelled metabolites in leaves and petioles were determined.

METHODS

We have modified a method of ^{14}C-valine incorporation into cassava which preserves source-sink relationships between leaves and roots (Hahlbrock et al., 1968; Tapper et al., 1962; Nartey, 1968; Butler and Conn, 1964). Uniformly-labelled

^{14}C-valine, the precursor of linamarin, was introduced into the fifth leaf from the apex in 0.5 mM valine while still attached to the plant. Leaves of high (CM996-6) and low cyanide (HMC-1) plants were exposed to ^{14}C-valine for 48 hours under continuous light (30° Celsius). Soluble compounds were extracted from frozen leaf and petiole tissues with boiling 80% CH_3OH followed by two extractions with $CHCl_3$ (Brimer and Dalgraad, 1984). Following lyophylization, the extracts were resuspended in CH_3OH:H_2O (1:1). The samples were loaded on thin-layer chromatography plates on the basis of tissue fresh weight, and were chromatographed using a solvent system of CH_3OH:$CHCl_3$:15% NH_4OH (2:2:1) (Selmar et al., 1988). The plates were exposed to X-ray film to identify ^{14}C-labelled compounds. ^{14}C-labelled compounds were removed from the plates and quantified by liquid scintillation counting.

In order to identify cyanogenic compounds, we sprayed TLC plates with a crude cassava extract (containing linamarase) and exposed them to a picric acid soaked filter paper for 24 hours. The release of cyanide from any compounds results in the generation of a red spot on the filter paper. Glycosides were also identified on the TLC plates using a colorimetric p-anisaldehyde reaction (Zitnak et al., 1977). Linamarin, linustatin and neolinustatin standards (provided by Dr. Ivan Palmer, South Dakota State University) were also run on TLC plates (Smith et al. 1980).

RESULTS AND DISCUSSION

Incorporation of radioactive valine into linamarin was assayed in high and low cyanide plants at various ages. In both varieties of plants, linamarin synthesis was highest during the initial 83 days of growth following seed germination. After that time, linamarin synthesis leveled off to near zero. During early plant growth, however, high-cyanide plants incorporated up to 2.2 times the amount of ^{14}C-valine into linamarin as in low-cyanide plants. The quantity of ^{14}C-labelled linamarin present in petioles at different times after seed germination showed similar trends as those seen in leaves. Early studies indicated that the steady-state pool size of linamarin in low and high cyanide varieties was similar (Mkpong et al., 1990). Since our results indicate that the rate of linamarin synthesis in high-cyanide plants is greater than in low-cyanide plants, it is apparent that linamarin transport from leaves is greater in high-cyanide than low-cyanide plants.

Selmar et al. (1988) have demonstrated that the transportable form of linamarin in germinating Hevea seeds is linustatin, the glucoside of linamarin. Significantly, linustatin is not hydrolyzed by the apoplastic enzyme, linamarase. No ^{14}C-labelled compounds corresponding to linustatin and/or neolinustatin were observed in either leaves or petioles. Two ^{14}C-labelled glycosides were observed in leaf and petiole extracts. One of these was linamarin. The second glycoside had an R_f value of 0.46 and has not been identified at the present time. It is not likely that this compound is cyanogenic since there was no release of cyanide from the corresponding region of the TLC plate when incubated with a crude protein extract. These findings suggest that linamarin is transported without conversion to a non-

hydrolyzable form. Furthermore, since linamarase is loaclized in the cell wall, it is proposed that transport of linamarin is restricted to the symplast.

From these results, it is proposed that linamarin is transported to the roots of cassava without conversion to the glucoside linustatin. Furthermore, since linamarin does not appear to be altered for transport, linamarin presumably remains in the symplast during transport to the roots. Finally, varietal differences in cyanogenic glycoside content in roots of high- and low-cyanide plants may be determined by age-dependent differences in the rates of synthesis and transport of linamarin in leaves and petioles.

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PARTIAL PURIFICATION AND CHARACTERIZATION OF HYDROXYNITRILE LYASE FROM CASSAVA

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An assay for hydroxynitrile lyase has been developed using acetone cyanohydrin. The resulting cyanide product was quantified spectrophotometrically using a cyanide detection kit. The protein was extracted from cassava leaf tissue and has been purified two-hundred fold. A specific activity of 1.8 mmol/mg protein/h has been obtained thus far. Cyanide evolution due to the protein's presence is significantly higher than that due to acetone cyanohydrin's decomposition alone, even at pHs (6.0) at which acetone cyanohydrin is unstable. The approximate molecular weight of the protein, determined by SDS Phastgel, is 25.5 kD. The pH optimum and the temperature optimum for enzyme activity are 5.0 and 30°C respectively. Two methods of partial purification greatly speed the purification process. These are activated charcoal and heat treatments.

INTRODUCTION

In cassava, linamarin is deglycosylated by linamarase producing acetone cyanohydrin. This compound can break down spontaneously or enzymatically to form acetone and hydrogerl cyanide. In the processing of cassava for food, hydrogen cyanide is volatilized. The acetone cyanohydrin and linamarin which remain, however, causes serious health risks when ingested. Since acetone cyanohydrin can break down spontaneously, hydroxynitrile lyase, the enzyme which catalyzes the conversion of acetone cyanohydrin to acetone and cyanide, has not been investigated as thoroughly as linamarase. Hydroxynitrile lyase has been isolated and partially characterized by Carvalho in 1981(1).

In a recent study in which the amounts of cyanogenic glucosides, cyanohydrins, and hydrogen cyanide in cassava flour were quantified, it was determined that cyanohydrins were the main source of cyanide when the cassava was only partially processed (3). This study proposed that the low pH of the medium in which the cassava was fermented slowed the spontaneous break down of acetone cyanohydrin.

Due to the importance of hydroxynitrile lyase for potentially removing acetone cyanohydrin, a purification scheme is being developed and the enzyme is being characterized in order to reveal its possible beneficial uses. The factors contributing to the breakdown of acetone cyanohydrin have been investigated as well.

MATERIALS AND METHODS

Purification Procedures

All steps were performed at 4°C unless stated otherwise. Crude extracts of leaf tissue were obtained by two different procedures. In both cases the leaf tissue

was homogenized in a blender with 0.1 M sodium phosphate buffer, pH 3.5, and filtered through Miracloth. In the first method, the solution was centrifuged at 100,000 9 for 1 hour and dialyzed versus 0.05 M sodium phosphate buffer, pH 6.0. In the second procedure, the solution was centrifuged at 24,000 9 for 30 minutes followed by ammonium sulfate precipitation, with the 40-60 percent ammonium sulfate fraction dialyzed versus 0.05 M sodium phosphate buffer, pH 4.5, for at least 16 hours. Dialysis was followed by centrifugation at 11,000 9 for 20 minutes to remove precipitants. The protein solution was then either treated with activated charcoal or a heat treatment. Activated charcoal was added to protein solutions at a concentration of 1.5 mg charcoal/ml. After 0-30 minutes, the solution was filtered to remove the charcoal. Heat treatment consisted of 45 minutes at 58°C in a water bath.

Assay for Hydroxynitrile Lyase

Aliquots of protein, approximately 0.05 μg/ml, and acetone cyanohydrin (10.95 μM final concentration) were added to 0.05 M sodium phosphate buffer, pH 4.5. These concentrations gave linear rate kinetics over a 20 minute period. A Spectroquant kit (EM Science) was used to determine cyanide generated.

Other Methods

Proteins were separated on SDS Phastgels and stained with Coomassie Blue. Protein was quantified by the Bradford method using BSA standards (2).

RESULTS AND DISCUSSION

Reaction mixtures containing only acetone cyanohydrin and buffer were used to determine the effects of temperature and pH on its spontaneous breakdown. In all cases the reaction mixture was added to a 4 ml volume of pH 4.5 buffer after 15 minutes and cyanide was quantified. For measurements of temperature optimum, the reaction mixtures were placed at the given temperatures for 15 minutes. The pH optimum was determined by using different pH sodium phosphate buffers only in the reaction mixture. As determined by others (1.3) acetone cyanohydrin breaks down more rapidly at higher pHs (Fig. 1A). Nearly 40 percent of the acetone cyanohydrin decomposed in under 20 minutes at pH 6.0. Temperatures above 38°C greatly increase its decomposition as well (Fig. 1B).

Although spontaneous breakdown is significant, the enzymatic reaction can greatly enhance cyanide production even at pH's at or above 6.0 (Fig. 2). After 30 minutes incubation in the presence of 10 llg of crude extract 100 percent of the acetone cyanohydrin was converted to cyanide, whereas the spontaneous reaction was only half complete after 1.5 hours.

Partially purified hydroxynitrile lyase that was prepared from crude leaf extracts followed by 100,000 g centrifugation, dialysis versus pH 6.0 sodium

phosphate buffer, and ammonium sulfate precipitation had a specific activity of 1.8 mmole/mg protein/hour. Crude extracts and those treated by the three purification procedures were visualized by SDS Phastgels. Hydroxynitrile lyase has a molecular weight of 25.5 + 2 kD. Previously, Carvalho had proposed a molecular weight of 15 kD (1). The difference in our determinations of its molecular weight may be due to the presence of different oligomeric forms of the enzyme (1). Protein content after different purification procedures was determined by Bradford assay (Table 1). The activated charcoal and heat treatments decrease the protein concentration over 60 and 70 percent, respectively. The partially purified enzyme preparation was then used for determination of pH and temperature optima (Fig. 3A and B). It was determined that the temperature optimum was 30 °C and that the enzyme lost substantial activity at temperatures above 50 °C. In difference to previous studies, we determined that the pH optimum for hydroxynitrile lyase was approximately 5.0 and not 6.0 (1).

In conclusion, we have demonstrated that acetone cyanohyrin is stable at low pHs and low temperatures; conditions which may exist in fermenting cassava. Increases in the level or activity of hydroxynitrile lyase during processing may reduce its cyanogenic potential and toxicity.

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Table 1. Yield of Protein from Partial Purification

PROCEDURE	YIELD (mg)
Crude extract	17.36
Ammonium sulfate	11.72
Activated charcoal	6.42
Heat treatment	5.03

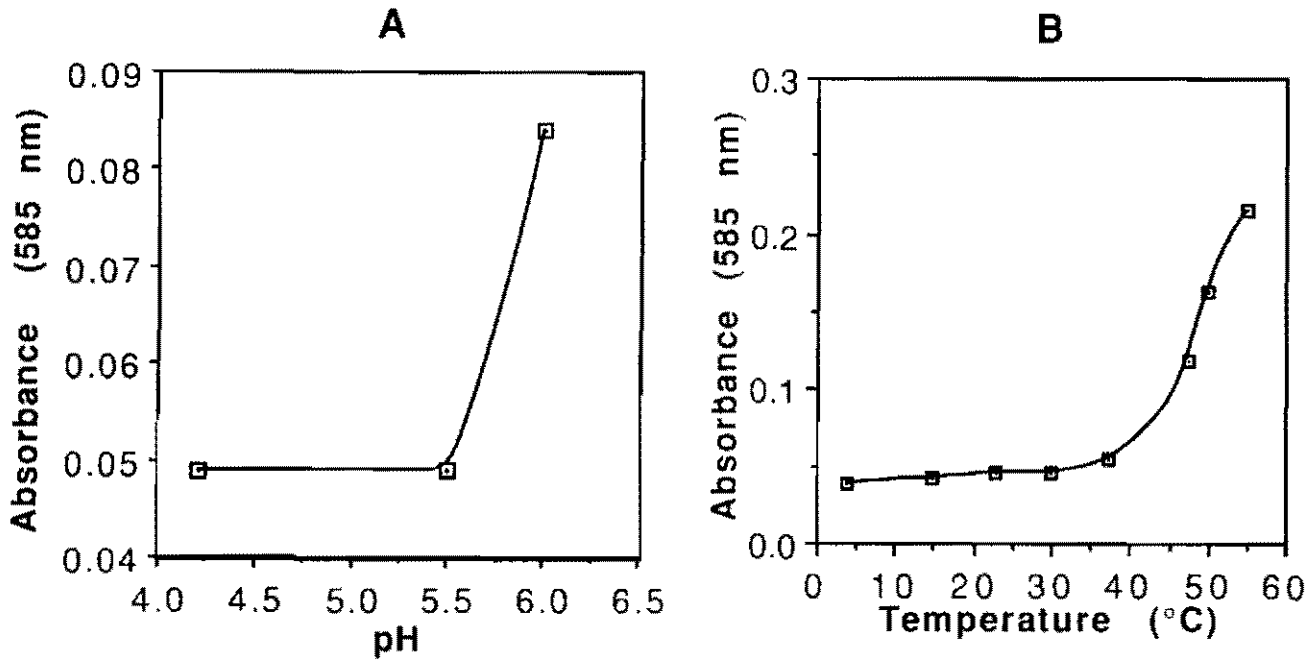

Figure 1. A. and B. The effects of pH and temperature on the decomposition of acetone cyanohydrin.

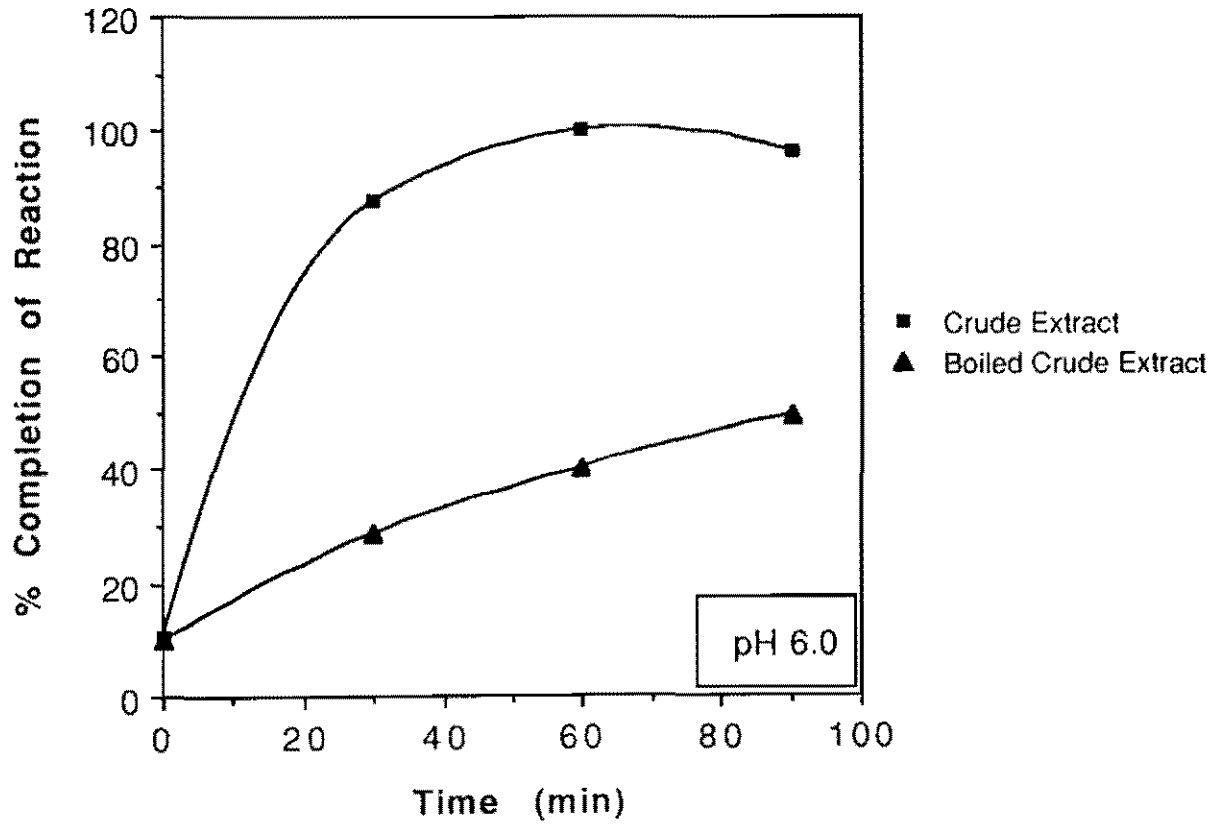

Figure 2. Enzymatic versus Spontaneous Degradation of Acetone Cyanohydrin.

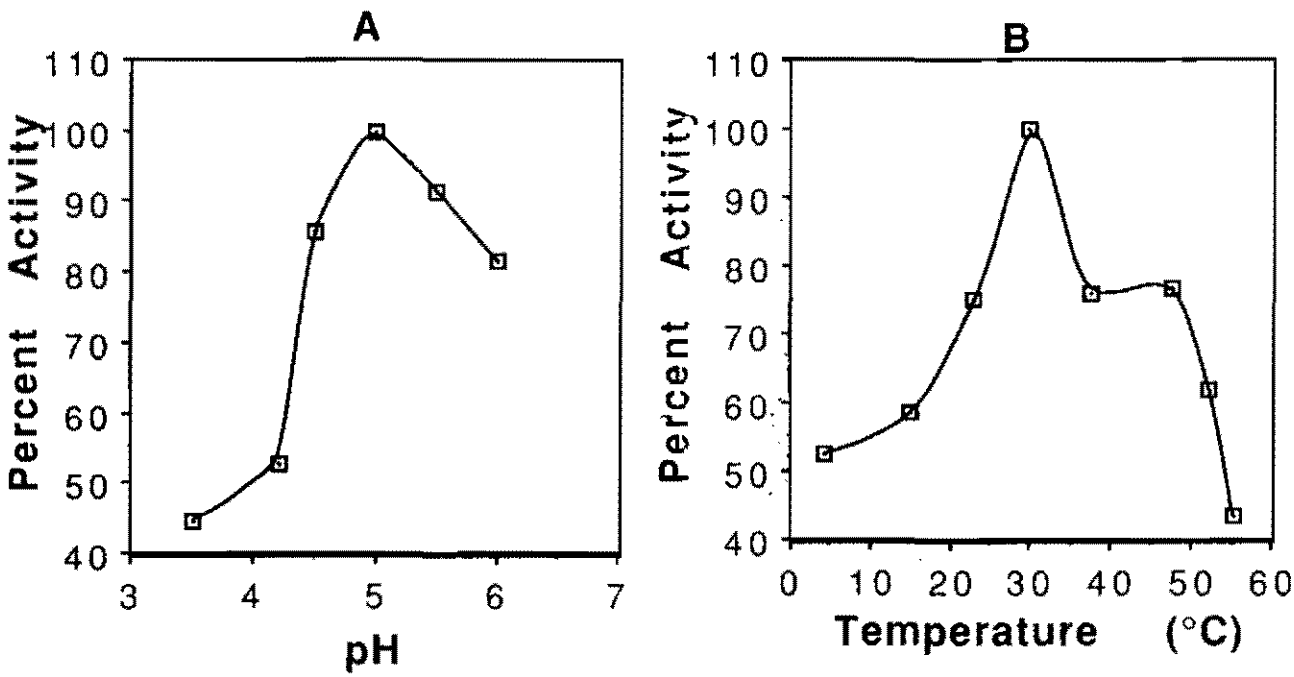

Figure 3. A and B. pH and Temperature Optima for Hydroxynitrile Lyase.

MOLECULAR STUDIES OF CYANOGENESIS IN CASSAVA

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Hydrocyanic acid (HCN) is released from all tissues of cassava (*Manihot esculenta* Crantz) following mechanical damage. HCN is produced by the breakdown of two structurally related cyanogenic glucosides by the sequential action of a ß-glucosidase (linamarase) and an α-hydroxynitrile lyase (HNL). The final stage in the biosynthesis of the cyanoglucosides is catalyzed by a UDP-glucosyltransferase. The cloning and expression of these three soluble enzymes has been approached by a variety of methods. A cDNA library prepared from cassava cotyledons has been screened for both the ß-glucosidase and glucosyltransferase genes. A linamarase cDNA clone from white clover was used to select the equivalent cyanogenic enzyme from cassava. The ß-glucosidase clone has been characterised and sequenced (Hughes *et al.*1991), and gene expression has been localized by non-isotopic *in situ* hybridization of mRNA (Pancoro and Hughes, 1992). Several glucosyltransferase clones have been isolated and sequenced, using a flavonoid glucosyltransferase clone from *Antirrhinum majus* as a heterologous probe. Comparisons between expression of these genes and enzyme activity in different developmental states and tissues of cassava may indicate potential cyanogenic glucosyltransferase clones which appear to represent single copy genes. Peptide sequences for HNL have been obtained preparatory to cloning. It has been confirmed that this enzyme is not glycosylated in cassava.

INTRODUCTION

The release of HCN from cassava tissues following mechanical damage is the result of the breakdown of two structurally related cyanogenic glucosides by the sequential action of two soluble catabolic enzymes, a ß-glucosidase (linamarase) followed by an α-hydroxynitrile lyase (HNL). The two cyanogenic glucosides found in cassava, linamarin and lotaustralin, are derived from valine and isoleucine respectively. The biosynthesis of the aglycone hydroxynitriles involves several membrane-bound steps (Koch et al, 1992) but the glycosylation of the unstable hydroxynitriles to give the stable cyanoglucosides takes place in the cytoplasm, and is catalysed by a soluble UDPG- glucosyltransferase (UDPG-GT). The molecular biology of the three soluble enzymes involved in cyanogenesis has been studied by a variety of methods.

The specific activity of the three enzymes (UDPG-GT, linamarase and HNL) in cassava cotyledons, hypocotyls and roots was determined at defined stages during the first twelve days of germination. Linamarase was the first of the enzymes to be cloned. Because increasing levels of linamarase activity were found in 10-day-old cotyledons,indicating active gene expression, this tissue was selected for the construction of a cDNA library in the lambda vector gt10 using EcoR1/Not1 adaptors. The same library was subsequently screened for glucosyltransferase clones and will also be used to select HNL clones, since these enzymes also have high levels of specific activity in young seedling cotyledons.

UDPG-GLUCOSYLTRANSFERASE (UDPG-GT)

A UDPG-glucosyltransferase consensus signature peptide sequence has been proposed, based on UDPG:flavonoid glucosyltransferase genes from maize and barley (Bairoch, 1991). Flavonoids, like cyanoglucosides, are secondary plant products found in the cell vacuole, and it has been shown that partially purified cassava UDPG-GTs which convert acetone cyanohydrin to the glucoside, linamarin, also glycosylate other secondary plant products, including flavonoids (H. Mederacke, personal communication). It was therefore assumed that there will be some sequence homology between flavonoid and cyanogenic lucosyltransferase genes. A BglII-AccI restriction fragment, containing the region with the consensus signature sequence, from a UDPG:flavonoid 3-O-glucosyltransferase clone from Antirrhinum majus (kindly provided by Dr. C. Martin, John Innes Institute) was used as a heterologous probe to screen the cassava cotyledon cDNA library. Five clones were selected which differed from each other on the basis of their restriction maps and their homology to the probe.

The DNA sequences of the five clones showed that two, pCGT.G2 and pCGT.G7 contained a deduced amino acid sequence with homology to the UDPG-GT consensus signature sequence, with 87% identity over 23 specified amino acid residues.

In order to identify a putative cyanogenic UDPG-GT the pattern of mRNA levels was compared with the pattern of UDPG-GT activity found during plant development.

The clones were used to probe mRNA extracted from different developmental stages and tissues of cassava seedlings, and from young cassava leaf material. The expression of pCGT.G2 in seedlings reflected that of UDP-GT enzyme activity, which shows tissue and temporal variation in seedlings. pCGT.G7 showed no differential expression during development. Expression in young leaf material was low for pCGT.G2 compared with that for pCGT.G7. These results suggested that pCGT.G2 was a possible candidate for the cyanogenic glucosyltransferase gene. The size of the pCGT.G2 cDNA clone was 1.1Kb whereas the transcript was 1.6Kb. In an attempt to obtain a full length pCGT.G2 clone the cotyledon cDNA library was re-screened using pCGT.G2 as probe. An additional four clones with homology to, and larger than, pCGT.G2 were selected. Northern blot analysis showed that these clones have a similar transcript size to pCGT.G2 (1.5-1.6Kb) but have different patterns of differential expression in developing seedling tissues. cDNA clones pCGT.M6 (1.2Kb) and pCGT.M4 (2.0Kb) have been fully sequenced and the deduced amino acid sequence confirms the presence of the UDPG-GT consensus signature sequence in both clones. pCGT.M6 has high homology to pCGT.G2 along its whole length but is not identical and must therefore represent a different glucosyltransferase gene.

Although all of the clones selected from the second screen of the cDNA library have some homology with each other, they have been shown to represent different genes (proteins) on the basis of patterns of expression, sequence data and homology with genomic restriction fragments.

We have to date at least three different clones that contain both the glucosyltransferase signature peptide and show differential expression. We hope eventually to have amino acid sequence data from purified cyanogenic glucosyltransferase protein which will enable us to determine which clone is the cyanogenic glucosyltransferase. In addition, there is evidence to suggest that the absence of glucosides from acyanogenic, ac ac, white clover plants is associated with the absence of the cyanogenic glucosyltransferase (Hughes and Conn, 1976). The putative cassava clones will be used to probe RNA from genetically characterised cyanogenic and acyanogenic white clover leaves. Absence of expression of white clover cognates in acyanogenic plants would help to clarify the nature of the putative cassava glucosyltransferase clones.

ß-GLUCOSIDASE, LINAMARASE

The similarity of the cyanogenic pathways in white clover (Trifolium repens L.) and cassava has been exploited in the cloning of the first of the catabolic enzymes involved in the breakdown of the cyanoglucosides, linamarin and lotaustralin, which are found in both species. A white clover ß-glucosidase had previously been cloned in this laboratory (Oxtoby et al, 1991), and a 704 bp Ssp1 fragment from the cDNA clone pTRE361 was used as a heterologous probe to screen the cassava cotyledon cDNA library. Six clones were selected and subsequent restriction mapping and sequencing showed that they all represented the same gene (Hughes et al, 1992). The identity of the gene as the cyanogenic ß-glucosidase, linamarase, was confirmed by the presence in the deduced amino acid sequence of two internal peptide sequences obtained from digestion of the purified enzyme by a-chymotrypsin, followed by SDS-PAGE, Western blotting and peptide sequencing (Protein Sequencing Facility, Leicester University). The derived amino acid sequence has considerable homology with that of the white clover linamarase cDNA clone pTRE104. In the region residue 44 to residue 354 (310 amino acids) 64% of the amino acids are identical or equivalent.

Affinity chromatography of the purified enzymes with the lectin concanavalin A, which binds high-mannose oligosaccharides, showed that both enzymes were glycoproteins. The higher concentration of a-methyl mannoside required to elute the bound cassava enzyme may indicate that it has more oligosaccharide residues than the white clover linamarase. Digestion with endoglycosidase H indicated that the enzymes are glycosylated with asparagine-linked M8 or M9 high-mannose oligosaccharides. This is a characteristic of many secretory proteins. The native cassava linamarase was found to have a relative molecular mass of 70K compared with 62K for white clover, as estimated by SDS-PAGE. After endoglycosidase H digestion the proteins were 65K and 59K respectively, the proportionally larger

reduction in cassava linamarase suggesting, again, that it has more carbohydrate residues than white clover. The predicted size of the unglycosylated cassava linamarase from the deduced amino acid sequence is 62K. This is smaller than the observed value (65K) and has been shown to be due to incomplete digestion by the endoglycosidase rather than the presence of O-glycoside linked oligosaccharides. Five putative N-linked glycosylation sites have been identified in the C-terminal region of the cassava sequence, only one of which is conserved in the white clover sequence, though this site may not be glycosylated due to an adjacent proline residue (Bairoch, 1991).

Recently, 2-deoxy-2-fluoro-ß-D-glucopyranoside has been used to chemically identify a glutamic acid residue as the nucleophilic residue in the active site of a ß-glucosidase from Agrobacterium (Withers et al, 1990). The sequence which contains this residue, I/V TENG, is conserved in a number of bacterial ß-glucosidases and ß-galactosidases and is also present in the cyanogenic ß-glucosidases from both white clover and cassava and a non-cyanogenic ß-glucosidase from white clover. In contrast to the bacterial enzymes, the plant enzymes have a prominent hydrophobic N-terminal region. It is known that an N-terminal peptide is cleaved from the white clover linamarase during co-translational processing (Dunn et al, 1988) and a similar signal peptide appears to be present in the cassava enzyme. In white clover the enzyme has been shown to be apoplastic (Kakes, 1985), and recent immunogold labelling studies proposed that cassava linamarase protein is associated with the cell walls of cassava leaves (Mkpong et al, 1990). However, in situ localisation studies of mRNA in young cassava leaves carried out in this laboratory suggest an alternative location for the expression of the linamarase gene. A non-isotopic digoxygenin labelling system was used to visualise the presence of mRNA in young cassava leaf sections. Antisense riboprobes, produced from the linamarase clone pCAS5 gave strong hybridisation signals only within the network of latex vessels (laticifers) found throughout cassava tissues. No signal was found with the equivalent sense probes. An antisense riboprobe of a putative glucosyltransferase clone, pCGT.G4, gave a signal in all leaf mesophyl cells except the epidermis and gave no significant signal within laticifers. In order to confirm the presence of linamarase mRNA in cassava laticifers, latex was taken from syringe needle wound sites on the mid leaflet vein, petiole and adjacent stem. A dot blot membrane of this material was hybridised to both digoxygenin labelled sense and antisense riboprobes from pCAS5. The clear signal produced with the antisense probe and absence of a signal with the sense probe confirmed the presence of linamarase mRNA within cassava latex (Pancoro and Hughes, 1992).

In addition latex was assayed for the presence of linamarase protein both by SDS-PAGE, where a prominent band in the complex mixture of latex proteins co-migrated with purified linamarase, and by enzyme assay, where high levels of linamarase activity were found. Specific activity in latex was higher than that of whole leaf extracts indicating that linamarase activity is primarily associated with the latex. Levels of linamarase activity in latex from leaf petioles fell significantly after removal of one or more leaflets from the attached leaf, suggesting that linamarase

synthesised in leaf laticifers is transported to other parts of the plants through the network of latex vessels. The in situ hybridisation and enzyme assay results show unambiguously that the linamarase gene is expressed within anastomising laticifers and that the protein has an intracellular location. Since other enzymes with a defensive role (chitinases) are found in latex, the presence of linamarase in latex is compatible with both its probable defence function and with the compartmentation required by cyanogenesis. There was no evidence for the presence of the cyanoglucosides, linamarin and lotaustralin, in latex, and very low levels of hydroxynitrile lyase activity were found in latex compared with whole leaf extracts, suggesting an alternative location for the expression of HNL.

HYDROXINYTRILE LYASE (HNL)

The second enzyme involved in the breakdown of cyanogenic glucosides, α-hydroxynitrile lyase (HNL), has been purified by Sephacryl S-300 and DEAE Sepharose column chromatography to a single band on SDS-PAGE. The purified enzyme was digested with V8 protease, the resulting peptides were separated by SDS-PAGE followed by Western blotting, and bands were selected for peptide sequencing (Protein Sequencing Facility, Leicester University). The N-terminus sequence proposed by Carvalho (1981) was confirmed, with one amino acid residue difference, and an additional internal sequence of fourteen amino acids was obtained. These peptide sequences will be used to design an oligonucleotide probe to screen the cassava cotyledon cDNA library for HNL clones.

α-Hydroxynitrile lyases from a variety of cyanogenic species have been analysed for glycosylation (Poulton, 1988). The results demonstrate that HNL proteins from some species, such as Prunus serotina, are glycosylated and from others, such as Sorghum vulgare, are not. Purified cassava HNL did not bind to concanavalin A and Caravalho (1981) found no evidence of glycosylation of the cassava HNL using periodic acid and Schiffs reagent to detect the presence of carbohydrate with SDS-Page. This result has been confirmed by the more sensitive method of Western blotting followed by biotin-streptavidin-alkaline phosphatase detection of oligosaccharides. It can therefore be concluded that cassava HNL is not a glycoprotein.

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EVALUATION OF A RAPID SEMI-QUANTITATIVE ASSAY FOR CYANOGENS IN CASSAVA

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A rapid qualitative test for hydrogen cyanide, involving color changes on paper pre-treated with the compound tetra base (4,4'-Methylene bis [N,N-dimethylaniline]), was recently modified. The modified method was subsequently proposed as a semi-quantitative screening assay for the cyanogen content of cassava root parenchymal tissue (Bradbury & Egan, in press). This test is being considered specifically for use by cassava breeding programs as a replacement for the now updated picrate test. Research is currently being undertaken at CIAT to evaluate the proposed assay, using a variety of cassava cultivars with widely varying cyanogen contents. A quantitative enzymic assay is being used as a control. Work to date has indicated that a broad differentiation of samples according to cyanogen content may be obtained. This differentiation was more readily obtained on the basis of the time taken for color development, than according to differences in colors obtained within a fixed test-time. No significant temperature-related differences were indicated in the assay, within the range 20-35 C. The reliability of the sampling method used in the assay has also been partially investigated. The sampling method employed involves the removal of a central disc from the root being assayed. The cyanogen content of the central disc was compared against that of the remainder of the parenchyma. An acceptable degree of correlation was indicated, implying that the sampling procedure is acceptable within the context of a semi-quantitative assay. The aims of further work being undertaken include the continued investigation of possible effects of temperature upon the assay as well as a closer definition of its accuracy, reproducibility and limitations. The endogenous enzyme linamarase is responsible for the evolution of hydrogen cyanide in a given sample of cassava parenchymal tissue. The tetrabase assay depends on this function of the enzyme. Further work is planned to investigate possible assay-related effects of variation in sample linamarase activity.

INTRODUCTION

For the quantitative assay of cyanogens in cassava, several methods exist. Most important among these are colorimetric (spectrophotometric) methods. Although capable of high precision and reproducibility, quantitative assays to date generally are not suitable for the rapid screening of large numbers of samples. For the purpose of rapid screening of cassava samples, the semi-quantitative alkaline picrate paper test has been in use for many years (Williams and Edwards, 1980). The test has been in use at the International Institute for Tropical Agriculture (IITA) and at the Centro Internacional de Agricultura Tropical (CIAT) since the mid-1970's. Certain investigations have cast some doubts upon the accuracy and reproducibility of the picrate reaction, citing lack of specificity (Zitnak, 1973). The assay has produced results several times greater than those obtained by other methods (Mendoza et al., 1984). There have also been reports of "false positive" results, indicating cyanogens in acyanogenic samples (Nahrstedt, 1980).

Recently, Bradbury and Egan (1992) have proposed an alternative rapid semi-quantitative assay, based on the qualitative method of Feigl and Anger (1966). The method involves a redox reaction in which the copper (II) ion is reduced to Copper (I) by reaction with HCN (free cyanide). An oxidation product of the reagent tetra base (4,4'- Methylene bis-[N,N-dimethylaniline]) is formed. This product is blue-violet in colour. The format of the proposed assay involves the use of paper strips saturated with the test reagent mixture. The procedure is thus somewhat similar to the use of litmus paper in the testing of pH. The purpose of this paper is to present the findings of an investigation of this proposed assay, using an established quantitative spectrophotometric assay (Cooke, 1978; O'Brien et al., 1991) as a control.

EXPERIMENTAL METHODS

Materials

A range of 10 cassava varieties were used, with total cyanogen contents ranging from 10 mg/kg to 456 mg/kg (fresh basis).

For the tetra base assay, the reagents used were tetrabase in acetone (0.3% w/v) and cupric acetate (Cu $[Ac]_2$) in acetic acid (0.3% w/v). For the quantitative assay of cyanogens and of linamarase activity, the reagents used were as reported in O'Brien et al, 1991 op. cit.)

Methods

1. Cyanogens and linamarase: Comparison of central disc with whole root.

For both the picrate paper test and the modified tetra base test, the sampling of cassava root parenchyma involves cutting a disc from the centre of the root (as measured longitudinally). The production of the volatile free cyanide (HCN), which causes the colour changes in both of the tests, is autolytic. The endogenous linamarase in the sample breaks down the cyanogenic glucosides to ultimately produce free cyanide (HCN). In order to verify whether this sampling method was representative of the whole root, roots of 7 different varieties were assayed. The assays involved comparison of the central disc of a root with the remaining parenchyma as a whole. Additionally, a comparison was made of the central disc with discs taken from the proximal and distal parts of a root of M Col 1684. Total cyanogens and linamarase activity were assayed, as both are equally important in any autolytic assay. The results are reported in Table 1.

2. Investigation of tetra base assay: comparative assays of cyanogens by tetra base and by colorimetry.

The tetra base method was compared against the reliable and accurate colorimetric assay already in use at CIAT (Cooke, 1978; O'Brien et al., 1991). Given

the variability of cyanogen contents from one root to another, even of the same variety and taken from the same plant, the investigation was carried out using a sample pool of one root for each comparison. A further consideration was the potential variability of linamarase activity from one sample to another. Since the proposed tetra base assay involved an autolytic procedure, the linamarase activity must also be of importance. Therefore both cyanogen content and linamarase activity were assayed.

Sampling for the tetra base assay involved the removal of the central portion of the root (1 cm thick), and boring 4 cylindrical plugs from this disc. These plugs were trimmed to 0.5 cm length before being used for assay. This whole procedure took less than 5 minutes. Prior to the actual assay, the plugs were kept in the tightly-sealed assay vials (without test papers) for one hour.

Sampling for the colorimetric assay of both total cyanogens and linamarase activity, involved finely dicing up the remainder of the parenchyma from the same root, and making extracts (at pH 2 and pH 6 respectively).

The three assays were thus carried out using the same peeled root in all cases.

It has been reported that the modified tetra base assay may be susceptible to fluctuations in ambient temperature, performing more rapidly at higher temperatures (Bradbury, unpublished observations). It was decided that this possibility should not be ignored, as cassava research stations situated at different locations in Colombia experience widely varying ambient temperatures, ranging between 14 and 35°C approximately. Consequently, a further variable, that of temperature, was built into the experimental work. Using an incubator, the tetra base assay was carried out at 10, 14, 20, 25, 30 and 35°C. The entire range of 10 varieties was assayed at each temperature, except at 10°C, where 4 varieties were assayed. At each temperature, the whole roots were held in the incubator for around 3 hours before sampling. Similarly, the plugs of parenchyma used for the tetra base assay were held in tightly stoppered vials in the incubator for an hour before the assay.

Colours obtained in the assay were enumerated using a comprehensive colour guide (numbers 1 - 10). Colour changes were noted during the first three hours and also overnight.

RESULTS

Table 1a. Comparison of central disc with rest of root.

Variety of root	Total CN (mg/kg fresh) Centre	Rest	% Diff.	Lin.se Activity (EU/g fresh) Centre	Rest
CM 507-37	202.7	161.7	+25.4	0.21	0.04
M Bra 12	121.3	113.5	+6.9	1.92	0.95
M Ven 77	123.1	94.7	+30.0	0.34	0.16
CM 2766	77.6	104.7	-25.9	0.61	0.27
CG 996-6	51.3	44.5	+15.3	0.56	0.11
M Col 1684i	474.6	400.0	+18.7	0.63	0.12
M Col 1684ii	284.9	296.7	-4.0	2.74	0.60
M Ven 25	332.2	302.9	+9.7	0.61	0.06

Table 1b. Cyanogen and Linamarase Distribution in a M Col 1684 Root.

Total Cyanogens (mg/kg fresh) Centre	Proximal	Distal	Rest	Linamarase Activity (EU/g fresh) Centre	Proximal	Distal	Rest
284.9	315.7	154.6	278.4	2.74	0.40	1.40	0.60
			314.9				0.60

Table 2a. Summary of results obtained in tetra base experimentation.

Total Cyanogens (mg/kg, fresh)	Linamarase (EU/g, f.)	Tetra Base Score		
10.1-456.7	0.03-0.63	10 min	30 min	60 min
		1.0-10.0	1.0-10.0	1.0-10.0
SEMI-QUARTILE RANGE, T.B.		5.5	5.5	4.5

NOTES

1. Data comes from first phase, i.e. all data regardless of assay temperature and sample linamarase activity; 91 cases.

2. Full set of raw data available from principal author.

Table 2b. Statistical parameters pertaining to data.

Data Phase	No. of cases	Spearman Correl.n	Level of Signif-icance	Double Entry table R 1	R 2	R3
First	91	0.746	0.060	94.9%	50.0%	65.0%
Second	72	0.773	0.065	96.7%	50.0%	71.9%
Third	43	0.851	0.069	100%	55.0%	84.2%

NOTES

Data 1st Phase: all data irrespective of assay temperature and enzime activity.

Data 2nd Phase: minus data from tetra base assays run at temperatures below 20°C.

Data 3rd Phase: as in phase two but minus data from samples with enzyme activity below 0.2 EU/g (fresh basis).

Range 1: 0 - 50 mg/kg as HCN (fresh basis)
Range 2: 50 - 100 " " " " "
Range 3: 100+ " " " " "

Spearman Correlation: indicates linear correlation, and also takes account of similarity in the order or ranking of data points produced by the two methods.

Level of significance: indicates the probability that there is no correlation, that the assertion of such a correlation existing between the two data groups is mistaken.

Double entry table data: indicate the percentage of results within a given range (R1 - R3) of cyanogen content, which were correctly interpreted by the tetra base assay.

DISCUSSION

1a. Comparison of central root disc with rest of root.

(i) Total cyanogens

The total cyanogen contents of the central root discs were in most cases fairly close to those of the remainders of the roots. Most had 1.1 - 1.3 times the cyanogen content of the remainder. One result was quite different, however: the CM 2766 root result indicates a total cyanogen content in the central disc around 26% lower than in the rest of the root. These results are not claimed to be conclusive. They emerge from work involving a very limited number of varieties, and only one root of each variety was sampled. It would be useful to extend such a survey to a wider range of varieties. Nonetheless, the indication is that the total cyanogen contents of the central discs, being of the order of $\pm$ 30% of those of the remainders, are acceptable within the context of a semi-quantitative assay.

(ii) Linamarase activity

The other aspect of importance in such an autolytic assay is that of linamarase activity. The analytical assay of any enzyme is normally carried out under "ideal" conditions (excess substrate, optimal temperature, etc.). Because of this, such an assay cannot be assumed to give an estimate as to how an enzyme will function within a given situation. However, an estimate of the potential activity of the enzyme may be gained. Legitimate comparisons between different samples may be carefully made on such a basis.

The linamarase activities of the central discs were consistently found to be higher than those of the remainders (2 - 10 times as high). There are two important points implied by the results. One is that the assay of linamarase by colorimetry, using the remainder of the root (see part 2), does not necessarily give a good indication of the activity in the central disc. The other point is that it is possible for samples with similar cyanogen contents to have widely differing linamarase activities (as in the MBra 12 and MVen 77 samples in Table 1a). This may pose problems with the reliability of any autolytic assay, including the one under investigation here.

2. Tetra Base Assay Experimentation

(i) Operating mode for tetra base assay

As stated in the Methods section (part 2), colour changes were noted during the initial three-hour period and also overnight. It was found that the most consistent pattern in the results obtained was as follows (cyanogens expressed as HCN, fresh weight basis):

Tetra Base score < 8.5 after 1 hour:	0 - 50 mg/kg total cyanogens (range 1)
TB Score < 8.5 after 10 minutes, but > 8.5 after 1 hour:	50 - 100 " " " (range 2)
TB Score > 8.5 after 10 minutes:	100 + " " " (range 3)

(ii) Comparison of tetra base assay with colorimetric assay

As may be seen in Table 2b, the results of this experiment were sorted into 3 distinct phases. In the first phase, all available data was used (91 cases), irrespective of tetra base assay temperature and linamarase enzyme activity. The second phase, with the elimination of data relating to 10 and 14°C, contains 72 cases. The third phase, with the further elimination also of results derived from samples with low linamarase activity (less than 0.2 EU/g, fresh basis), has 43 cases.

The statistical parameters indicated in Table 2 show a marked increase in correlation between the TB and colorimetric methods, as one progresses from phase to phase. Although the third phase of the results shows the best correlation of the three, it has only 43 cases, less than half the original number. Interestingly, though, the level of significance changed only slightly. Hence, the probability of there being an error in the assertion that a correlation exists, is almost equally small in the third phase as in the first and second phases.

There is also an important practical consideration. The elimination of certain results on the basis of low linamarase activity (as in the third phase) implies the necessary routine assay of this enzyme in samples. This would effectively defeat the purpose of a rapid assay. Therefore, the second phase of results is proposed as an acceptable compromise between the best correlation observed in the third phase, and the lower (but still good) correlation of the first phase. The removal of results from TB assays run at 10 and 14°C produced an increase in correlation. This is apparent from the Spearman result, and the increased yield of correct results in the double entry table. Of the 19 results rejected because of low assay temperature, 11

were lower than expected, 1 was higher, and the rest were in keeping with expectations. This is not surprising. The temperature of assay for cassava linamarase has been set at 30°C (Cooke et al., 1978) and at 37°C (Yeoh, 1989). At low temperatures, the endogenous linamarase in a sample is likely to be adversely affected, producing a depressed result. The limitation of the tetra base assay at low temperatures would be a disadvantage only in two of the ecosystems where cassava is grown: the high altitude tropics during the coolest parts of the day (early morning and late evening), and the sub-tropics during the winter months.

One important cause for concern is the low (50%) yield of successful results in range 2 (50-100 mg/kg) as evidenced in the double entry table (Table 2b). It must be borne in mind, however, that the number of cases in this range was very small, with just 10 cases. Further, of the 5 "false" results obtained, 2 results were from samples with cyanogen contents very close to the cut-off points of the range. One had a cyanogen content of 50.1 mg/kg, the other, 98.9 mg/kg. In each of the two, the result crossed the respective boundary into the neighbouring range. Hence, a successful yield of 70% may be claimed. Obviously, more data is required within the cyanogen range of 50-100 mg/kg.

Even if such further work should produce other problematic results, the adoption of a two-range system would be possible. The two ranges could be Range 1 (0-50 mg/kg) and Range 2 (50+ mg/kg). The results from the second phase indicate that the yields of successful results in these ranges would be 96.7% for Range 1 and 85.8% for Range 2.

(iii) Correlation between cyanogen content and linamarase activity

During the tetra base assay experiment,both total cyanogens and linamarase activity of samples were assayed using the quantitative colorimetric procedure. This offered an opportunity to examine the results for evidence of any correlation between the two. Using the first phase set of results (91 cases), the Pearson correlation was estimated at 0.081. Effectively, no correlation was indicated. This requires further investigation over a wider range of germplasm.

CONCLUSIONS

1. The tetra base assay proposed as a rapid semi-quantitative cyanogen assay for cassava is under investigation. It has been compared with a reliable accurate colorimetric assay. Correlation was good, despite possible errors arising from differences in the cyanogen content and linamarase activity of the central root disc as compared with the remainder of the root.

2. Linamarase activity varied widely among root samples (range: 0.05 - 0.63 EU/g, fresh basis), and was not correlated with cyanogen content of the same roots. Despite this variation, the overall performance of the tetra base assay was little affected.

3. The assay was apparently not significantly affected by temperature within the range 20 - 35° C. At temperatures below 20°C (10 and 14°C), there was an apparent depression effect upon the assay. This was probably due to inhibition of the endogenous enzyme linamarase. The low temperature limitation would only be a problem at the high altitude extreme of cassava cultivation in the tropics: a relatively minor ecosystem. In the sub-tropics, the assay should be performed in the summer months.

4. The assay is proposed as a rapid screening method capable of distinguishing cyanogen contents in three ranges: 0 -50 ; 50 - 100; and over 100 mg/kg (expressed as HCN, fresh weight basis). The weakest of the three ranges in terms of successful results, is range 2 (50-100 mg/kg). However, data within this range is very limited. The acquisition of more data (from cassava samples with cyanogen contents within the range) should improve this situation.

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DEVELOPMENT OF HPLC TECHNIQUES FOR CYANOGENESIS IN CASSAVA

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The application of high performance liquid chromatography (HPLC) to the analysis of cassava cyanogens has been investigated. Qualitative analysis has identified the major carbohydrates including linamarin present in cassava root and leaf extracts. Initial studies involving chromatographic resolution, reproducibility and accuracy indicate the possibility of the application of this technique for routine analysis of the cyanogenic potential of cassava tissues.

INTRODUCTION

Certain higher plants contain cyanogenic glucosides which hydrolyse upon tissue damage to release hydrogen cyanide (HCN). This has been postulated as an effective deterrent to herbivory. These compounds are also involved in the general metabolism of the plant in the synthesis of ß-cyanoalanine and subsequently asparagine (Miller & Conn, 1980). In some cultivars of cassava (Manihot esculenta) significant quantities of cyanogenic glucosides are present. The consumption of inadequately processed high cyanide cassava has been shown to be a causative factor in a number of human disorders including acute intoxication, Konzo (a paralytic disease) and aggravated goitre (Rosling, 1987). Hence the need for routine analytical procedures to accurately determine the potential toxicity of cassava roots, leaves and processed products.

Linamarin and lotaustralin are the cyanogenic glucosides present in cassava, linamarin predominates at 97-98 % of the total amount (Bissett et al., 1969). Both compounds are hydrolysed by the endogenous glucosidase, linamarase (3.2.1.21) which in cassava leaves is located in the cell walls. It is postulated that linamarin is converted into a non-hydrolysable form for transportation from the site of synthesis in the leaves to the site of storage in the roots (Mkpong et al., 1990). This has been observed in Hevea brasiliensis where the diglucoside, linustatin has been associated with the mobilisation and metabolism of the cyanogenic glucosides (Selmar et al., 1988). The possible presence in cassava of a linamarase non-hydrolysable, cyanogenic glycoside would be overlooked by analytical methods that utilise this enzyme.

Routine and accurate quantitative screening methods for cassava cyanogens are based on the hydrolysis of cyanogenic glucosides by linamarase. The resultant cyanohydrins are subsequently broken down to HCN by attenuation of pH. The final product, HCN, can then be quantified by standard colourimetric techniques (Cooke, 1978). Such methods provide an indirect and sensitive method by which individual cyanogens can be quantified in cassava roots and processed products.

In a variety of plant tissues HPLC has been applied for preparative and qualitative studies of cyanogenic glucosides (Frehner et al., 1990 and Leiberie et al., 1986). This paper reports on both qualitative and quantitative HPLC methods that have been developed by NRI to analyse directly cassava cyanogenic compounds.

MATERIALS AND METHODS

Qualitative analysis of cassava roots and leaves.

Cassava roots were supplied by Central Tuber Crops Research Institute (CTCRI), India and leaves were courtesy of the University of Bath, UK. Extracts were made using 100% cold methanol which were subsequently concentrated using air, reconstituted in water and filtered (0.45 μm Millipore filter).

Standards of linustatin, neolinustatin and lotaustralin were provided by the Botanisches Institut der Technischen Universität, Germany. Linamarin, glucose, sucrose, fructose and acetone cyanohydrin were obtained from Sigma.

The HPLC system used consisted of a Varian RI-4 refractive index (RI) detector, a Spectra-Physics SP8800 ternary HPLC pump, a 20 μl sample loop, a Hewlett-Packard 3390A integrator and an Applied Research Laboratories Ltd. oven control unit. The analytical HPLC columns were the Bio-Rad Aminex HPX-87H and HPX-87P columns (300 x 7.8 mm) in conjunction with appropriate micro-guard columns. HPLC running conditions were varied to obtain optimum separation of the component saccharides (Table 1).

Quantitative analysis of linamarin in cassava roots.

Duplicate extracts of imported low cyanide cassava were prepared using the methodology described by O'Brien *et al.* (1991) using 0.1 M orthophosphoric acid as extraction medium. The extracts were then spiked with a known amount of linamarin within a concentration range corresponding to 500-2500 mg HCN equivalent per kg on a dry weight basis. Each sample was analysed in duplicate by colourimetry (O'Brien *et al.*, 1991) and HPLC.

The Aminex HPX-87P HPLC column was used to analyse the samples which were filtered through a 0.45 μm filter prior to analysis. Maximum resolution was obtained using the following running conditions: Column temperature of 80°C; water as eluent; 0.3 ml/min flow rate. The reproducibility of retention time and detector response was ascertained by analysis of results from repeated runs of a known standard. The linearity of the detector response over a concentration range equivalent to high and low cyanide cultivars was observed by plotting of duplicate determination of linamarin standards.

RESULTS AND CONCLUSIONS

Qualitative analysis of cassava cyanogens.

Comparison of the retention times obtained for known standards (Table 1) allowed identification of all the major chromatographic peaks present in extracts of cassava roots and leaves (Figures 1 and 2). Positive identification of the linamarin peak was supported by spiking extracts with a known standard. Linustatin, neolinustatin and lotaustralin could not be positively identified although minor peaks with similar retention times were apparent. Insufficient quantities of acetone cyanohydrin were present to enable HPLC detection.

Quantitative analysis of linamarin in cassava.

A linear relationship was found between the RI detector response and the linamarin standard concentration range used (Figure 3). Extracts of bitter and sweet cassava cultivars may therefore be analysed by HPLC directly without dilution.

In determining the limits of detection, the peak height response was observed for fiminishing linamarin concentrations. The peak height response was linear down to loadings of 0.64 μg of linamarin (0.032 mg/ml or 41 mg HCN/kg dry weight), below this level a non linear correlation existed, where values of linamarin could only be an estimate of the true value. This was due to the presence of the neighbouring glucose and sucrose peaks which were maintained at a constant concentration in the model standards. The limit of detection was 8 ng (4 μg/ml or 5 mg HCN/kg dry weight) at this level the peak was no longer integrated under the conditions described.

Repeated runs of a constant linamarin concentration enabled the reproducibility of the retention time and detector response to be evaluated. Consistent retention times were observed for all chromatograms run within each batch. The detector response contributed only $\pm$ 0.2 % standard deviation (n = 6).

A strong correlation was observed between the linamarin concentration determined by colourimetry and HPLC and the actual amount present (Figure 3). The observed coefficients of determination (R-squared) were 99.95 % and 99.92 % respectively. The gradient of the slope was marginally less than one in each case. Some divergence between HPLC and colourimetry determinations observed was probably due to the need to dilute samples for colourimetric absorption linearity. Across the concentration range HPLC gave determinations closer to the actual amount present in the sample.

DISCUSSION

Linamarin, glucose, sucrose and fructose have been positively identified as the major glucosidic constituents of cassava roots and leaves. The presence of lotaustralin which has been reported as being present in approximately 2-3 % of the total cyanogenic glucoside as determined by gas chromatography (Bissett et al., 1969) was not identified. Linustatin proposed as being present for translocational purposes (Selmar et al., 1988) was also not identified. The absence of detection of these glucosides may possible be due to the lack of sensitivity/resolution in this HPLC method at micro-levels or the possibility that they were the minor peaks observed, but with slightly modified retention times compared to the standards.

The potential of the HPLC method for routine, accurate and reproducible analysis of linamarin has been demonstrated. In addition the need to use toxic chemicals which are required for a number of the colourimetric methods and are often difficult to obtain in developing countries would be eliminated. HPLC analysis can readily be automated for routine analysis and thus a rapid method for the quantification of linamarin could be developed. This is of potential interest to plant breeding programmes for screening cassava cultivars for their cyanogenic potential.

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We thank Professor G.G. Henshaw, University of Bath, UK for the supply of cassava leaves, Dr. D. Selmar, Botanisches Institut der Technischen Universität, Germany for the cyanogenic glucoside standards, linustatin, neolinustatin and lotaustralin and C. Gay for his assistance on statistical analysis of the results.

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Table 1. Retention times for glucoside standards for Aminex columns HPX-87H and HPX-87P.

Standard	Retention time (min) HPX-87H[1]	HPX-87P[2]
Linustatin	8.9	20.6
Sucrose	9.3	21.8
Neolinustatin	9.4	-
Glucose	10.9	25.8
Linamarin	11.5	24.8
Fructose	12.1	39.4
Lotaustralin	12.9	-
Acetone cyanohydrin	20.6	-

Key: HPLC running conditions: 1. Water eluent; column temperature at ambient; flow rate at 0.5 ml/min. 2. Water eluent; column temperature at 70°C; flow rate at 0.3 ml/min.

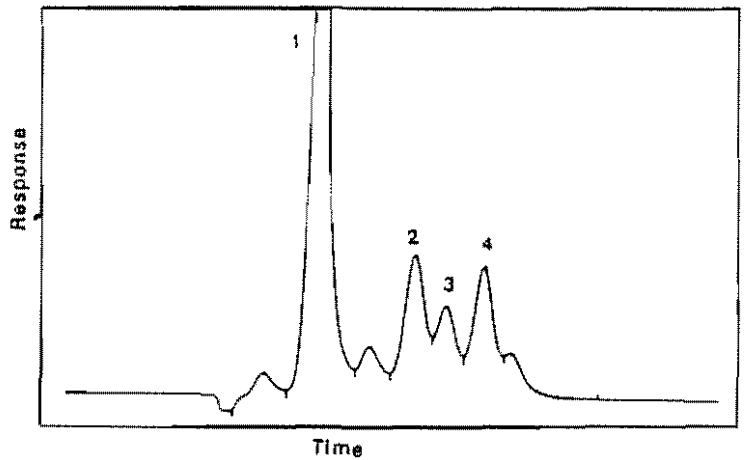

Figure 1: Carbohydrate constituents present after methanol extraction of cassava roots, using the HPX-87H column.

Key: 1 - Sucrose; 2 - glucose; 3 - linamarin; 4 - fructose.

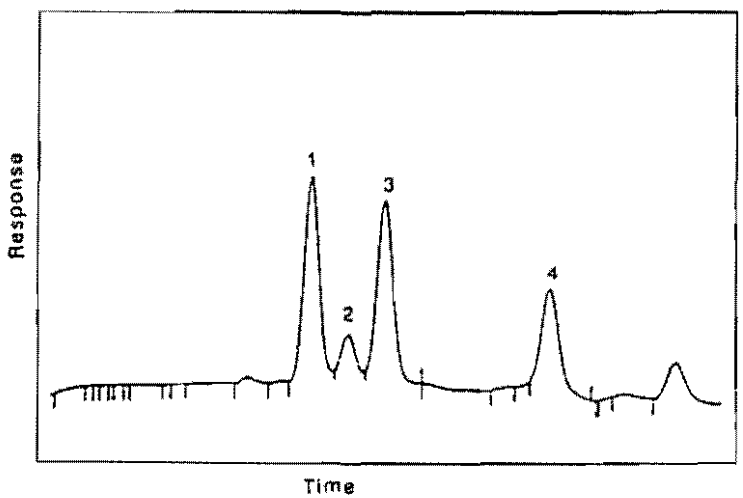

Figure 2: Carbohydrate constituents present after methanol extraction of cassava leaves, using the HPX-87P column.

Key: 1 - Sucrose; 2 - linamarin; 3 - glucose; 4 - fructose.

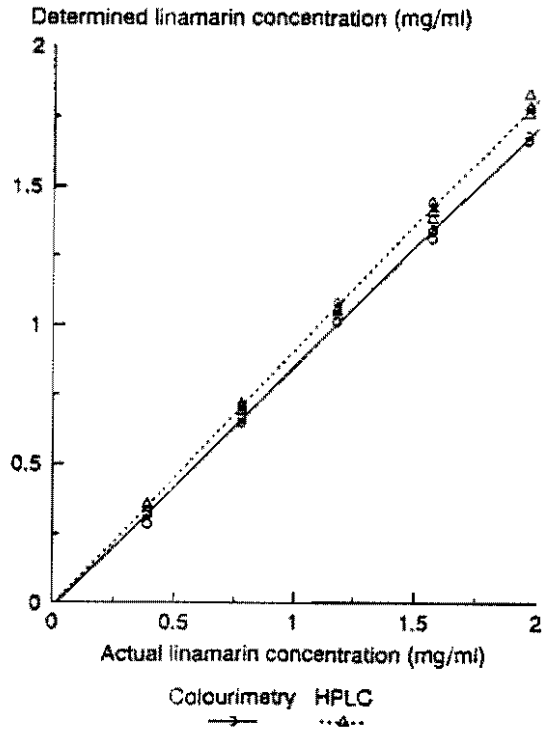

Figure 3: HPLC and colourimetric analysis of cassava extracts across a concentration range.

THE POSSIBLE ROLE OF HCN IN THE BIOLOGY AND FEEDING BEHAVIOR OF THE CASSAVA BURROWING BUG (CYRTOMENUS BERGI FROESCHNER: CYDNIDAE: HEMIPTERA).

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The burrowing bug, *C. bergi* (*Hymenopetra: Cydnidae*) was first recorded as a cassava pest in Valle, Colombia, in 1979. Nymphs and adults of this subterranean sucking insect feed on cassava roots by means of a thin, strong stylet. As it feeds, it inoculates the roots with soil-borne pathogens such as *Diplodia*, *Fusarium*, *Phytopthora* and *Phythium* spp. Brown or black lesions develop on the white, fleshy root, rendering it commercially unacceptable. Cassava root damage can reach 70 to 80% of total roots with more than 50% reduction in starch content. Additional hosts include onion, peanuts, maize, sorghum, sugar-cane, coffee, pasture grasses, potatoes and numerous weed species. Studies show that *C. bergi* develops faster on maize than on cassava, and prefers maize over cassava in free choice test (78 vs 22%). Field trials suggest resistance to *C. bergi* may be related to HCN content of the roots. In laboratory tests adults and nymphs fed on a high HCN clone had larger nymphal development, reduced adult longevity, reduced egg production and increased mortality. On CMC 40 (low HCN) nymphal mortality was 56%, while on high HCN clone MCol 1684, mortality reached 84%. On CMC 40, 50% mortality occurred at 35 days ($r^2 = 0.96$); for MCol 1684, at 28 days ($r^2 = 0.98$). Highest mortality occurred during the first two instars when nymphs primarily feed on the root peel. These results constitute one of the few documented cases where HCN content in cassava may be related to arthropod resistance.

INTRODUCTION

Cassava (**Manihot esculenta** Crantz) is attacked by a wide range of insects and mites (Bellotti and Schoonhoven 1978). The great majority of these pests are leaf and stem feeders and their damage is often indirect in that the commercial part of the plant, the swollen root, is not directly fed upon. Few pests directly feed upon the roots; termites, grubs and certain species of borers are reported as occasional root feeders.

The burrowing bug, **Cyrtomenus bergi** Froeschner, was first recorded as a pest in Caicedonia, Valle, Colombia (García & Bellotti 1980). Nymphs and adults of this subterranean sucking insect feed on cassava roots by means of a thin, strong stylet. As it feeds, the bug inoculates the roots with soil-borne pathogens such as **Aspergillus**, **Diplodia**, **Fusarioum**, **Genicularia**, **Phytophthrora** and **Pythium** spp. (Bellotti **et al.** 1988). Brown or black lesions develop on the white fleshy root 12 to 24 hours after feeding is initiated, resulting in serious reduction in commercial value.

Surveys in Colombia have revealed that onion **Allium fistulosum**), peanuts (**Arachis hypogea**), maize (**Zea maize**), sorghum (**Sorgum vulgare**), sugar cane, coffee, coriander, pasture grasses, potatoes asparagus and numerous weed species are also hosts of **C. bergi**. Yield losses in peanuts and onion are considerable and require

repeated pesticide applications for effective control, since other control measures are presently not available.

C. bergi populations are present in the soil throughout the cassava crop cycle and root damage increases with plant age (Arias & Bellotti, 1985). Damage can reach 70 to 80% of total roots with more than a 50% reduction in starch content. **C. bergi** has five nymphal instars. The life cycle lasts more than one year, and cassava roots may be the only food source exploited (García & Bellotti, 1980). Recent studies indicate that **C. bergi** develops faster on maize than on cassava (Riis, 1990) and prefers maize over cassava in free-choice feeding tests (78 vs. 22%). Oviposition was 300 times greater on maize than on cassava (105 vs 0.4 eggs/female). Oviposition was greater on onion than in cassava but less than on maize. The LD_{50} on maize was 95 days, compared to 69 on onion and 66 and 64 days respectively on sweet (CMC 40) and bitter (M Col 1684) cassava clones. Nymphal development was shortest on maize 92 days, longest on onion (119 days) and intermediate on cassava (111 days) (CIAT 1989; Riis, 1990).

MATERIAL AND METHODS

Field trials suggest that **C. bergi** feeding preferences may be related to HCN content of the root. Preference studies were done at Santander de Quilichao, Valle, Colombia employing two planting designs and three varieties, CMC 40 (low HCN content), M Mex 59 (intermediate HCN content) and M Col 1684 (high HCN content). In one design the three clone were planted in a completely randomized block so that there was no grouping of any one clone. In the second design, the three clones were planted in randomized blocks of 36 plants each with four replications.

RESULTS

Results from the first design show a high feeding preference for the low HCN clone CMC 40 (6%) over the intermediate variety M Mex 59 (46%) and the high HCN clone M Col 1684 (12.5%). Results from the second design using 36 plant block show a greater range in feeding preference. Damage to roots of CMC 40, M Mex 59 and M Col 1684 were 85%, 20% and 4.2% respectively (CIAT 1984). These results indicate that **C. bergi** is capable of distinguishing between high and low HCN content in cassava roots.

Laboratory studies showed that adults and nymphs fed on a high HCN clones. Recent studies under controlled conditions compared nymphal mortality and feeding on a low (CMC 40) and a high (M Col 1684) clone. On CMC40, nymphal mortality was 56%, and occurred during the first two instars. Nymphal mortality on the high HCN clone in two trials was 96 and 84% respectively (Table 1). All mortality occurred during the first two instars. Fifty % mortality on CMC 40 occurred at 35 days ($r^2=0.96$) and for M Col 1684 at 28 days ($r^2=0.98$). The earliest instars are most susceptible to mortality due to root feeding. Nymphal development time for the first through third instar was 43.5 days on CMC 40 and 81.7 days on M Col 1684., Measurements were made of nymphal stylet length and the thickness of cassava root peel. Results show that feeding during the first two nymphal instars is confined primarily to root peel (Table 1) (Riis, 1990). The third

though fifth instars and adults can consistently feed on the root parenchyma. Although CMC 40 as low HCN content (43 ppm) in the parenchyma, its root peel contains a high HCN level (707 ppm). The clone M Col 1684 contains higher levels of HCN in both the roots (276 ppm) and peel (627 ppm).

This support previous observations that cassava is not a preferred host for **C. bergi**. However, these results also indicate that additional or other components in the root peel may be linked to **C. bergi** clone preference and nymphal mortality.

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Mortality of *Cyrtomenus bergi* nymphs feeding on cassava roots with relation to stylet length

INSTAR	% Mortality		Stylet	Peel
	CMC-40	MCOL 1684	Average Length(mm)	Thickness (mm)
I	26	40	1.4	
II	30	42	1.9	
III	0	2	2.9	2.0-2.5
IV	0	0	3.5	
V	0	0	3.9	
Total	56	84		

CYANOGENIC GLUCOSIDES IN CASSAVA AND SORGHUM: THE BIOSYNTHETIC PATHWAY AND THE ENZYME SYSTEM INVOLVED

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The biosynthesis of the cyanogenic glucosides linamarin/lotaustralin and dhurrin has been studied *in vitro* using microsomal enzyme systems obtained from etiolated seedlings of cassava (*Manihot esculenta* Crantz) and sorghum (*Sorghum bicolor* L.). A prerequisite to obtain active preparations from cassava seedlings is the complete removal of the endosperm pellicle covering the cotyledons before seedling homogenization. The enzyme system in cassava seedlings is located in the cotyledons and petioles. The linamarin and lotaustralin produced in these tissues is actively translocated to the hypocotyl and roots. In sorghum, the enzyme system is localized in the coleoptile and primary leaf and the dhurrin produced here is not translocated to other parts of the plant.

The biosynthesis of linamarin, lotaustralin and dhurrin follows a general pathway involving the conversion of the respective parent amino acids valine, isoleucin and tyrosine to the corresponding N-hydroxyamino acid, α-nitro-carboxylic acid, *aci*-nitro compound, E-aldoxime, Z-aldoxime, nitrile and α-hydroxynitrile before final glucosylation to produce the cyanogenic glucoside. The pathway is highly channeled as demonstrated by double-labelling experiments. The substrate specificity of the enzyme system is high at the level of the amino acid but less pronounced at the level of the aldoxime and nitrile intermediates. The first N-hydroxylation step is the rate limiting reaction in the overall conversion. The biosynthetic pathway encompasses three hydroxilation reactions. The N-hydroxylation reaction resulting in the formation of the N-hydroxy amino acid and C-hydroxylation reaction producing the α-hydroxynitrile are dependent on cytochrome P-450 as demonstrated by photoreversible inhibition by carbon monoxide. The innvolvement of cytochrome P-450 is also demonstrated by inhibition of the *in vitro* biosynthetic reactions by mono-specific polyclonal antibodies raised towards NADPH-cytochrome P-450 oxidoreductase isolated from sorghum. The C-hydroxylation reaction converting P-hydroxy-phenyl-acetonitrile to p-hydroxymandelonitrile has been reconstituted in an *in vitro* system containing partially purified cytochrome P-450 from sorghum, NADPH-cytochrome

P-450 oxidoreductase and L- α-dilauroylphosphatidyl choline. The establishment of such reconstituted systems will be a necessary prerequisite for further characterization of the individual enzymes catalyzing the conversion of amino acids into cyanogenic glucosides.

INTRODUCTION

A high content of cyanogenic glucosides is found in a number of plants of which several are important food crops (21). Most important in this respect is cassava (*Manihot esculenta* Crantz) which is extensively cultivated in the tropics for its large tuberous roots, the starch content of which forms a valuable foodstuff (9, 27). Upon disruption of plant tissue containing cyanogenic glycosides, these are degraded with concomitant release of hydrogen cyanide (6). Inadequate detoxification of food products derived from cyanogenic plants constitutes a nutritional health hazard (24). Traditional breeding programs have produced low

cyanide cultivars of *e. g.* cassava and sorghum (*Sorghum bicolor* (L.) Moench) but so far it has not been possible to obtain a totally acyanogenic variety of these (9, 27). The aim of our research is to understand the physiology, biochemistry and molecular biology of cyanogenesis in cassava and sorghum. The final goal of the project is to use a molecular approach to regulate at the cellular level the key processes related to biosynthesis, transport or accumulation of cyanogenic glucosides and thereby to control the level of individual cyanogenic glucosides in specific parts of crop plants. The main applicational aspect of our work addresses cassava. Sorghum has proven to be an easier experimental plant to work with and therefore serves as our model plant.

EXPERIMENTAL RESULTS AND DISCUSSION

Cassava contains the two cyanogenic glucosides linamarin and lotaustralin in an approximate ratio of 93:7 (22). Sorghum contains a single cyanogenic glucoside, dhurrin (2). Cassava (22) and sorghum (8) seeds have a very low content of cyanogenic glucosides compared to young seedlings (12, 22) demonstrating that seed germination is accompanied by a net synthesis of cyanogenic glucosides. From etiolated seedlings of sorghum (18, 19) and cassava (16), it has been possible to isolate microsomal syntems which in the presence of the parent amino acid and NADPH catalyze the *in vitro* synthesis of the corresponding aglycons *i.e.* all but the last step in the pathway. The aglycons may be converted to the respective cyanogenic glucosides by addition of a soluble UDP-glucose glucosyltransferase present in the supernatant during the preparation of microsomes (18, 23). The microsomal preparations obtained from etiolated seedlings of sorghum are far more active compared to those from cassava (16). In sorghum, the inhibitory seed coat is easily separated from the seedlings by germination of the sorghum seeds between layers of gauze on metla screens (12). In cassava, the pellicle covering the endosperm remains attached to the primary leaves of the seedlings. The pellicle contains a strong inhibitor of the biosynthetic activity and has to be manually removed from each individual cassava seedling before an active enzyme preparation can be obtained (16). Several attempts have been made to isolate biosynthetically active microsomal preparations from green plant tissues (see *e.g.* M. Bokanga *et al.*, these proceedings). The preparations obtained have had little or no activity compared to preparations from etiolated plant material. The difference is probably due to the presence of high amounts of phenolics in green tissue, the inhibitory effect of which are not completely circumvented by the addition of polyvinylpolypyrrolidone, hydrophobic beads or reductants. Methodological aspects related to the isolation of active microsomal systems and to the analyses of intermediates have recently been reviewed (21).

The biosynthetic pathway for cyanogenic glucosides has been elucidated using the highly active microsomal enzyme system obtained from etiolated sorghum seedlings (18, 19). Biosynthetic *in vitro* systems subsequently obtained from a number of plant species have shown identical pathways. The pathway is clearly more complex than originally thought (18) and involves an *N*-hydroxyamino acid, an

α-nitrocarboxylic acid, an *aci*-nitrocompounds, and (*E*)-oxime, a (*Z*)-oxime, a nitrile and an α-hydroxynitrile as obligatory intermediates (Figure 1) (11, 13, 15, 19). Using microsomal *in vitro* systems, the substrate specificity of the enzyme system may be investigated. In sorghum, tyrosine is the only amino acid metabolized (18). In cassava, the microsomal system prefers valine and isoleucine as substrates but 2-cyclopentenylglycine is also converted to some extent (16). The conversion rates are highest with valine, the precursos for the predominant cyanogenic glucoside (linamarin) of cassava. A similar relationship between the enzymatic activity of the microsomal system towards different substrates and the *in vitro* content of cyanogenic glucosides is reported in flax (7) and white clover (5), with linamarin being predominant in flax and lotaustralin in clover. The observed ability of the cassava system to metabolize 2-cyclopentenylglycine prompted us to investigate whether cassava contains cyclopentenyl-type cyanogenic glucosides not hitherto reported. The cyanogenic glucoside content of cassava seedlings was analyzed by HPLC analysis using authentic reference compounds (Figure 2). The only fractions from which cyanide was liberated upon treatment with snail glucoronidase were those corresponding to linamarin and lotaustralin. The observed metabolism of 2-cyclopentenylglycine by the *in vitro* system most likely reflects its structural similarity to valine and isoleucine. The specific activity of the cassava microsomal system is 5 to 10% of the activity obtained with the sorghum system when measured using amino acids as substrates (16). In both systems, the conversion of the parent amino acid to the *N*-hydroxyamino acid is the rate limiting step (16). The substrate specificity of the microsomal systems is less pronounced when subsequent intermediates like oximes are tested (16).

The pathway resulting in the biosynthesis of cyanogenic glucosides is highly channelled as demonstrated in biosynthetic experiments using the sorghum microsomal system and simultaneous administration of ^{3}H- and ^{14}C-labelled intermediates (20). Additional support for the channelling hypothesis has recently been obtained by the almost quantitative incorporation of $^{18}O_2$ into the oxime using the *N*-hydroxyamino acid as substrate (11). This precludes the possibility of free rotation around the C-N bond of the α-nitrocarboxylate ion (Figure 1). An equilibrium between the nitro compound with free rotation around the C-N bond and the *aci*-nitro tautomer during the enzymatical conversion to the oxime is similarly precluded.

Partial purification of the sorghum microsomal system has been obtained using sucrose gradient centrifugation and gel filtration chromatography (12). Although the polypeptide profile of the purified fractions is clearly much simpler than that of the microsomal system, the specific activity of the preparation was not increased due to a partial loss of specific components necessary to retain biosynthetic activity. The pathway involves two N-hydroxylation steps and a single C-hycroxilation step (13). At least two of these hydroxylations are catalyzed by cytochrome P450 as demonstrated by carbon monoxide inhibition and reversal of the inhibition by 450 nm light (11, 14). Cytochrome P450 dependent monooxygenases are small electron transport chains consisting of a flavin-containing NADPH-cytochrome P450 oxidoreductase transferring electrons from NADPH to the cytochrome P450 which

is responsible for substrate binding and O_2 activation (10). Purification of each of these components requires initial detergent solubilization of the microsomal system. The NADPH-cytochrome P450 oxidoreductase has been isolated from sorghum after solubilization with Renex and chromatography using ion-exchange anf affinity columns (14). Partial amino acid sequences have been obtained and used for the design of oligonucleotide probes. Using these and a monospecific antibody raised against the isolated sorghum protein, partial cDNA clones have been isolated and characterized. The antibody towards NADPH-cytochrome P450 oxidoreductase isolated from sorghum cross-reacts with the NADPH-cytochrome P450 oxidoreductase of cassava as demonstrated by western blotting (4). This demonstrates the feasibility of using antibodies raised towards purified sorghum proteins in the identification of the corresponding proteins in cassava. In sorghum, the rate of tyrosine conversion obtained in the presence of NADPH is increased 15% when NADH is added (18). This indicates that an additional electron transport chain consisting of cytochrome b_5 and NADH-cytochrome b_5 oxidoreductase is involved in electron donation to cytochrome P450. Cytochrome b_5 and NADH-cytochrome b_5 oxidoreductase have been isolated from sorghum (26). When the two isolated proteins were combined, a reconstituted electron transport system catalyzing electron transport from NADH to cytochrome c was obtained. A partial purification of cytochrome P450 from from sorghum has also been achieved (26).

Physiological studies concerning sites of cyanogenic glucoside synthesis and accumulation as well as cyanogenic glucoside transport have also been carried out. In etiolated sorghum seedlings, dhurrin and the dhurrin-synthesizing enzyme system are primarily located in the seedling tip and evenly distributed between the coleoptile, primary leaves and the upper part of the hypocotyl (12). In etiolated cassava seedlings, the enzyme system responsible for linamarin and lotaustralin synthesis is found only in the cotyledons and their petioles. Linamarin and lotaustralin are present in all tissues with the highest amounts found in hypocotyl and roots (16). This demonstrates hat in cassava, cyanogenic glucosides are synthesized in the cotyledons and transported to other tissues, primarily the root. A transport of cyanogenic glucosides from the green leaves of the mature cassava plant to the root tubers has been observed by ringing the stem of adult cassava plants and demonstration of cyanogenic glucosides accumulation in the cortex above the incision (17). Etiolated cassava seedlings may therefore constitute an efficient experimental system for studying cyanogenic glucoside transport. Mobilization and transport of cyanogenic glucosides from one tissue to another has also been demonstrated in *Hevea brasiliensis* (25). In this plant, mobilization and transport of linamarin involves its prior conversion to a diglucoside, linustatin (25). Most likely, a similar linustatin pathway is operating in cassava. Biosynthetic incorporation studies using ^{14}C-valine may permit the detection of low amounts of linustatin not detectable by the HPLC method (Figure 2). The parallel distribution of the enzyme system and dhurrin in sorghum indicates that dhurrin accumulates in the same cells in which it is synthesized. Dhurrin synthesis in sorghum has been shown to occur in both light and dark photoperiods and the rate of synthesis was found to be faster in the dark (1). Although dhurrin appears to accumulate at its site of synthesis, the

accumulated dhurrin is subject to continuous turn-over with the break-down rate amounting to approx. 30% of the synthetic capacity (1). Evidence for cyanogenic glucoside turn-over in cassava has also been presented (3). From the turn-over studies it is apparent that accumulation of cyanogenic glucosides is not a static process. Among other purposes, the stored cyanogenic glucosides may serve as a rapid source for provision of carbon and nitrogen.

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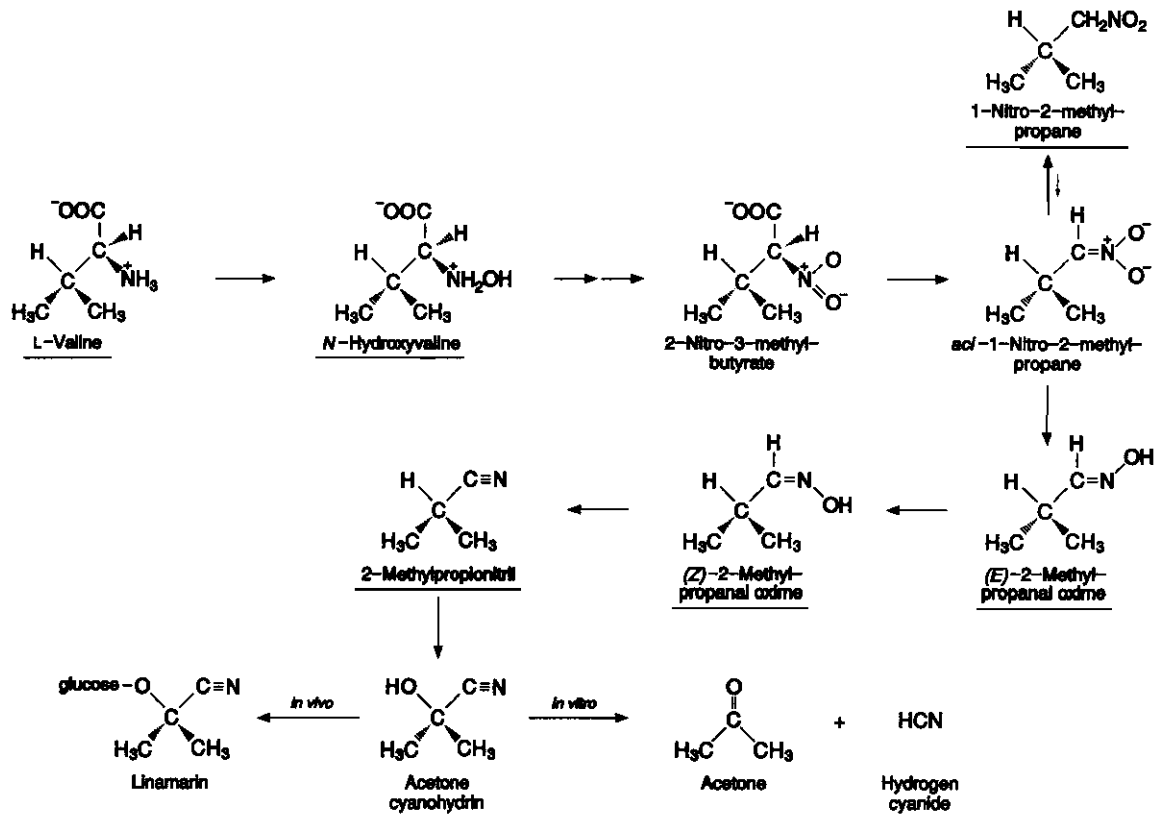

Figure 1: The biosynthetic pathway for the valine-derived cyanogenic glucoside linamarin in cassava (*M. esculenta* Crantz)

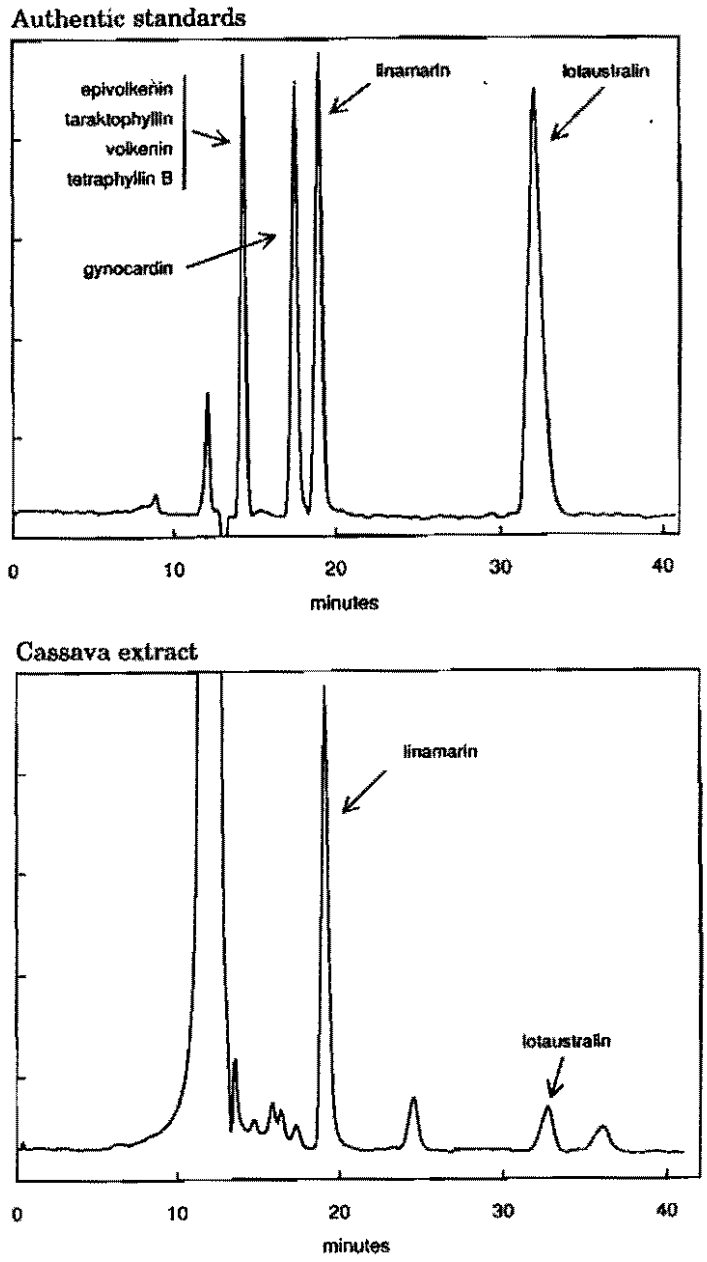

Figure 2: Cyanogenic glucoside content of cassava (*M. esculenta* Crantz) seedlings. The plant material was extracted with boiling 80% MeOH and the cyanogenic glucosides separated by HPLC (column: Lichrosorb RP18; elution: 20% MeOH (isocratic), size: 250 mm x 16 mm i.d.) coupled to a refractive index detector. Identification of cyanogenic glucoside is based on (1) co-elution with authentic standards, (2) mass spectrometry and (3) requirement for cyanide release upon treatment with snail glucoronidase.

STUDIES ON THE BIOSYNTHESIS OF CYANOGENIC GLUCOSIDES IN CASSAVA

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The biosynthesis of cyanogenic glucosides in cassava has been investigated in vitro using a microsomal system extracted from young etiolated seedlings of 3 cassava varieties. The rate of conversion of L-valine to acetone cyanohydrin, the immediate precursor of linamarin, was 1.16, 1.15 and 0.17 nmol HCN/(μg protein x hr) for TMS 30555, TMS 4(2) 1425 and 82/00058 respectively. The biosynthetic activity did not correlate with the cyanogenic potential of these varieties. Attempts to demonstrate in vitro the biosynthetic activity in young leaves from mature plants were not successful. The biosynthetic activity was demonstrated in 4-day old green sorghum seedlings using tyrosine as the substrate to produce p-hydroxybenzaldehyde cyanohydrin. With older seedlings (8 days), the biosynthetic activity could not be demonstrated.

The endosperm pellicle covering the cotyledons of the cassava seedling contains an inhibitor of the biosynthetic activity. The inhibitor precipitates with the microsomal pellet, is heat resistant, is not removed by dialysis and not extracted by diethylether. The cassava inhibitor also inhibits the sorghum enzyme system. Polyclonal antibodies raised against sorghum NADPH-cytochrome P450 oxidoreductase, the enzyme responsible for supplying electrons in the hydroxylation reactions of the biosynthetic pathway, cross-react with the cassava enzyme on a western blot, indicating that the two enzyme systems may have a high degree of homology. The cassava enzyme has a lower slightly molecular mass compared to the sorghum enzyme.

INTRODUCTION

Cassava *(Manihot esculenta* Crantz) is one of over 2000 plant species known to synthesize cyanogenic glucosides (Poulton, 1988). In all those species, only five hydrophobic amino acids (L-valine, L-isoleucine, L-leucine, L-phenylalanine and Tyrosine) and a single non-protein amino acid (cyclopentenylglycine) serve as primary precursors for cyanogenic glucosides (Halkier et al., 1988). Cassava uses L-valine and L-isoleucine to produce linamarin and lotaustralin, respectively (Nartey, 1969). These two compounds accumulate in all parts of the cassava plant, particularly in the roots and leaves which form an important part of the diets of over 500 million people in the tropical world (Cock, 1985).

The presence of cyanogenic glucosides in cassava is a serious health hazard, particularly in some parts of Africa where cassava is the main staple and when supplementary foods are in short supply (Rosling, 1987). One of the priorities of the plant breeding program at the International Institute of Tropical Agriculture (IITA) is the development of cassava varieties with a reduced or lacking ability to produce cyanogenic glucosides. Up until now screening for low cyanogenic potential is conducted at harvest by determining the concentration of cyanogenic glucosides in the roots. A new strategy consists of searching for varieties with a low biosynthetic

capacity for cyanogenic glucosides. These varieties may then be crossed in a conventional breeding program to produce offsprings with an even more reduced capacity to synthesize cyanogenic glucosides.

The biosynthetic pathway of cyanogenic glucosides in etiolated cassava seedlings has recently been elucidated (Koch et al., 1992). The methodology for obtaining a microsomal fraction capable of converting L-valine to acetone cyanohydrin, the immediate precursor of linamarin is now available. The endosperm covering the cotyledons of germinating cassava seedlings has a strong inhibitory effect on the biosynthetic activity of the microsomal fraction.

In this study, we have investigated the biosynthetic activity of microsomal fractions of etiolated seedlings from 3 different varieties and in green tissues. Attempts have been made to determine the chemical nature of the inhibitor of the biosynthetic activity, and to show the similarity between the sorghum *(Sorghum bicolor* Moench) and cassava biosynthetic enzymes.

MATERIAL AND METHODS

Plant material. Cassava seeds were obtained from IITA in Ibadan, Nigeria. They were from open-pollinated female plants of three breeding lines TMS 30555, TMS 4(2)1425 and TMS 81/00058. The seeds were germinated in the dark as previously described (Koch et al., 1992). Young green cassava leaves were obtained from mature plants of unknown variety growing in the glasshouse of the Plant Biology Institute of the Royal Veterinary and Agricultural University, Copenhagen, Denmark. Sorghum seeds were obtained from Seedtec International Inc. (Hereford, Texas) and were germinated as previously described (Halkier and Moller, 1989).

Preparation of the biosynthetic microsomal enzyme system. Sixteen seedlings from each cassava clone were used to prepare the microsomal fraction as previously described (Koch et al., 1992). The biosynthetic activity was determined as HCN production as described (Halkier and Möller, 1989) using L-valine as the substrate for the cassava system and L-tyrosine for the sorghum system.

Preparation of the cassava endosperm inhibitor. The cassava endosperm inhibitor was present in microsomal preparations obtained from cassava endosperm following the procedure for obtaining the microsomal enzyme system. The inhibition-containing fraction was called cassava endosperm microsomal fraction (CEMF). The supernatant after precipitating the microsomes was dialyzed in 50 mM Tricine buffer (pH 7.9) under nitrogen atmosphere and was called cassava endosperm supernatant fraction (CESF).

The inhibition assay was carried out by substituting 10 μL of the buffer in the activity assay with 10 μL of either one of the endosperm preparations.

SDS-PAGE and western blotting. Analytical SDS-PAGE was performed using 8 to 25% linear gradient gels prepared as described by Fling and Gregerson (1986). After separation by electrophoresis, the protein of the SDS-solubilized microsomes were transferred onto nitrocellulose paper and probed with anti-(sorghum NADPH-Cytochrome P-450 oxidoreductase) antibodies. The antibodies fixed on the nitrocellulose paper were revealed by swine to rabbit antibodies linked to alkaline phosphatase which was reacted with 5-bromo-4-chloro-3-indolylphosphate.

RESULTS AND DISCUSSION

The biosynthetic activity of etiolated seedlings from 3 cassava varieties and from sorghum is indicated in Table 1. The cyanogenic potential of the three parent cassava varieties in the 1990-91 season is also indicated. The seedlings obtained from TMS 81/00058, a variety which had the highest cyanogenic potential showed the lowest biosynthetic activity. However, a larger number of varieties need to be screened before definite conclusions about a correlation between the cyanogenic potential and the biosynthetic activity can be drawn. Moreover, since the seeds were obtained by open pollination, only half the genome of the seedlings is similar to the genome of their respective parent variety.

The biosynthetic activity of the microsomal fraction from etiolated seedlings is much higher in sorghum than in cassava (Table 1). This is in agreement with previous observations (Moller and Halkier, personal communication).

Previous attempts to obtain *in vitro* biosynthetic activity from green tissues were not successful (Kojima et al., 1979). We have succeeded in isolating a biosynthetically level of activity that we obtain from the green seedlings are substantially lower than the activity we obtain from the etiolated seedlings (Table 2). This difference in activity might reflect the difference in levels of detrimental phenolics in green and etiolated seedlings rather than actually difference in biosynthetic activity. An active preparation could not be obtained from 8-day old seedlings or from very young cassava leaves from mature plants. The endosperm covering the cotyledons of the germinating cassava seedling contains an inhibitor of the biosynthetic activity (Koch et al., 1992). The inhibitor from the cassava endosperm is shown to be heat-stable, strongly bound to the membrane fraction, not extracted by a non-polar solvent such as diethylether, and is absent in the cell soluble fraction.

The data also show that the cassava endosperm inhibitor inhibits the sorghum enzyme system (Table 3). The similarity between the enzyme system from the two species is further demonstrated by a western blot of microsomal fractions of cassava and sorghum (Fig. 1) using polyclonal antibodies raised against purified NADPH-Cytochrome P450 oxidoreductase from sorghum. Both the cassava and sorghum fractions reacted with the antibodies. The electrophoretic migration of the bands recognized by the NADPHCytochrome P450 oxidoreductase antibodies indicate that the cassava reductase has a slightly lower molecular mass compared to the sorghum reductase (Fig. 2). The sensitivity to the same inhibitor and to the

same antibody is a good indication of some degree of homology in the enzymes from the two species, and suggests that findings made on the sorghum enzyme system will be applicable to the cassava system.

CONCLUSION

We have for the first time demonstrated in vitro biosynthesis of cyanogenic glucosides in green plant tissue. The tissue was derived from light-grown sorghum seedlings. The level of biosynthetic activity obtained from the light-grown sorghum seedlings was substantially lower than the levels found in etiolated seedlings. Low levels of biosynthetic activity were demonstrated in etiolated seedlings of different varieties of cassava. No measurable activity could be obtained from young leaves of mature cassava plants.

An inhibitor of the biosynthetic activity in both sorghum and cassava was found in the microsomal fraction of the endosperm of etiolated cassava seedlings. The nature of the inhibitor is unknown. NADPH-cytochrome P450-reductase from cassava is recognized by sorghum reductase antibodies and has a molecular mass slightly lower than the sorghum reductase. Sensitivity to the same inhibitor and antiserum indicates a good homology between the enzyme systems from sorghum and cassava, suggesting that advances made in the study of the sorghum system are applicable to the cassava system.

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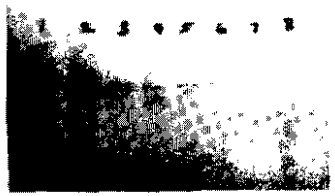
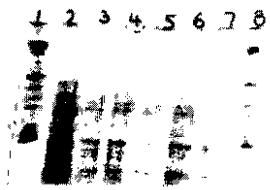


Western blot of NADPH-cytochrome P450 oxidoreductase (**rabbit** antibodies to the sorghum enzyme, revealed by **swine-to-rabbit** antibodies coupled to alkaline phosphatase)

Lane 1: Protein standards
2: Etiolated sorghum microsomes (partially purified)
3: Etiolated cassava microsomes (TMS 30555)
4: Etiolated cassava microsomes (TMS 4(2)1425)
5: Etiolated cassava microsomes (TMS 81 00058)
6: Etiolated cassava microsomes (from mixed seeds)
7: Green sorghum microsomes (active preparation)
8: Protein standards

Fig. 1



SDS-PAGE of cassava and sorghum microsomal fractions.

Lane 1: Protein standards
2: Etiolated sorghum microsomes (partially purified)
3: Etiolated cassava microsomes (TMS 30555)
4: Etiolated cassava microsomes (TMS 4(2)1425)
5: Etiolated cassava microsomes (TMS 81/00058)
6: Etiolated cassava microsomes (from mixed seeds)
7: Green sorghum microsomes (active preparation)
8: Protein standards

Fig. 2

CYANOGENESIS IN CASSAVA

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The distribution pattern of linamarin and its major catabolic enzymes - linamarase, hydroxinytrilelyase (HNL) and B-cyanoalanine synthase (B-CAS) was studied in cassava tissues. In all cultivars studied higher accumulation of linamarin was observed in leaves, stem and rind tissues as compared to tuber. All three enzymes were present in cassava tissues, indicating active turnover of linamarin in the plant.

The distribution of linamarin in tissues was found to vary in different growth stages. Studies on the metabolism of linamarin in early growth phases showed that during germination, almost all the linamarin present in stem was utilized. The levels remained unchanged initially till roots and leaves developed and then decreased rapidly. Partial translocation of linamarin to roots was observed. However, the presence of linamarase, HNL and B-CAS activity at all stages suggests partial mobilization and utilization of linamarin as noncyanogenic compounds. Total HCN potential decreased initially but later increased due to subsequent biosynthesis in leaves. Detailed studies on cassava linamarase indicate the role of certain inhibitors, which could prevent linamarase action, thereby facilitating transport of linamarin. These studies would throw light on further understanding the metabolism and mechanism of transport of linamarin in the plant, which is necessary for effective control of cyanogenesis in cassava.

INTRODUCTION

Linamarin is the major cyanogenic glucoside present in cassava, and is distributed in the plant tissues in varying concentrations. The presence of linamarin hydrolysing enzymes along with cyanide detoxifying enzymes in cassava tissues suggests that linamarin is metabolised in the plant and utilized by converison into non-cyanogenic compounts (1). It is not clear how the linamarin level in tuber is related to its level in other tissues, and whether translocation of linamarin from other tissues to the tuber takes place. Studies on the biosynthesis, tissue distribution, degradation and transport of linamarin in cassava would help in understanding its physiological role in the plant and in devising methods for its control.

EXPERIMENTAL METHODS AND RESULTS

1. Cyanogenic potential of tissues in high and low cyanide cassava varieties

The distribution of linamarin and the activity of its hydrolysing enzymes linamarase and acetone cyanohydrin nitrilase were studied in four cassava cultivars grown under uniform fertility conditions and normal climatic environment. Linamarin content was determined by the method of Nambisan and Sundaresan (2) Linamarase activity was assayed by the method of Sorbo (4) ß-Cyanoalanine synthase (ß-CASa) as given in Miller and Conn (5) acetone cyanohydrin lyase (HNL) by the method of Selmar et al (6).

Tubers of low cyanide varieties M4 and H 1687 contained <150 µg CN/g dry weight while tubers of high cyanide varieties H 165 and H 226 contained >450 µg CN/g. Leaf, rind and stem tissues in all varieties had much higher linamarin levels than the tubers. The pattern of distribution of linamarin in different tissues was similar in high and low cyanide varieties. There seemed to be no direct relation between the content in tuber and that of other tissues.

The linamarase activity was very high in stem, leaf and rind tissue as compared to that in tuber (Table 1a). A similar pattern of tissue distribution was observed in high and low cyanide varieties. Comparison of linamarase activity in tubers showed that activity was higher in the high cyanide cultivars H.165 and H.226. In general, tissues with high linamarin content also had high activity.

Cyanohydrin lyase activity was detected in all tissues (Table 1b). Highest activity was observed in leaf tissue. In stem and rind tissues activity was much lower, and comparable with that of tuber. The tissue distribution pattern appeared to be different from that of linamarase, the significance of this difference is not clear.

2. Cyanide tetoxifying enzymes in cassava tissues

Rhodanese was detected only in leaf tissue. Mature leaves contained higher activity than young and old leaves. No activity could be detected in the other tissues in any of the varieties.

The enzyme ß-CAS was present in all tissues (Table 2) Activity in leaf, stem and rind tissues was comparable, while higher activity was observed in tubers. A similar pattern was seen in high and low cyanide cultivars.

The presence of the enzymes linamarase, HNL and ß-CAS suggests that linamarin is probably degraded in cassava tissues and utilized. This is particularly important in tuber tissues, where HNL and ß-CAS appear to be high.

3. Changes in linamarin content in cassava tissues at different growth stages

The pattern of distribution of linamarin in tubers and leaves of high and low cyanide varieties was studied at various stages of plant growth.

Results indicated that accumulation of cyanoglucoside was initiated in roots as early as the first month stage. Maximum cyanide potential in tubers was observed around the third month stage. Decline in cyanide levels was associated with the active tuber bulking phase and levels were minimal at maturity when dry matter accumulation was maximum. Dry periods caused increase in tuber cyanoglucoside content. The increase was associated with accelerated leaf senescence and leaf fall during this period, which probably increased translocation. Linamarin content in mature leaves remained fairly constant during the active growth phase but decreased

during the dry period. Changes observed in the pattern of accumulation of linamarin in tubers during the different growth stages was similar in high and low cyanide cultivars.

4. Accumulation of linamarin in cassava stems during girdling

The accumulation of linamarin in cassava stems due to girdling or ringing of stem bark has been reported by Bruijn (7) and Ramanujam and Indira (8). Girdling experiments were done in plants of high and low cyanide cultivars (viz. H 226 and M4). Stem bark of width 1 cm was removed as a complete ring from the stem at a distance of about 30 cm from the base level. The linamarin levels in the stem bark above the girdled region was monitored at different intervals after girdling.

High and rapid accumulation of linamarin took place in the stem tissue just above the girdled region. The rate of increase was highest during the first two days. Subsequently there was slow steady increase and after 1 week the linamarin levels were 5-6 fold higher than the original content. The extent and rate of increase in accumulation of linamarin was comparable in both high and low cyanide cultivars. The results suggest that translocation of linamarin is possible. But a similar high accumulation of linamarin is not observed in tuber tissue which implies that there must be a differential transport between rind and tuber or an efficient metabolising mechanism in tuber which prevents high accumulation of any transported linamarin.

5. Mobilisation of linamarin in cassava stems during germination

The metabolism of linamarin in stems during the process of germination was investigated. Uniform stem cuttings (M4) were planted in pots and allowed to germinate in growth chamber under controlled conditions. The linamarin content in stems did not change during the early stages of germination but later decreased rapidly, reaching about 15% of the original content at the twentieth day after the onset of germination. The total linamarin content of the germinating stem decreased by about 50% after 15 days and then increased after 30 days following root and shoot growth. The initial decrease in linamarin levels in stem tissue corresponded with an increase in root tissue, suggesting that translocation of linamarin to the newly formed tissue may be possible. The linamarin metabolising enzymes - linamarase, HNL and ß-CAS are present in stem tissue at all stages of germination indicating that mobilisation of linamarin and its utilisation by conversion into asparagine is also possible.

CONCLUSIONS

Linamarin and its metabolising enzymes linamarase, acetonecyanohydrin lyase and ß-Cyanoalanine synthase are present in all tissues of cassava plant.

The rapid and high accumulation of linamarin in girdled stems indicates the possible transport of linamarin. However, a similar high accumulation of linamarin is

not observed in tubers of low cyanide or high cyanide varieties, suggesting the operation of other control mechanisms in tuber itself. Studies on the mobilisation of linamarin in stems during germination further indicates that linamarin is utilised during this process as well as translocated to newly formed root tissue. The presence of linamarin degrading enzymes in all tissues necessitates the operation of suitable mechanisms in cassava which can protect linamarin from being degraded during transport.

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Table 1a. Linamarase activity in tissues.

Variety	Linamarase (μg CN/min/100 mg protein)					
	Young leaf	Mature leaf	Old leaf	Stem	Rind	Tuber
M4	88.2	50	45.2	200	281	5.3
H-1687	76.5	43.8	40.9	178	111	7.5
H-165	79.5	45.2	39.6	155	126	14.6
H-226	84.5	50.1	36.5	196	394	15.7

Table 1b. Acetone cyanohydrin lyase activity in tissues.

Variety	Acetone cyanohydrin lyase (μg CN/min/100 mg dry wt.)			
	Leaf	Stem	Rind	Tuber
M4	237	17.8	17.8	10.6
H-226	147.6	16.6	28.3	15.6

Table 2. Cyanide detoxifying enzymes in tissues of cassava varieties.

Variety	Rhodanese μg SCN/min/100 mg protein	B-CAS μg H_2S/min/100 mg protein			
	Mature leaf	Mature leaf	Stem	Rind	Tuber
M4	71.0	24.2	21.7	20.1	57.2
H-1687	75.1	24.1	28.4	19.0	50.7
H-165	82.0	24.5	22.2	22.5	55.1
H-226	80.0	23.5	29.2	21.5	60.1

LEAF CYANOGENIC COMPOUNDS IN INNOCUOUS AND TOXIC CASSAVA

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In cassava, the leaves and tubers contain linamarase, beta-glucosidase which catalyses the hydrolysis of linamarin, which result in the release of hydrogen cyanide. Previous authors reported no differences between linamarin content and linamarase activity in leaves of high and low cyanide varieties. Linamarase activity and linamarin content were determined as a function of the age of the leaves in four different cultivars. In previous measurements of HCN in roots parenchyma, the values obtained were: 9, 33, 69 and 115 μg HCN/g of parenchyma for cultivars 06, 37, 08 and 32. Linamarase partially purified was obtained by acetone precipitation. The linamarase activity and linamarin content were determined according to the method used by Eksittikul and Chulavatnatol. Results were presented in Table 1.

Significant reduction in linamarase activity of old leaves (14-17 weeks old), was observed in cultivars with innocuous HCN contents in their roots, but not in the toxic cultivars. Furthermore, the linamarin content is higher in young fully expanded leaves (2 weeks old) in the 4 cultivars studies. These results suggest differences in translocation of linamarase from leaf to root, being higher in innocuous than in toxic cassava cultivars. This difference is not observed in the substrate measured.

The correlations between the studied parameters are shown in Figs. 1, 2, and 3. As previously reported (Mkpong et al, cited) the linamarin content and linamarase activity in young fully expanded leaves (2 weeks old) are independent of the cyanide content of the root parenchyma. It seems that synthesis of enzyme and substrate are similar in sweet and bitter cassava. No change in substrate concentration of leaves was observed as a function of time. The significant correlation between the leaf linamarase activity and root HCN concentration shows differences in translocation or inactivation of linamarase from leaves in cultivars with different HCN content in the root parenchyma, being higher in innocuous than in toxic cassava cultivars.

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Table 1. Linamarase activity and HCN content in young and old cassava leaves.

CULTIVARS	YOUNG LEAVES (1-2 WEEKS)		OLD LEAVES (14-17 WEEKS)	
	LINAMARASE $\frac{U \times 10^3}{min\ g\ fw}$	HCN $\frac{\mu g}{g\ fw}$	LINAMARASE $\frac{U \times 10^3}{min\ g\ fw}$	HCN $\frac{\mu g}{g\ fw}$
06	118.0	31.0	7.1	19.3
39	22.1	8.2	5.4	7.6
37	35.0	37.7	9.0	19.1
08	38.0	37.3	39.8	29.8
32	43.0	64.5	40.3	13.4
01	16.7	12.8	14.5	15.3

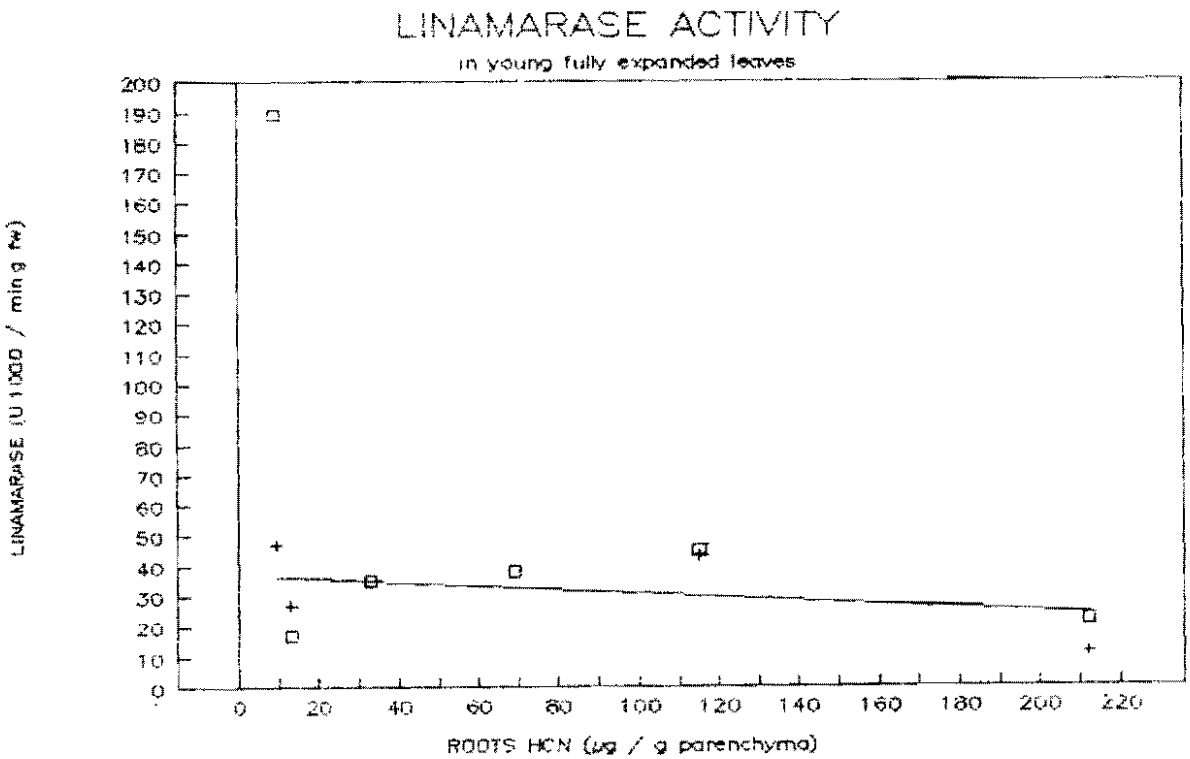

Figure 1. Presents the linamarase activity in young fully expanded leaves (1-2 weeks) against the HCN contents in roots of the six cultivars analysed.

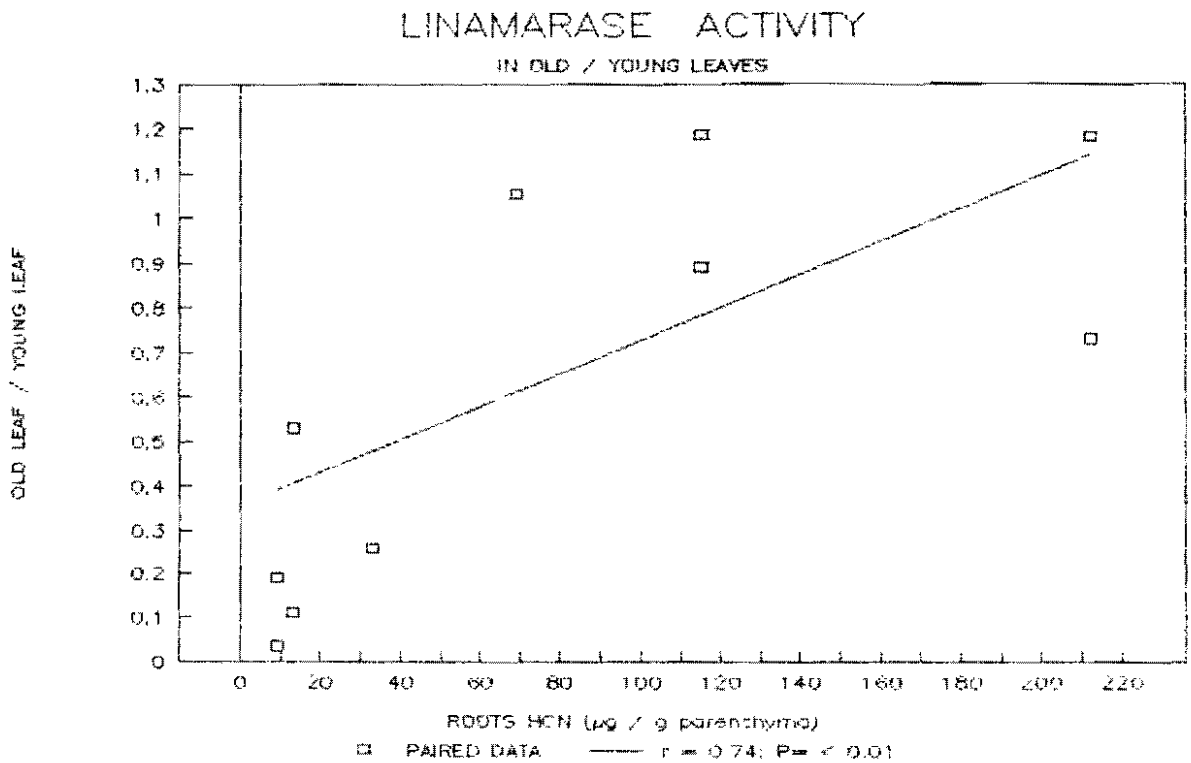

Figure 2. Presents the relationship of linamarase activity between old (14-17 weeks) and young (1-2 weeks) leaves against the HCN contents in roots of the six cultivars analysed.

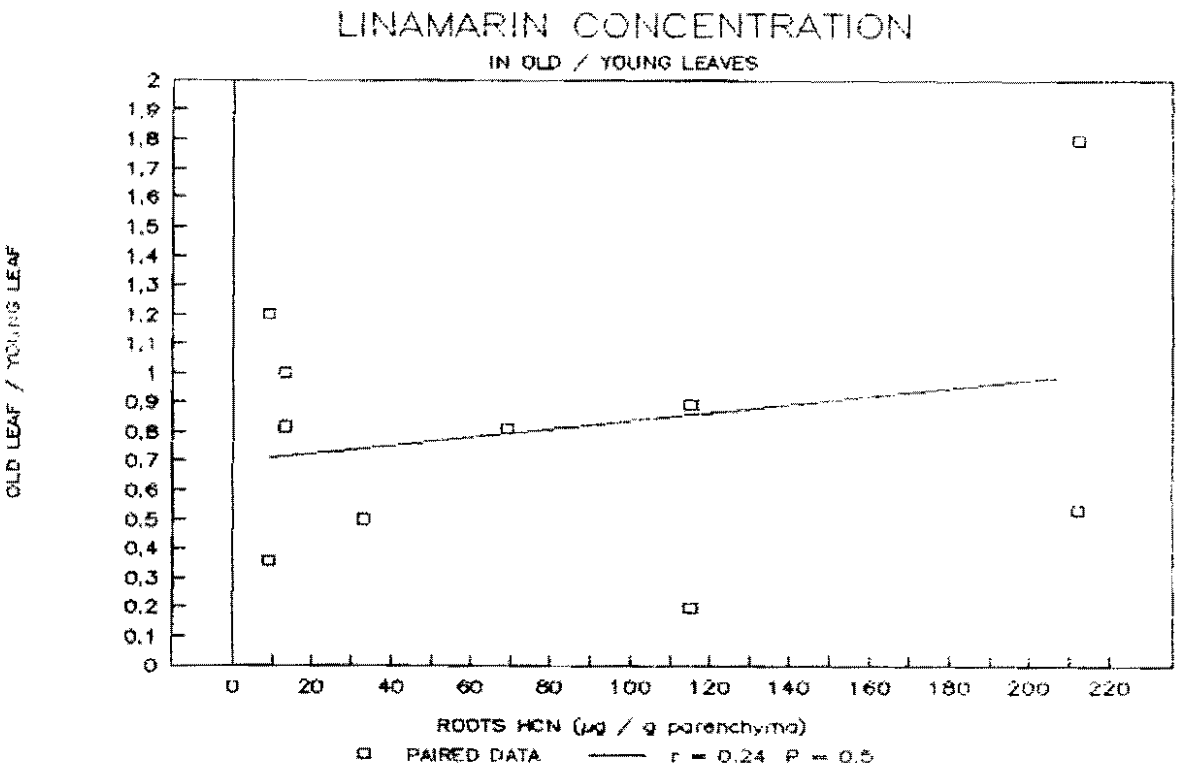

Figure 3. Presents the relationship of linamarin concentration between old and young leaves against the HCN contents in roots of the six cultivars analysed.

EFFECT OF RAINFALL ON HCN CONTENT IN CASSAVA ROOTS

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Environmental conditions can modify the cyanide content in cassava roots. A given cultivar with low cyanide could result in an increased HCN content under different environmental conditions (Dominguez *et al*, 1979). Regulation of HCN content by selecting climatic conditions could be used according to the end product desired. Two plant groups of the same cultivar were used to analyze the effect of rainfall distribution during plant cycle on the HCN content. Plants were produced *in vitro*, kept in soil for one month under controlled conditions in green houses and subsequently transferred to the field. After eight months in the field, the roots were harvested and cyanide content were evaluated (Ikediobi *et al*, 1980). In Bolivar State, the rain starts in March, reaches a maximum in July and decreases until December, accumulating a total of 67.2 inches. The first group was transferred to the field in April and harvested in January. The second was transferred in June and harvested at the end of February. A significant higher value of HCN content (174 ± 18 μ HCN/g fresh parenchyma) was obtained in plants harvested after two months of drought as compared to those harvested in the middle of January (42 ± 5 μg HCN/g fresh parenchyma). A second comparison of the effect of total rainfall was performed with other cultivar using the location described above and a different location in Cojedes State. In Cojedes the rainfall starts in March, has two maxima in July-August and October and gradually decrease until January, accumulating a total of 42.4 inches. When rains last longer, no significant effect of total rainfall on the HCN content was observed in the cultivars studied. In Bolivar State the HCN content was 69 ± 15 μ/g fresh parenchyma with 67.2 inches of rain while in Cojedes State was 61 ± 8 μ/g fresh parenchyma with 42.4 inches. These results suggest that HCN content depends more on the lasting humidity in the soil during the plant cycle than on the total rainfall. On these bases it is possible to decide the date when the plants must be transferred to field, according to the last use of the roots to be produced. This work was partially supported by CAF.

Since 1985, several field tests have been performed at diverse adapho-climatic regions of Venezuela using promissory cassava cultivars (Villegas and Bravato, 1988). In addition to root yields, starch content and number of stakes, the HCN content of roots parenchyma was evaluated in each assay. Table 1 shows the locations where the adaptation tests were carried out.

In vitro plants propagated in the laboratory were used in the tests. Plants were adapted under greenhouse conditions and subsequently transferred to the field.

The plants were transferred from the test tubes to fiber pots with a soil mixture which had been chemically disinfected. The pots were placed under a translucid chamber to keep a high relative humidity during 5 days. The chamber was removed gradually leaving the plants exposed to the greenhouse conditions. Greenhouse conditions were as follows: 35°C temperature during the day and 18°C at night, about 60% of the outside light intensity and over 50% of relative humidity. During this period fertilizer was applied and phytosanitary control was performed.

Each field test was done consistently following the same design consisting of doing hazard blocks with at least two replicas per cultivar. The selected soil was prepared with one plowing and two rakings. One foot high ridges were made with 3 feet of separation between them. The vegetal material was planted on the ridges, 3 feet apart from each other, reaching a density of 10,000 plants/Ha. The plants were harvested after 8 months in the field. An enzymatic assay for total cyanide content in the roots parenchyma was performed (Ikediobi et al, 1980): Partially purified cassava root linamarase was prepared. The root cortex was extracted in acetate buffer, filtered and precipitated with acetone. The precipitate was extracted in acetate buffer and dialyzed against buffer to remove the remaining linamarin. Activity was assayed for activity using linamarin as a substrate. The linamarin was obtained from Sigma. The parenchyma tissue was homogenized in HCl, filtered and centrifuged in order to measure linamarin content in the roots. The supernatant extract was incubated with the linamarase preparation in a phosphate buffer of pH 6.8. Alkaline picrate was added and the resulting solution was incubated at 95°C. Upon cooling to room temperature, the absorbance was read at 490 nm and compared against and standard curve for cyanide estimation. The cyanide concentration was expressed in μg cN-/g fresh weight.

The HCN contents in roots of the four groups of plants studies is shown in Table 2.

Two plant groups of the same cultivar, planted in the same year, were used to analyze the effect of rain distribution on the HCN content (Test 1). The first group of plants was transferred to the field at the start point of the rainy season and harvested at the end of the rains. The second group was transferred to the field two months later and harvested after two months drought. A significantly higher value was obtained in plants harvested after two months of drought ($P < 0.01$). (Fig. 1)

A second comparison was established using two locations with similar distribution and different total rainfalls (Test 2). No significant effect of total rainfall on the HCN content ($P > 0.10$) was observed, when the rainfall remains over the limit of 1,000 mm per cycle (Table 2).

These results suggest that HCN content depends more on the humidity lasting in the soil during the plant cycle than on the total rainfall.

By using the following guide for acute toxicity of cyanide (Coursey, 1973):

Innocuous:	< 50 ppm
Moderately toxic:	50 - 100 ppm
Dangerously toxic:	>100 ppm

It is possible to determine that a cultivar could be innocuous when harvested at the end of the rainy season and dangerously toxic when harvested after two months of drought.

Based on the results presented here it is possible to decide better on the date on which plants should be planted and harvested depending on the end use of the roots to be produced.

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Table 1. Location of cassava field tests.

Venezuela state	Location	Latitude Degr:Min W	Longitude Degr:Min N	Altitude masl
Anzoátequi	Anaco	64:29	09:27	220
Apure	Biruaca	67:31	07:51	45
Aragua	El Mácaro	67:29	10:14	470
Barinas	Barinas	70:13	08:37	190
	Dolores	69:34	08:16	80
Bolivar	Caicara	66:10	07:40	30
	Guarataro	64:48	07:00	31
	Hato Gil	63:45	08:15	29
	La Vergareña	63:32	06:46	220
	Parupa	63:36	06:30	1,100
	Pozo Oscuro	61:14	07:08	175
Carabobo	Belén	67:40	10:00	670
	Guigue	67:35	10:06	440
Cojedes	El Milagro	67:58	09:14	105
	San Carlos	68:35	09:40	155
Dtto. Fed.	Carayaca	67:07	10:32	1,350
	Chuspa	66:10	10:37	10
Lara	Barquisimeto	69:24	10:07	545
Miranda	Yare	66:44	10:12	150
Monagas	Marutín	63:10	09:45	65
	Tonoro	63:44	09:26	55
Táchira	Rubio	72:21	07:43	850
Trujillo	Agua Viva	70:39	09:32	150
	Santa Isabel	70:46	09:39	60

Table 2. Effect of rain distribution on HCN concentration in root parenchyma.

	Rainfall (mm)			Difference	P
	1714	1461	1081		
	- CN µg / g root parenchyma -				
Test 1	42 ± 5	174 ± 15		132 ± 18	< 0.01
Test 2	69 ± 15		61 ± 8	- 8 ± 17	> 0.10

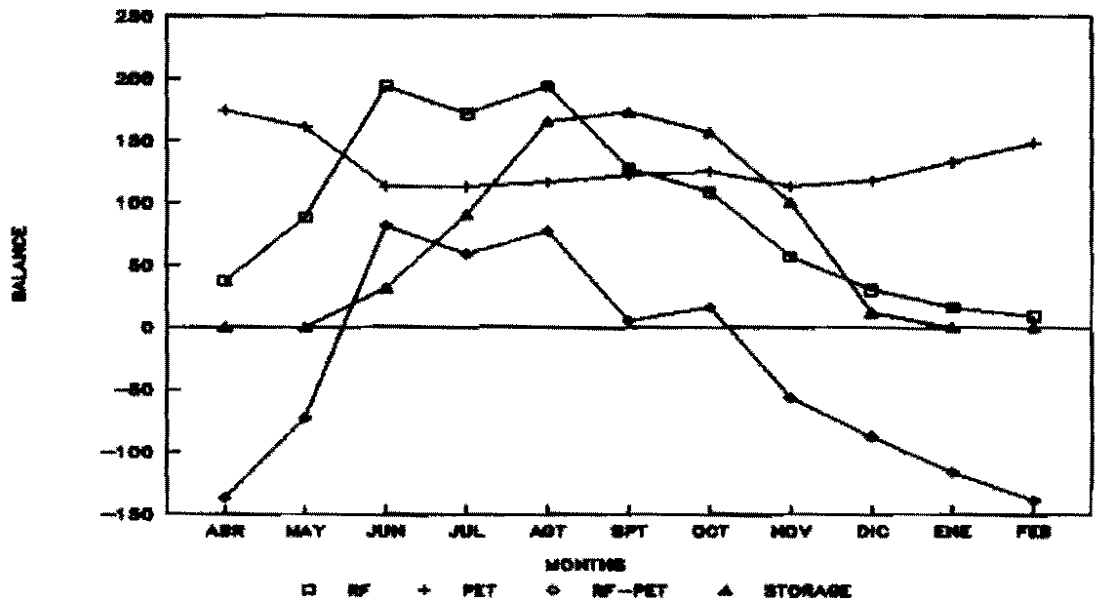

Figure 1. Water balance (mm) in the location used for the tests analyzed: RF = rainfall; PET = potential evapotranspiration; Storage = water stored in soil.

CASSAVA PROCESSING, CONSUMPTION AND HUMAN HEALTH

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Results from our laboratory indicated that students from O.A.U. Ile-Ife are the highest consumers of cassava products (64 % eat cassava products at least once a day) but serum thiocyanate (SCN) was higher in the cassava processors.

Preliminary studies on kwarshiokwor and healthy children showed that exposure to cyanide depletes the body's sulfur amino acids and exacerbates malnutrition. The high urinary SCN (220μg/dL) level in the kwarshiokwor subjects also indicate the possible occurrence of hypothyroidism and other related diseases in these subjects.

Investigations in Akungba and Oke-Agbe, Akoko area (where goiter is endemic) showed that exposure to cyanide coupled with low iodine intake and protein malnutrition act in synergism to cause goiter while the control and other populations in Nigeria are at high risk of hypothyroidism and goiter.

INTRODUCTION

The toxicity of cassava is attributable to the presence of two related cyanogenic glucosides - linamarin and lotaustralin -which on hydroysis yield HCN (Conn, 1979). Intact cyanogenic glycosides are relatively innocuous and their toxicity depends on ß-glucosidase/glucuronidase distribution in the body and the intestinal microflora (Adewusi, 1983, Brimer et al, 1983). Accidental and often fatal cyanide poisoning due to the ingestion of apricot kernels (Rubino et al, 1979), bitter almond (Shragg et al, 1979) and the quack drug-laetrile (Moertel et al, 1982) have been reported.

In addition to the occasional acute toxicity, chronic cyanide toxicity from dietary and environmental sources is very common in the tropical countries where cassava is a staple food. Some of the diseases associated with chronic toxicity include abortion, stillbirth, congenital anomalies, increased perinatal and infant mortality, retarded mental growth, juvenile hypothyroidism and goiter (Akindahunsi, 1991), spastic paraparesis (Essers et al, 1992), tropical ataxic neuropathy and the inability to achieve full working potential before the onset of muscle pain, cramps and tenderness (Taylor et al, 1992). In research and process development, emphasis has been placed on detoxification techniques to make cassava safer for consumption. This paper summarizes the recent findings from our laboratory indicating that both cassava processing and consumption may constitute a health hazard.

MATERIALS AND METHODS

Experiment 1: A preliminary survey on cassava consumption was carried out among 200 respondents - 25 students from O.A.U., 25 cassava processors in Ile-Ife

and 150 students from the Federal University of Technology, Akure (a non-traditional cassava consuming area). Blood and urine samples were collected analysed for SCN.

Experiment 2: Anthropometeric measurements were carried out on 20 kwashiokwor and 20 healthy patients aged 2-5. Urine samples were collected and analysed for creatinine, SCN and inorganic sulfate.

Experiment 3: Chemical and nutritional causes of goiter among women of child-bearing age were investigated in Akungba and Oke-Agbe (Akoko area) and compared to Erinmo and Ifewara (control) in Ijesha area; all in South Western Nigeria. Drinking water and food samples were collected and analysed.

Analytical procedures: SCN was estimated by the Pettigrew and Fell method, (1972), creatinine by the Vasiliades method, (1976) and inorganic sulfate according to Lundquist et al (1980). Iodine in food was estimated by the Belling method, (1983), in drinking water and salt by the method of Lambert et al (1975). Energy and crude protein content were quantified by standard AOAC (1984) methods and statistical analysis according to Zar (1984).

RESULTS AND DISCUSSION

The results presented in Table 1 show that cassava consumption is high in the three groups with 64, 48, and 30 % of respondents eating cassava based products at least once a day. Serum but not urinary SCN was significantly higher in cassava processors (0.57mg/dl) than either student population (0.38). Since on average, cassava processors eat less of their products, the higher serum SCN level could only be attributed to occupational cyanide exposure. Contrary to Jackson, (1988), the similar urinary SCN level among the groups would lead to suggest that renal clearance is a limiting factor in the excretion of SCN in the body.

Anthropometric measurements shown in Table 2 confirm the clinical diagnosis and show distinct differences between kwarshiokwor and healthy children. The inorganic sulfate concentration in the urine which is an indicator of the body's sulfur amino acid content (Sabry et al, 1965, Lundquist et al (1980), was significantly lower in kwarshiokwor compared to healthy subjects (Table 2). The significantly higher urinary SCN level in kwarshiokwor subjects (Table 2) suggests that these malnourished children are probably weaned on cassava based diets or exposed to other dietary and environmental sources of cyanide/SCN. Since cyanide has the first call on serum methionine for its detoxification in vivo (Delange et al, 1980), this would definitely lead to a deficiency of serum sulfur amino acids (as indirectly confirmed by the low urinary inorganic sulfate level) and hence kwarshiokwor in the children.

Excretion of iodine was significantly lower while the SCN level was significantly higher in Akungba and Oke-Agbe compared to the control locations (Table 3). The

nutrient intake in all populations studied is about 60, 20, and 7 % of RDA values for energy, protein and iodine respectively. Low iodine intake and high SCN level exacerbated by protein malnutrition could therefore explain the goiter endemia observed.

We have shown that exposure to cyanide from dietary, occupational and environmental sources poses health hazards to the millions of people dependent on cassava for food and source of income in the tropics, including children whose brain development could be impaired. Since cyanide concentration in cassava varies with environmental conditions (Bruijn 1971, Bokanga and Bradbury, unpublished results), the best way to remove this health hazard is to produce biotechnologically engineered low/cyanide free cassava cultivars. In the meantime, a detailed investigation into the level of health hazard from occupational/environmental exposure to cyanide should be carried out and measures to reduce such risks put in place.

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Table 1: Frequency distribution of the cassava consumption/week (%) Serum and urinary thiocyanate (SCN) content (mean ± SEM).

No. of respondents	25	25	150
Category label /week	Gari processors (%)	OAU Students (%)	FUTA Students (%)
2-3	24.0	28.0	21.4
4-6	20.0	8.0	27.3
≥7	48.0	64.0	30.0
No response	8.0	0.0	21.3
Serum SCN (mg/dL)*	0.57 ± 0.08^a	0.38 ± 0.07^b	0.37 ± 0.02^b
Urine SCN (mg/dL)*	0.34 ± 0.04^a	0.38 ± 0.05^a	0.35 ± 0.02^a

* Figures with the same superscript are not significantly different at 95% confidence level.

Table 2: Anthropometric and chemical/clinical assessment of urine from Kwarshiokwor and healthy children aged 2-5 years (mean ± SD).

Parameter	Kwarshiokwor			Healthy		
	Male	Female	Total	Male	Female	Total
Weight (kg)	6.8±1.8[a]	5.4±0.9[a]	6.3±1.7[a]	16.0±3.2[b]	15.6±2.7[b]	15.8±3.0[b]
Height (m)	0.7±0.1	0.7±0.1	0.7±0.1	1.0±0.1	1.0±0.1	1.0±0.1
Weight/Height (kg/m)	9.4±2.1	7.8±0.7	8.9±1.9	15.6±1.3	15.5±2.8	15.6±2.1
Weight/age (kg/yr)	0.4±0.1	0.4±0.2	0.4±0.1	0.4±0.3	0.3±0.1	0.4±0.2
Inorganic sulfate/ (mg/dL)	1.6±0.7[a]	1.1±0.5[a]	1.5±0.5[a]	4.7±0.7[b]	3.9±0.7[b]	4.3±0.8[b]
Inorganic sulfate/ creatinine (mg/g)	85.4±28.4	40.9±6.5	71.7±24.0	289.0±12.6	196.6±12.0	246.4±9.8
SCN (μg/dL)	220±90	230±100	220±90	60±8	50±8	60±8
SCN/creatinine) (mg/g)	22±8.8[a]	50.3±4.9[b]	31.0±9.7[a]	2.3±0.8[c]	3.1±1.3[c]	2.7±0.9[c]

* Figures with the same superscript in a row are not significantly different at 95% confidence level.

Table 3: Chemical parameters in the urine, incidence of goiter, energy, protein and iodine contents of food and water samples in Akungba and Oke-Agbe in comparison with Erinmo and Ifewara control (mean ± SEM). No. of respondents in parenthesis.

	Location			
Variable	Akungba	Oke-Agbe	Ifewara	Erinmo
A. Urine	(104)	(28)	(48)	(56)
Lodine μg/dL	9.6±0.4	8.2±0.9	13.8±0.6[b]	13.6±0.5[b]
Range	0.8-15.5	0.5-13.8	0.5-17.5	0.5-17.3
SCN(mg/dl)	1.1±0.1[a]	0.8±0.1[b]	0.6±0.1[c]	0.7±0.1[b]
Range	0.2-47	0.2-1.4	0.2-1.5	0.2-1.6
I/SCN (≤ 7μg/mg)*[1]	30.6	24.6	4.2	3.6
I/SCN (≤ 4 μg/mg)*[1]	15.3	16.3	4.2	1.8
Incidence of goitre[2]	~20	~20	0	
Incidence of goitre (Cassava processors)*[2]	~25	-	-	
B. Food /Day	(12)	(18)	(8)	(8)
Energy Intake(Kcal)	1473.4	1483.0	1496.0	1490
Protein Intake (g)	10.7	12.2	13.0	11.4
Iodine Intake*[3] (μg)	10.6	12.3	11.7	9.3

Cont...

Cont...

C. Iodine Content
(in water)

well	3.1 ± 0.2^{a}	3.0 ± 0.4^{a}	3.2 ± 0.2^{a}	3.3 ± 0.2^{a}
Rain	2.1 ± 0.4^{b}	1.8 ± 0.3^{b}	2.1 ± 0.3^{b}	2.0 ± 0.3^{b}
Stream	2.7 ± 0.3^{c}	2.6 ± 0.4^{c}	3.3 ± 0.1^{c}	3.1 ± 0.2^{c}

(in common salts)

English Dendrite salt (cassava brand) 0.4 ± 0.1^{a}. Fine table salt 0.4 ± 0.1^{a}

Dicon table salt 3.0 ± 0.1^{b}

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*1 Figures refer to percentage of respondents.
*2 Incidence of goiter (%) estimated from a detailed survey-individual values for Akungba and Oke-Agbe are not available and for cassava processors among gari processors/sellers in mass markets in Akoko area.
*3 Iodine intake from main meals - breakfast, lunch and dinner alone.

MULTI-DISCIPLINARY RESEARCH AND FARMER PARTICIPATION: ESSENTIAL IN TECHNOLOGY DEVELOPMENT FOR SMALL-SCALE FARMERS

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From the point of view of small-scale farmers in developing countries, the record of technology development and transfer has been rather disappointing. Despite good intentions, the innovations rarely reached the end-user; when they did, it was often with negative side-effects.

One of the main problems in technology development for small-scale farmers is that there is usually little association between research units and potential clients. Consequently, researchers and policy-makers are often ignorant of the problems and needs of small-scale farmers, and priorities and goals are set through conclusions drawn from own theoretical models and value systems of what out to be appropriate. As a result some of the most impeccable "practical" pieces of research end up with disappointingly few adopters. Researchers blame the farmers for their failure to transfer innovations which have shown so much promise under experimental conditions. Farmers often perceive researchers as being incapable of providing answers to their problems and needs. Although the potential of biotechnology to address the problems of small-scale farmers are often mentioned, the technology has not "built-in" guarantees to do any better than previous technologies. If the gap between research and application is not successfully bridged, biotechnology research will do little of practical worth for small-scale farmers.

In my presentation, I will discuss the achievements and pitfalls of several approaches which have been developed to bridge this gap. On the basis of this analysis I will argue that a thorough knowledge of farmers'needs and constraints is a prerequisite to successful technology development. However, if effective mechanisms for joint project design and implementation are not integral part of technology development as well, information on farmer's needs and farming systems will not be used effectively; no matter how many surveys, experiments or demonstrations are carried out. If farmers do not fully participate in the whole process of technology development, the resulting innovation is unlikely to meet their needs.

INTRODUCTION

The Cassava Biotechnology Network (CBN) has been established with the aim of coordinating and stimulating biotechnology research and development (R&D) on cassava. Cassava is a so-called 'poor man's crop'; it is mainly grown by resource-poor, small-scale farmers and receives little attention from formal R&D. The ultimate objective of CBN is to contribute -through technology development- to the improvement of food security and income of small-scale farmers in the Third World. This is a highly commendable goal which I fully support. However, we should be realistic; technology development for small-scale farmers, particularly the resource-poor, has a bad performance record to such an extent that a directorate-general of an international agricultural research institute recently stated: "*So many agricultural R&D projects for small-scale farmers have failed that a fatigue has developed amongst donors. We need some good results soon, otherwise there won't be any financial support available anymore for these kind of projects*" (pers. comm.).

There is very little disagreement about the importance of technological change for (agricultural) development. As early as the 1950s, it was found that only 10% of economic growth in the USA could be explained by growing investments, the rest had to be explained by either technological change or improvements in productivity (Sande *et al*, in press). Technological change has also played an important role in the development of Third World agriculture; e.g. the success of the Green Revolution in Asia. However, from the point of view of resource-poor, small-scale farmers in developing countries, the record of technology development and transfer has not been impressive; the innovations aimed at small-scale farmers rarely reached them, and when they did, it was often with negative side-effects. There are no indications that biotechnology has any 'built-in' guarantees to do any better than previous technologies. The central question is: how can biotechnology be of benefit to small-scale farmers? In answering this question, important lessons can be learnt from past experiences.

In my paper, I will argue that failure of technology development projects is generally not caused by the attitudes of small-scale farmers or the potential of the technology itself, but that the approach to technology development is the main problem. I will discuss the achievements and pitfalls of several approaches to technology development: from the conventional top-down approach to the bottom-up approach of participatory technology development. It will be largely a chronological discussion reflecting the active learning process which has taken place. I will conclude that if a project has the objective to
improve food security and the income of small-scale farmers, multi-disciplinary research and farmer participation should be an integral part. If the conventional approach to technology development is taken, biotechnology R&D will do little of practical worth for small-scale farmers.

TOP-DOWN APPROACH

The conventional approach of technology development is in essence a centralised, top-down approach. Universities and research institutes are the principal sources of new technology, which then spreads (or does not spread) to the peripheries. The R&D approach is based on model systems in which the homogeneous conditions are completely controlled with only one or two variables. There is a one-way flow of 'new' or 'improved' technologies: highly trained technical experts develop technologies in laboratories and experiments stations, and then pass them over to extension services to transfer them to 'end users' (Chambers and Ghildyal, 1985; Horton and Prain, 1989). The end users are really at the 'end' of the development line; the flow of information from end users to researchers is of decidedly secondary importance. Implicitly it is assumed that technical experts adequately understand the principal problems of the end users.

This top-down approach has its origins in the industrialised countries where it has worked very well. Its performance has been particularly impressive in industry and agriculture with resource-rich groups. The approach is deeply embedded in the

thinking of many technical experts as well as policy-makers, both at the national and international level. It is part of the structure of centralised knowledge in which power, prestige and professional skills are concentrated in 'centres of excellence'.

When in the 1970s small-scale farmers in developing countries became a specific 'target group' for technology development, it was not more than logic that the top-down approach was applied, unfortunately without much success. A review study in the 1980s revealed that most of the agricultural R&D projects directed to small-scale farmers did not achieve their objectives; generally resource-poor, small-scale farmers did not adopt the new and improved technologies (Cassen, 1986).

The initial premise was that if small-scale farmers did not adopt the innovations which had shown so much promise under experimental conditions, it was because they did not know about them, or did not know enough about them. Thus, the solution was that "*The farmer should be educated*". It took a while before it was realised that it was not ignorance preventing farmers from adopting the innovations, but the inappropriateness of the technologies; the technologies did not fit the needs and conditions of the farmers.

The centralised, top-down approach may have worked for large-scale farming systems, it did not for small-scale farming systems in which production takes place under completely different conditions. In large-scale agriculture the environment and production factors are controlled, thereby very much resembling the R&D situation. In those farming systems technology development has become the major determinant of agricultural development (Röling, 1989). In small-scale agriculture the situation is different. Farming is characterised by incomplete control over environmental conditions and production factors, and the farming systems are highly complex, therefore the R&D situation which is based on homogeneity and simplicity is inappropriate for small-scale farming systems. A further complicating factor is the difficulty small-scale farmers face in translating new technologies into economic benefits.

This requires appropriate policies and infrastructure which are often lacking for small-scale farmers. Technology, therefore, has a less dominant role in the development of small-scale farmers.

It was realised that in case of technology development for small-scale farmers there is a need for knowledge on the social, political, cultural and institutional environment of farmers. Technical experts have been trained to look at the technical, 'hard' elements and not at these so-called 'soft' elements. Due to their dominance in project design and implementation, the highly important 'soft' dimensions of technology development projects for small-scale farmers are very much neglected. As a result some of the most impeccable 'practical' pieces of research end up with disappointingly few adopters.

MULTI-DISCIPLINARY RESEARCH

From these earlier experiences, it was concluded that technology development directed towards small-scale farmers requires that explicit attention is paid to the socio-cultural, political and institutional context in which the farmers operate. Since social scientists are experts in the analysis of this context, it was thought that they should be involved in technology development projects. They could provide the necessary sociological knowledge and expertise both on the micro-level -farmers' needs and constraints- and on the macro-level -the policy environment (Cernea, 1985; Dusseldorp and Box, 1990, Horton and Prain, 1989).

The initial cooperation between technical experts and social scientists was not very fruitful. On the one hand, social scientists -who had until then primarily endeavored to explain and describe past or existing social structures- had little experience with providing input to technology development projects. On the other hand, the technical experts -who were still dominant in project design and implementation- were unprepared to cooperate with the social scientists; they did not know what to ask from social scientists or what to expect from them, and were afraid that social scientists might question the social relevance of their research (Cernea, 1985; Dusseldorp and Box, 1990). In practice, the contribution of social scientists was mainly limited to the end of the project cycle to provide information on how to refine and facilitate the transfer of the technology to end users and to conduct ex-post evaluation and social impact assessment. In any way their contribution was too late to affect the design and implementation of the project. The discipline was used simply to validate a given technology rather than to shape the structure and sequence of actions (Cernea, 1985).

At the beginning of the 1980s it became evident that knowledge about the context of small-scale agriculture is not only important during implementation of a technology development project, but particularly needed during the design phase as a starting point (Bunders and Broerse, 1991; Cernea, 1985; Dusseldorp and Box, 1990). Thus, social scientists are needed at all stages, as a continuum, and should make different contributions tailored to the internal logic of each stage. With the emergence of new areas in social sciences such as development anthropology, sociology of agriculture, peasant studies, crop sociology and farming systems research, social scientists are able to produce operationally usable knowledge. Technology development thus becomes multi-disciplinary. This is by no means easy to achieve given the differences in background of the natural and social scientists; they usually think differently and speak a different language. Measures should be taken to overcome the constraints in cooperation.

FEEDBACK THROUGH FARMER PARTICIPATION

To complicate the situation even more, involvement of social scientists only has proven to be insufficient. Although involvement of social scientists did to some extent lead to more appropriate innovations, the situation was still far from

satisfactory. Evaluation studies learnt that social scientists -just like technical experts- may assume *a priori* that they know what is best for the people involved (Cernea, 1985). The way in which sociological information is sought and assessed can itself be very much 'top-down'. The value of the information is likely to be reduced accordingly if there is no process to correct misinterpretation of data, and to remedy the problems and attitudes delineated by the outside studies (Uphoff, 1985; Chambers and Ghildyal, 1985). The people who are experts in the provision of this kind of information are, of course, the users of the innovations -the small-scale farmers. It was realised that participation of farmers in technology development projects was essential; through participation the design and implementation of projects is tailored to the needs and capabilities of people who are supposed to benefit from them. Farmers have a wealth of knowledge of their environment and they have developed specific skills to use their environment (ILEIA, 1989). Their knowledge and capabilities could be used as a starting point for formal R&D.

Farmers can provide feedback on sociological information collected, priority setting, ideas on possible solutions, proposed sequence of actions, project evaluation, etc..

To be effective, farmer participation should have an organisational base. It isbest to begin by looking at what channels already exist whereby local people mobilize resources and solve problems. Social scientists could help to collect this information. Proposals for various kinds of participation should be checked out with the intended beneficiaries to ascertain whether they are feasible and acceptable.

It is important to recognize that 'participation' can refer to many different things, not all of which are relevant or desirable in any specific project context. Technical experts, policy-makers and social scientists may readily concede that information from rural people is useful in project planning and operation, but still be very reluctant to actually engage farmers in decision-making, assuming that poorly educated persons have little to contribute. The attitude of project designers towards farmers is well illustrated by the following quotation: "*Following review within [the central government] and decision on the form of organisation to be adopted, there would be a short public information campaign to advise the farmers of what is intended and ensure their willing participation*" (Uphoff, 1985).

Of course, small-scale farmers can be wrong, but then so can technical experts and social scientists. Although more participation by farmers in project design is no guarantee to produce a successful project, it would make the project more realistic and increase the people's commitment to it.

DISCUSSION AND CONCLUSION

Technical experts are usually dominant in the initiation, design and implementation of technology development projects. Technical experts are knowledgable on a project's 'hardware' -the technical elements. The success of

technology development projects for the benefit of small-scale farmers, however, commonly turns as much (if not more) on the 'software' -social, political, cultural and institutional elements- which makes a new technology utilised and productive. Social scientists and small-scale farmers are experts on this 'software'. Without the participation of social scientists and farmers, many projects remain socially underdesigned and register a high rate of economic, technical and socio-political failure. It may be argued that multi-disciplinary research and farmer participation will slow down the project and will increase project costs, but they make the rest of the time and expenditure much more productive.

With this in mind, I will return to the present time. One would expect that the learning process of the past has resulted in a much more balanced design of technology development projects by the end of the 1980s. However, when evaluating recent R&D projects for the benefit of small-scale farmers, it can be concluded that the majority is still set-up along the lines of the top-down approach. Still technical experts are the primary initiators of technology development projects and they are still reluctant to include social sciences research and intended beneficiaries in project design and implementation. Since this approach simply does not work when addressing small-scale farmers in developing countries, the same lessons are learnt over and over again.

I think the past experiences provide very interesting lessons for the Cassava and Biotechnology Network. It is important to realise that the top-down approach is deeply embedded in the thinking of many technical experts. This does not pose a problem when more fundamental research such as mapping of the cassava genome or developing cassava transformation protocols is conducted, or when addressing resource-rich (large-scale) farmers. But this way of thinking does become a constraint when conducting applied, problem-oriented R&D for the benefit of small-scale farmers. It is also important to note that biotechnology, notwithstanding its enormous technological potential, has no 'built-in' guarantees to do any better than previous technologies. Therefore, if the CBN is serious about its objective to improve food security and the income of small-scale farmers through biotechnology R&D on cassava, it should pay explicit attention to project design and implementation and should involve both social scientists and small-scale farmers from the start. Otherwise, the CBN will do little more than repeat the injustice and damage of earlier technological revolutions, and thus waste scarce financial resources and the hopes and aspirations of people.

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PROBLEMS AND OPPORTUNITIES IN CASSAVA BIOTECHNOLOGY

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Although world cassava production has been increasing, it is shown that considerable cassava yield gaps still exist. Constraints are divided according to being endogenous and exogenous to the (plant and) production system. It is indicated that besides these constraints, considerable "adoption constraints" exist. The latter relate to fundamental inherent cassava plant characteristics. It was argued that these fundamental plant constraints severely limit both the R&D and adoption process.

It is shown that several modern biotechnology techniques have a comparative advantage over more traditional methods to solve these fundamental limitations. Significant progress has already been made at CIAT, in developing novel approaches towards solving these. It is pointed out that still much work needs to be done to fine-tune these methods and make them appropriate for end-users. While for in-vitro propagation techniques, this is the case, for the development of True Cassava Seed (TCS), significant basic and applied research still needs to be conducted. For the latter technology, a serious commitment and development of an appropriate research strategy is urged.

INTRODUCTION

World cassava production increased from 137 million MT in 1985 to 150 million MT in 1990 (FAO, 1991). Although average cassava yields have increased, this has been camouflaged to a certain extent by the continuing shift of cassava cultivation to more marginal and fragile production zones. There continue to exist wide gaps between the average yields of cassava on farmers' fields and that achieved in on-farm tests and research stations (Sarma and Kunchai, 1991).

Cassava's biotic and abiotic constraints together with adverse socio-economic and government policy conditions are the major limitations to yield improvement. In addition, its low priority in the majority of national programs has earned it an "orphan" status and subsequent underinvestment in national R&D. Also, not enough attention has been paid to the tremendous potential cassava offers due to its high adaptability to adverse climatic and soil conditions and its multiple end uses.

As such, the role that the CGIAR centres and the CBN can play, is of vital importance to link cassava R&D to improve the well being of small-scale farmers and urban poor in LDCs. Increasing attention has been paid to the integration of biotechnology in the cassava R&D portfolio. In this paper cassava constraints are re-assessed in order to show that the majority of constraints (and research opportunities) are related to the inherent characteristics of cassava planting material. It is shown that modern biotechnologies have a specific comparative advantage over more traditional approaches in solving these problems.

CASSAVA CONSTRAINTS

Limitations to cassava production, processing, marketing and consumption aspects, can be classified as 1) **biotic** (plant interaction with pests and diseases); 2) **abiotic** (plant interaction with soil, water, nutrients and temperature); 3) **endogenous agro-socio-economic** (production system and techniques, labor, management); 4) **exogenous socio-economic** (markets, policies). In general, national programs have allocated the majority of resources to research activities in pursuit of alleviating biotic and abiotic constraints.

Besides the above constraints for which technical improvements are developed, there exist a wide array of limitations regarding the "technology transfer", or better said, "technology adoption process". Following Henry (1991), these can be divided into five groups: (1) **multiplication**, (2) **diffusion**, (3) **institutional**, (4) **political**, and (5) **structural** constraints.

Multiplication constraints are inherent to the biological makeup of the cassava plant. As shown in Table 1, the average growing cycle of cassava is 12 months. However, there are many regions in the tropics and subtropics (Northeastern Brazil, Paraguay, Northeastern Colombia, South China) where cassava is harvested after 18-24 months. When comparing with crops such as beans, rice, and maize, which have growing cycles of 3-4 months, it becomes obvious that cassava's long growing cycle turns into a disadvantage with respect to its (annual) multiplication rate.

Another aspect that has a direct adverse effect on cassava's multiplication rate is its vegetative reproduction system. The stems of the cassava plant form the new planting material. On the average, 8-12 planting stakes can be cut from one (mature) cassava plant. When comparing the multiplication rate (the number of hectares that can be planted after one year, starting with one hectare of planting material) of the various crops, the differences are striking. While cassava can generate planting material for just 10 hectares in one year, both rice and maize can multiply into 1,600 hectares, or 160 times as much (Table 1).

The second constraint regards **varietal diffusion**. In other words, these are aspects that hinder an optimal flow of improved cassava varieties after release. One of the very marked issues here is the weight and bulkiness of cassava planting material. As demonstrated in Table 1, to plant one hectare of cassava, a farmer needs 100 kg of stakes, whereas in the case of maize only 25 kg are needed. The practical difference is renting a truck or horse and cart versus carrying the seed home in a shopping bag!

These first two groups could be called "internal" constraints, since they deal with the inherent cassava plant characteristics. The remaining groups could be labeled as "external" constraints, since researchers in general will not be able to influence these (at least not to any significant extent and only in the longer run).

It is straight forward to distinguish a high correlation between the internal constraints of the first and second set of constraints. The common denominator is cassava planting material and its inherent characteristics. In order to demonstrate how important this issue is, Table 2 shows a comparison of technology adoption parameters for several crops[1]. The original table was constructed for the purpose of calculating ex-ante future research pay-offs. The data for the table was partially generated by a Delphi survey. Parameter estimates were then used to calculate a "k-factod cost-effectiveness of research through the integration of selected biotechniques into strategic germplasm development efforts for: (i) characterization and screening of germplasm; (ii) broadening the genetic base of the crops; and (iii) identification of entry points for genome manipulation aimed at overcoming major constraints in plant-biotic/abiotic interactions. Similarly, the Virology Research Unit supports the germplasm development programs by identifying, characterizing and developing methods for cr rice or bean technologies, is directly dependent on each one of the constraints shown in Table 1. The same reasons apply to the lengthy adoption time. The probability of success to achieve the adoption rate over this time period incorporates, besides the already-mentioned constraints, an assessment of political, institutional and structural constraints, which as was argued before, must be treated as exogenous factors of influence.

Another constraint to cassava's traditional propagation method is its implications for germoplasm conservation and exchange. Cassava field collections are not only extremely costly, but incorporate the danger of loosing valuable germplasm, due to biotic stresses. In addition, germplasm exchange among researchers has been fraught with complications and inefficiencies.

Hence, any cassava technology development i.e. drought tolerance, disease or insect resistance, higher yielding, better eating quality, higher starch or lower HCN contents, etc, will be, by definition, subjected to growing cycle and multiplication constraints, which implies considerably longer (than maize, rice, beans, etc.) research lag times. In addition, once any of these technology components is in the transfer process, it will be, again by definition, subjected to even more constraints. It seems justified to argue that any possible level of alleviating these constraints would have a tremendous impact on research resources and cassava technology adoption time and rates. In the end, the compounded benefits will directly accrue to our clients of cassava R&D: small-scale farmers/processors and urban poor in LDCs.

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[1] The original table was constructed for the purpose of calculating ex-ante future research pay-offs. The data for the table was partially generated by a Delphi survey. Parameter estimates were then used to calculate a "k-factor", in order to shift each commodity's supply curve. For a more detailed treatise of the methodology, see Janssen et al., 1991.

Research on alleviating these constraints, for many years was conducted, employing traditional techniques. However, novel biotechnology approaches have introduced techniques that will enable cassava scientists to considerably speed up this R&D process.

POSSIBILITIES FOR BIOTECHNOLOGIES

For more than five years, the Biotechnology Research Unit (BRU) at CIAT has made a tremendous effort in applying novel techniques in an attempt to solve the fundamental limitations of traditional vegetative cassava propagation. The BRU developed several vegetative in-vitro multiplication techniques and made considerable progress with supportive research towards True Cassava Seed (TCS) development (Roca et al.,1992):

Enhancement of in-vitro propagation through meristem culture

A set of techniques was developed during the 70's to allow a safe movement of cassava germplasm among countries. It includes meristem culture, chemo- or thermo-therapy and a range of virus indexing techniques that ensure the elimination of viruses and other pathogens from cassava clones (Roca et al., 1989). Field results have shown a high probability of reinfection of a cleaned clone within a few years after its transfer to the field under normal growing conditions. In order to benefit from in-vitro cleaned and indexed varieties a continuous and large flow of planting material must be ensured each planting season. This will involve large and costly lab facilities coupled to an efficient rapid multiplication scheme (Cock et al., 1976) and planting material diffusion. This method has been implemented with success in Venezuela by private enterprises producing planting material for their own cassava production fields, and in other countries like Cuba and China.

In-vitro propagation through somatic embryogenesis

Somatic embryogenesis in cassava has been studied for many years with variable results (Szabados et al., 1987). The development of a reliable methodology for profuse induction of somatic embryos could open new avenues for solving cassava propagation constraints. The use of "artificial seed" produced from encapsulated somatic embryos is already being used or developed in several crops (Redenbaugh et al., 1987). This
methodology will probably lead to high levels of sophistication in management of planting material increasing the cost for the production of a low-value crop like cassava.

True Cassava Seed (TCS)

No major cassava virus has been demonstrated to pass through sexual seeds, therefore, each generation of TCS propagation would serve as a filter for most viruses present in a given plantation. Other reasons for proposing TCS as an

alternative propagation system have been: higher multiplication rate, easier storage and handling of planting material and the development of more efficient plant types (Iglesias, 1992). Increased variation in root quality is one of the principal limitants for the acceptability of TCS among cassava producers, intermediaries and consumers. Therefore, one of the targets is to develop TCS populations that are phenotypically uniform for root quality traits.

Production of di-haploid cassava lines through pollen or microspore culture is still being studied by CIAT's Biotechnology Research Unit. If those lines could be successfully produced and used for F1 hybrid seed production, still a great deal of inbred selection and testing for combining ability will be required for a crop that has not been systematically subjected to inbreeding.

One of the possibilities for cassava biotechnology to contribute to the development of TCS as a viable propagation system will be in the area of transferring apomixis genes from other species, and/or the induction of apomixis in interspecific crosses. Research in the area of apomixis induction and its genetic manipulation could lead to the production of uniform TCS crops. Early vigor, flowering capacity and plant architecture are other traits where gene tagging, isolation and transferring might be useful tools in the future of TCS.

BIOTECHNOLOGY'S EDGE

In-vitro techniques largely surpass traditional methods in propagation potential (Roca et al., 1992). Single node culture and multiple shoot culture can produce 60,000 and 600,000 stem cuttings per plant per year, respectively. This in comparison with 8-12 cuttings through traditional propagation.

Micro-propagation of disease-free stocks has been tested and applied by national programs in Brazil, China, Cuba, Panama, Paraguay, Peru and Brazil. In addition, traditional cassava cultivars show significant yield improvement when the planting material is generated and multiplied through micro-propagation (Roca et al., 1992). In South China, 30-70% yield increases over local varieties were obtained with an improved CIAT clone. In addition, since 1988, more than 30,000 HA have been planted through this system of massive propagation (CIAT, 1992).

Over the last decade, CIAT has started to build an in-vitro cassava germplasm collection. Currently this holds 95% of the live (in field) collection. This has greatly improved the management and efficiency of maintaining a germplasm collection. In-vitro maintenance costs are half that of a field collection (Roca et al., 1992). In addition, the speed, volume and management of (inter) national cassava germplasm exchange has been greatly improved by in-vitro techniques.

The integration of biotechnologists, especially with socio-economics and agronomy scientists becomes even more important than before, since particular innovative technologies like TCS, require a very intensive and extensive farmer-

participatory research, development and transfer approach. Moreover, besides on-farm research, as in the case of TCS, in-depth knowledge of seed production systems, markets and marketing becomes fundamental for successful acceptance, adoption and impact.

CONCLUSIONS AND DISCUSSION

Although world cassava production has been increasing, it was shown that considerable cassava yield gaps still exist. Constraints were divided according to being endogenous and exogenous to the (plant and) production system. It was indicated that besides these constraints, considerable "adoption constraints" exist. The latter relate to fundamental inherent cassava plant characteristics. It was argued that these fundamental plant constraints severely limit both the R&D and adoption process.

It was shown that several modern biotechnology techniques have a comparative advantage over more traditional methods to solve these fundamental limitations. Significant progress has already been made at CIAT, in developing novel approaches towards solving these. It was pointed out that still much work needs to be done to fine-tune these methods and make them appropriate to fit users production, processing, and marketing systems.

The aforementioned has made a case about the importance of fundamental cassava plant limitations and has shown progress made. The question that arises, is how much priority has been assigned to these research issues, vis-a-vis other biotechnology research items, currently and in the future?

First, at CIAT past emphasis has been placed on the development of novel in-vitro propagation techniques. As shown, several relevant options are already (experimentally) being used by some national programs. Given this, for the immediate future, the focus should shift to making these techniques more economical and more user-friendly at the national program level and subsequently, appropriate for adoption by farmers. As such, the emphasis should now be placed on the end-users. To this end, an appropriate research approach should be developed (and funds be identified), integrating technologists and end-users in a multidisciplinary fashion with national programs.

Secondly, although TCS has been identified by the CBN as one of the highest priorities (DGIS, 1991), no (major) funds have been allocated towards TCS research activities. Some research has been conducted at CIAT (Iglesias, 1992; Moreno and Sanchez, 1992), and a TCS research proposal has been developed. However, the potential of TCS and the fact that a long research lag time is envisioned should warrant an immediate and substantial financial commitment to commence this research on all the different research aspects that are required. This will involve basic, applied and user participatory research at various levels, as was already mentioned.

Especially in the area of biotechnology which embodies various novel facets of research, a continuous monitoring of new information, results and appropriate feed back are crucial in (re) assessing priorities and subsequent (re) focussing among the many issues and possibilities. There is no doubt that specific biotechnologies have a tremendous potential (and edge) towards solving specific problems, generating significant scientific, economic and social impact.

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Table 1. Production, agronomic, and multiplication constraints of cassava and other crops.

Crop constraints	Cassava	Rice[a]	Beans	Maize[b]
Growing cycle (months)	12	4	4	4
Rate of multiplication[c]	10	1600	400	1600
Weight of planting material for 1 ha (kg)	1000	100	50	25
Commercial seed availability	--	***[d]	**	***
Crop yield related to seed yield	no	yes	yes	yes
Crop area intensiveness	--	***	**	**

a. Irrigated rice
b. Lowland maize
c. For cassava: 1 cycle, for other crops, 2 cycles
d. Asterisks indicate relative importance, *** = most importance, ** = medium importance

SOURCE: Henry (1991)

Table 2. Parameters for technology adoption coefficient estimation of selected crops, 1990.

Parameter	Fresh cassava	Processed cassava		Beans	Rice[a]
	LA	LA	Asia	LA	LA
Research lead time (years)	8	8	6	4	4
% Yield increase	35	70	30	40	15
% Adoption	20	20	50	40	85
Adoption time (years)	30	30	30	30	6
Probability of success	0.5	0.5	0.5	0.8	1.0

a. Irrigated rice only; LA = Latin America

SOURCE: Henry, (1991)

ROLE OF AN NON-GOVERNMENTAL ORGANIZATION IN THE IDENTIFICATION OF RESEARCH CONSTRAINTS THAT MAY BE SOLVED THROUGH BIOTECHNOLOGY

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In several developing countries like Colombia, Brazil and Ecuador, to mention only those in Latin America, cassava is increasingly becoming a cash crop, overcoming those times when it was only a subsistence crop of secondary importance researchwise. The key factor of this transformation, specially in Colombia and Ecuador, has been the involvement of the farmers on an integrated project, which included the transformation of the end product, and the opening of new markets. As a consequence, there is now more and more willingness to adopt technology and demand for it to increase yield and for varieties with specific characteristics, to meet specific demands.

On the other hand, official institutions, national and international had identified cassava as one of the subsistence crops which could help to solve the energy intake of a large and poor population, therefore National Cassava Programs were set up to do research addressing the usual constraints on yield (i.e. varieties, fertilization etc), and they were living the (as well common) frustration of not getting an impact within the farmers, and, like in Ecuador, even closing down the research program because the yield, without technology, was superior to the existing demand. Eventually the Program in Ecuador is relocated and is ironically trying to catch up with the actual needs of farmers. From this picture, clearly emerge two roads for the future of a cassava program: on one side the need to identify new markets and new derived products will ensure its long term viability; on the other side, research should adopt modern biotechnology to accelerate the process of solving many constraints which the above mentioned demand, is putting over the crop for farmers and research scientist alike. The farmer's organization, as a private NGO, naturally will take the first road and at the same time participate on institutions and research workers in charge of taking the right decision on the other road; in both cases, the linkage provided by and NGO like FUNDAGRO with its IEE philosophy, provides means to identify problems and alternatives to solve them.

To establish appropriate criteria to identify problems on yield, processing and uses of a crop like cassava, it is necessary to have in mind the whole problem and to find an equilibrium between the farmer's needs, the national research capabilities and the international capacity available. This paper gives some examples of criteria to take into account to solve some constraints already identified (high fiber varieties, quantity and quality of seed, drying process, pellets production, etc) and which could be better solved with the help of biotechnology.

A scientifically based agriculture should be supported by a series of public and private institutions that function as an integrated system that sustains farmers' activity. It should be reliable for those farmers so that they accept the risk of changing their traditional farming system for a technical one. However one of the main problems that faces many developing countries is the fact that several of those Institutions, which are components of that system, try to "sell" to producers, independently, one from the other, various ways to improve their productivity and yield. As a consequence, most research and extension efforts in agriculture in LDC's

have emphasized the production of varieties and technologies, not taking into account the socio-economic environment of the farmer.

FUNDAGRO, as a non-governmental organization (NGO) develops what is called the IEE Project (Investigation-Extension and Education Project), with USAID-Quito funds. The aim of this project is to develop a system of coordination between the various components of agriculture within Ecuador, incorporating medium and small farmers into the processes of diagnosis, planning and execution of research and extension activities.

From its beginning in 1987, FUNDAGRO has established the liaison units between research-extension and education for crops defined for its activities as a priority. These are the two main objectives of those units:

1. Define production constraints, the solution of which will increase yield or reduce production costs; and

2. Identify appropriate technologies that may contribute to solve problems of a specific crop (Program).

Initially, as part of the activities of the project, several agro-socio economic surveys were made within the areas and among the crops selected, which allowed the main constraints to be considered. The needs of farmers were defined, and directed to objectives and targets of ongoing research and extension, changing and/or adapting them to adequately fit those needs.

Within this model, the role of FUNDAGRO, by means of structures that include specialists and advisers in different areas, and by means of the trial and error mechanism, opportunity targets are identified and actions are taken to follow a strategy to develop the small and medium farmer in a scientifically based agriculture.

Cassava was one of the four priority crops selected. This product has multiple uses, has potential for the domestic and international market and is used not only as direct food for human and animal feed, but produces several products based on starch and flour which are obtained through adequate processing.

In Ecuador, where in 1984 approximately 24.000 ha (equal to 240.000 TM of fresh cassava) were produced, as in other countries cassava shows characteristics of a "traditional crop of low input" widely described in literature both in its socio-economic and agronomic aspects.

Cassava has always been an important component in the diet of people from a large region of the Equadorian coast, and its production was maintained to a subsistence level up to the arival of the cassava integrated project as a joint venture of CIAT and INIAP (The National Institute of Agricultural Research) to one of the

most depressed areas, with a high human concentration and very scarce production alternatives due to a characteristically long dry season.

Actually, though direct consumption is tending to decline, cassava has become a "cash crop", improving the general wellbeing of the people involved and generating sustainable activity in an area formerly subject to migration.

As a consequence of cassava processing, the opening of new markets and farmers' interest, there is now a larger demand for technology to increase yield and productivity. The "modus operandi" of the IEE-Project has allowed the identification of some production constraints, which in relation to the biological and agronomic aspects of the crop could be summarized as follows:

- There is a deficit in the supply of energy-producing food at national level.
- Improved varieties are required for (a) different agroclimatic zones; (b) mite resistance; and (c) higher dry matter content.
- Sustained production throughout the year.
- To extend the period of fresh cassava perishability.
- Deficit of technology within the reach of farmers' resources.
- Lack of available planting material in quantity and quality.

Despite advances in research in Ecuador -including the release of a variety tested for local conditions- there are still no answers to most of the above questions and Ecuador will need to participate in international relationships to accelerate the process of finding answers. It draws attention the fact that these constraints coincide with Dr. J. Nickel's (1990) expression in relation to the strategy required to generate varieties suitable to the need of producing in "marginal land, without irrigation, in the hands of farmers with limited resources or scarce or nil access to credit and income markets":

- To adopt a genetic rather than chemical solution to production constraints.
- To develop technologies that use few supplies efficiently.
- To emphasize sustainable yield throughout the year.
- To develop appropriate technology for different agricultural systems.
- To select crops and develop genotypes for specific ecosystems.

The former criteria help to define research priorities in countries like Ecuador and as one may infer, these coincide with what has already been established by the Cassava network and some of them may be solved sooner and with best results using biotechnology, particularly:

- Resistance to diseases (Bacterial blight) and pests (mites).
- Improving nutritional quality.
- Improving quality and quantity (dry matter) of starch.
- Reducing perishability of post harvest roots, and

- Improving photosynthetic capacity of cassava under drought conditions and poor soils.

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ROLE OF PLANT BIOTECHNOLOGY IN CROP IMPROVEMENT

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Plant biotechnology includes three interacting technical components, (a) microbial bioprocessing techniques, (b) techniques for culturing somatic and reproductive cells, tissues and organs, and (c) molecular and celular techniques for the characterization and modification of genomes, including techniques for the identification, recombination, cloning, transfer and expression of genetic material.

Cell and tissue culture techniques have been available and continously improved since very early. Current genetic modification technologies are being used to improve the effectiveness of microbial strains. Short-term applications of tissue culture technology involve clonal propagation, production of virus-free stocks, interspecific and somatic hybridization and haploid and double haploid plant production to enhance plant breeding efforts. Intermediate term applications involve technologies which are based on the integration of cell culture and molecular genetic techniques, e.g. generation of transgenic plants either by Agrobacterium-, and direct gene transfer techniques for genetic transformation. Long-term applications of plant biotechnology will be realized only after more complete understanding of basic plant physiological and genetic mechanisms, and on the structure and function of agronomically important genes, among others. Biotechnology is bound to affect virtually all crops, and cassava will be no exception. Potentially every aspect of cassava production, processing and utilization can be amenable to biotechnological approaches, but actual applications have to take into account the comparative advantages of biotechnology to enhance the attributes, minimize the drawbacks, and open new opportunities for cassava, as well as the crop's socio-economic and environmental setting. Research should also focus on technological bottlenecks precluding the full utilization of biotechnology to cassava, e.g. plant regeneration from protoplast and cell cultures, and efficient genetic transformation technology. At the same time, basic information on critical processes such as photosynthesis, starch biosynthesis, cyanogenesis, stress tolerance, root deterioration, etc. will be necessary for using biotechnological approaches in cassava.

INTRODUCTION

Improved plant breeding methods coupled with judicious selection strategies were responsible for the phenomenal increase in agricultural productivity during the last 2-3 decades. While this could be considered as a matter of great comfort, the predicted increase of world population to the 6 billion mark in the year 2000 and over 10 billion in 2050, most of which expected to occur in the developing countries, necessitates additional gains in global food production. Most of the major agricultural crops, in addition to possessing narrow genetic base, are approaching a plateau in yield component and spectacular gains in yield as occurred in the 60s may not be achievable. Agricultural systems will be required that remain productive in the presence of increasing biotic, edaphic and climatic stress without degrading the natural resource base. This, coupled with the impossible task of expanding agricultural land base due to the pressures of civilization and urbanization trends, warrants that other avenues should be pursued to meet the ever increasing and

challenging need for more food and fiber. In this context, plant biotechnological approaches become more relevant and attractive. Specifically, plant biotechnology's mission is to develop novel, exploitable methods to genetically control and alter plant development, performance and products in concert with other technologies both biological and socio-economical. Plant biotechnology is a multidisciplinary field; the two major interacting components being Cell Technology and Gene Technology (recombinant-DNA technology). While cell technology involving the *in vitro* culture of isolated cells, tissues, and organs is well established and has found applications in crop improvement now for over 3 decades, the gene technology is of recent origin. The ability to precisely manipulate DNA, the development of efficient vectors and methods of transformation led to the development of genetic engineering strategies. The first transgenic plants were produced less than a decade ago; already the list has grown to include over 50 species (Gasser and Fraley, 1992). The applications of plant biotechnology for crop improvement may be arbitrarily grouped into the following three categories based on the time-frame.

CURRENT APPLICATIONS

Short-term or immediate applications of plant biotechnology are those based on established procedures or methodologies. In the domain of plant tissue culture, a number of such technologies are currently being used extensively for crop improvement.
These are as follows:

1. Propagation, Virus Elimination and Germplasm Preservation

Plant tissue culture, especially meristem culture or shoot-tip culture has been extensively used for rapid clonal multiplication, virus elimination and germplasm preservation. The most preferred system for clonal multiplication is either the direct regeneration or somatic embryogenesis. In the direct regeneration system involving differentiation of pre-existing shoot meristems into plantlets unmediated by an intervening callus phase, or proliferation of axillary meristems, the genetic fidelity of the regenerants is fairly assured. This is in contrast to induced differentiation pathways such as organogenesis and even somatic embryogenesis. A number of commercial firms all over the world are employing meristem or shoot-tip culture techniques for the clonal propagation of a range of ornamental plants, fruit, vegetable, medicinal and even tree species (George and Sherrington, 1984, Debergh and Zimmerman, 1990). A recent adjunct to micropropagation in order to increase the efficiency and reduce the production cost is automation (Levin et al. 1988) and bioreactor technology. Mass induction of somatic embryos in carefully controlled bioreactors can considerably increase both the embryo proliferation and synchronization of embryo maturation frequency. Such systems could lend themselves to the production of artificial (synthetic) seeds.
Viral diseases are present virtually in all food crop species and, depending upon the severity of infection and the nature of hosts involved, can cause serious yield losses. Meristem culture has been extensively used for the production of virus-free plants in

such major crops as cassava, potato, yams, sweet potato, sugarcane, strawberry etc (Kartha, 1986). This is one of the areas of plant cell technology which has gained immediate acceptance from a crop improvement perspective. Since the virus-free plants originating from meristem culture are equally susceptible as the mother plants if exposed to viral pathogens, the technology can only be used for the establishment of virus-free nuclear foundation stocks and as replacements for infected planting material. In recent years, recombinant-DNA technologies are being developed to produce crop species resistant to viral pathogens (Powell-Abel et al. 1986).

Preservation of all valuable germplasm of cultivated crops is an integral part of any plant improvement programs. While this can be readily achieved in the form of seed storage for a number of crop species, those types of crops which are clonally propagated such as cassava, sugarcane, potato, sweet potato, yams and strawberry, to name a few, or those with recalcitrant seeds such as coffee, cocoa, rubber, oil palms, pose serious storage constraints. During the last decade, two principal approaches have been successfully pursued to ameliorate the situation - storage of plantlets *in vitro* under growth limiting conditions as a means of short-to-medium term storage and storage under cryogenic conditions (cryopreservation) for long-term preservation. The latter technology is still under developmental phase, but appears to hold great promise for application in the near future.

Relating the technologies discussed above to cassava improvement, considerable progress has already been made on all fronts. An efficient and genotype-independent plant regeneration system has been well established for cassava using meristem culture (Kartha et al. 1974, Roca et al. 1985) and this technique has been successfully used for the production of mosaic and frog-skin disease-free plants (Kartha and Gamborg, 1975, Roca, 1985). This technique is now routinely used in most of the cassava growing regions of the world. Over 5000 accessions of cassava are being maintained *in vitro* at CIAT using shoot-tip culture and growth-limiting conditions. The technical feasibility of storing cassava meristems under cryogenic conditions has been successfully demonstrated (Kartha et al. 1982) and the methodology has been further developed at the CIAT during the last 4 years. Similarly, somatic embryogenesis is now well established for cassava (Stamp and Henshaw, 1987, Szabados et al. 1987) and attempts to cryopreserve them were successful both at the CIAT and the University of Bath, U.K. Somatic embryogenesis is also being used at CIAT and elsewhere as a regeneration system for transformation experiments with cassava.

2. Haploidy and Embryo Rescue

Any time saved in the process of plant breeding would result in considerable savings in input costs including labor. Anther and/or microspore culture has been extensively used in the production of homozygous lines in one step especially from the F_1 hybrids. The technique involves culturing anthers, microspores and ovaries to stimulate the development of haploid gametophytic cells to form either regenerable callus or embryos. These are induced to form haploid plants and upon doubling of

chromosomes, these plants become fertile homozygous diploids. The haploidy system is in use in several countries as an adjunct to classical breeding programs. The application of this technology has resulted in the production of new cultivars of rice, wheat, maize, tobacco and oil seed rape. It also appears that the haploid microspores could become ideal gene recipient systems in transformation experiments utilizing accelerated particle bombardment. For cassava improvement, the haploidy technology needs to be further developed. Considering the heterozygous nature of cassava, haploid systems will be very valuable for hybrid production, interspecific crossing as well as generation of additional genetic variability.

Sexual incompatibility is a major problem in several crops where interspecific crosses are attempted. The incompatibility could arise either at the pre- or post-fertilizational levels. In the latter case, although fertilization takes place, the developing embryos abort at any developmental stages of their maturity. In such instances, these embryos could be rescued and reared to form plants *in vitro* . This method has been used for the production of viable hybrids in several cereals and legumes. In cassava, embryo rescue could be used in cases of difficult interspecific crosses.

3. Protoplast Culture and Somatic Cell Hybridization

In the early 70s, protoplast culture and somatic cell hybridization generated a great deal of excitement. It was touted as the 'technology of the decade' capable of overcoming all incompatibility problem encountered in intergeneric and interspecific sexual hybridization. However, it became apparent in less than a decade that somatic hybridization has only limited application since chromosome instability, chromosome elimination and lack of fertility started appearing in the somatic hybrid plants. Successful examples on the production of 'useful' somatic hybrids are very few, e.g. transfer of disease resistance (*Phoma lingam*) in the somatic hybrid *Brassica naponigra* produced by the fusion of *Brassica napus* and *B. nigra* protoplasts (Sjodin and Glimelius, 1989) and transfer of viral disease resistance in the somatic hybrid between *Solanum tuberosum* and *S. brevidens* (Gibson et al. 1988). A new tobacco variety *Delfield* was produced incorporating fungal disease (blue mold and root rot) resistance and elevated levels of nicotine by fusing protoplasts of *Nicotiana tabacum* and *N. rustica* (Pandeya et al. 1991). The possible use of somatic hybridization will be in the production of cybrids which contain the complete genomes of one parent and only the cytoplasmic genome of the other. This approach has merit in transferring cytoplasmic male sterility for the production of hybrid crops.

Another development that occurred subsequent to the development of protoplast technology was the use of protoplasts as recipient systems for the incorporation of genes by direct gene transfer techniques such as, electroporation, microinjection or PEG-mediated gene transfer. This has been now attempted in several crop species including cereals. For cassava improvement, protoplast

technology has not reached a stage where plants could be regenerated. Until this task is accomplished, although very strategic, protoplast systems cannot be used in cassava for any mode of direct gene transfer.

MEDIUM-TERM APPLICATIONS

The rapid advances being made in the development of gene transfer technologies coupled with the development of efficient plant regeneration systems from cultured protoplasts, cells, tissues and organs even for such recalcitrant species as cereals and grain legumes suggest that some aspects of crop improvement through the application of biotechnology will be realized in the medium-term, 5 to 10 years. This view is supported by the fact that the first transgenic plants appeared on the scene only in 1983, and in less than a decade already over 50 species have been successfully transformed; these species include such important crops as corn, rice, wheat, cotton, soybean, potatoes, oilseed rape, tomato and strawberry. The majority of the crops that have been successfully transformed are dicotyledonous species employing the *Agrobacterium* system. The recent discovery of accelerated microprojectile bombardment (Sanford et al. 1987) as a means of directly introducing genes into totipotent cells and organs has aided the genetic transformation of important cereal crops such as, corn (Gordon-Kam et al. 1990, Fromm et al. 1990), rice (Christou et al.1992) and wheat (Vasil et al. 1992, Kartha et al. 1992) which were once thought to be not amenable to genetic engineering owing to several technological constraints including the inability of *Agrobacterium* to effect transformation of these species. Even those species which fall within the host range of *A. tumefaciens* or *A. rhizogenes*, direct introduction of genes into the L_2 cell layer of shoot apical meristems has resulted in increased frequency of stable transformation as has been demonstrated for soybean (Christou, 1989).

It appears that the methods of gene transfer are now fairly well established. Using various gene transfer approaches, a few simple genes of commercial interest have been successfully transferred to agricultural crops. The choice of candidate genes for successful incorporation into agricultural crops are currently dictated by the availability of fully characterized genes, the traits which are under the control of single genes and the technological constraints involved in targeting and achieving high levels of stable expression. Nevertheless, considerable progress has been made in the incorporation of genes conferring resistance to several modern and ecologically safe herbicides (Padgette et al. 1989, Gasser and Fraley, 1992), a number of insect pests (Vaeck et al.1989, Delanny et al.1989) and viruses (Stark and Beachy, 1989). Although controversy exists regarding some of these approaches especially from an ecological perspective, considerable investments are being made by several multinational corporations and government laboratories in these areas of research. Transgenic plants of corn, soybean, potato, cotton, tomato, tobacco, and oilseed rape carrying one or more of the above genes have already been produced and are currently undergoing extensive field evaluation.

In addition to the above examples, several novel approaches are being pursued to modify agricultural crops with other kinds of agronomically useful genes. The Keystone Symposium on Crop Improvement via Biotechnology held in April 1992 at Keystone, Colorado, highlighted some of the major achievements made to-date in this direction. For example, Monsanto scientists demonstrated several-fold increase in starch content in potato by introducing a bacterial ADP-glucose pyrophosphorylase gene under the control of the tuber-specific patatin promoter. High starch levels are desirable for producing high fructose syrup and bio-ethanol. Furthermore, high starch potatoes are cheaper to process for French fries and chips (Kishore et al. 1992). In a similar approach to redirect plant biochemical pathways, scientists at Calgene have successfully produced two different types of oilseed *Brassicas* with unique fatty acid compositions. These workers employed anti-sense technology to suppress the activity of a specific endogenous desaturase to produce high stearate *B.napus*. High laurate *B. napus* was also produced through the insertion of a gene isolated from the California Bay tree. These products have potential uses in food and detergent industries (Knauf, 1992).

A recent major breakthrough in plant biotechnology is in the genetic engineering for fertility control for the production of novel hybrid systems. Plant Genetic Systems, a Belgium-based Company, has pioneered this technology. It involved the isolation and expression of a TA 29 gene from tobacco which is characterized by its extreme cell specificity in the tapetal cells of immature anthers. The 5' regulatory region of TA 29 was used to target the expression of ribonucleases such as barnase in the tapetal cells resulting in the precocious degeneration of tapetal cells, arrest of microspore development and thus the induction of male sterility. The induced male sterility could be reversed by the simultaneous expression of an inhibitor of barnase, known as barstar. This new hybrid system has been successfully applied to various crops including corn, oilseed rape, *Brassica* vegetables and cotton (Leemans, 1992).

In other developments, genes that control fruit ripening employing anti-sense technology have been successfully employed to delay fruit ripening process in tomato (Sheehy et al. 1992). Both biotic and abiotic stress components are being manipulated at the molecular level. Use of chitinase genes, ribosome inhibiting proteins (RIP) (Logemann et al., 1992), PR protein genes involved in systemic acquired resistance (SAR) (Glascock et al. 1992) and manipulation of osmo-regulatory compounds such as glycine-betaine at the molecular level (McCue and Hanson, 1990; Selvaraj-personal communication) are being investigated to produce crop plants with resistance to fungal diseases and desiccation and salinity stress, respectively. The current decade, in other words, is experiencing a great deal of excitement and it is anticipated that crop species with some of the valuable traits discussed above will be commercially available by the turn of the century.

A number of opportunities exist for cassava improvement employing transgenic technology. These include control of cyanogenic glucoside biosynthesis, augmentation of nutritional quality, incorporation of both biotic and abiotic stress

tolerance, control of such physiological processes as flowering and photosynthesis, production of hybrids, to name a few. One of the major impediments to applying certain biotechnology to cassava
improvement is primarily due to the non-availability of efficient plant regeneration systems from *in vitro* cultured cassava cells and protoplasts. This area of work, therefore, should be accorded top priority.

FUTURE APPLICATIONS

As discussed above, the current progress in plant biotechnology is attributable to the choice and incorporation of traits under the control of single genes. While this approach is bound to result in considerable gains in food production, some of the important traits such as yield and tolerance to stress conditions are under multigenic control. It is time now that attention be focused on the identification of such genes. The emerging technologies such as RFLP, RAPD and PCR are in fact trying to address this issue. At the biochemical and molecular level, more remains to be learnt about plant growth and development, structure, function and co-ordinated expression of agronomically useful genes. Even in cases where single genes have been expressed, there exists gaps in our understanding of the stability of gene expression as a function of transgene x environment interaction, position and copy number effects and even on spatial and temporal expression. In other words, regulation of gene expression would continue to assume a high priority status. In regard to transgenic technology, diversification of agriculture more attuned to the production of value-added compounds such as pharmaceuticals, immunotherapeutical compounds, growth hormones, neuropeptides etc. could become commercial realities of the next century. Finally, plant biotechnology would continue to assume an ever increasing role in concert with classical breeding techniques for the improvement of crop species.

CONSTRAITS IN APPLYING PLANT BIOTECHNOLOGY TO CROP IMPROVEMENT

In addition to the technical constraints briefly discussed above, a number of other constraints could be identified which could limit the wider use of biotechnology especially in the developing countries. Several policy-related issues have been and will be discussed in this meeting. Some of these include heavy resource investment required to develop the technology, the ownership issue or intellectual property rights, lack of dissemination of scientific results in strategic areas especially for crops like cassava which is mostly grown and consumed in the tropics by resource-poor small farmers and consumers. Other constraints relate to the lack of adequately trained scientific personnel, lack of regulatory guidelines for the release of transgenic material in most countries, adverse ecological concerns, and most importantly, public fear on the acceptance of biotechnology-derived products, especially food. Until such time that some of the above issues are addressed, the wider use of biotechnology for crop improvement in the developing countries may not be fully

realized. The meeting such as this of the Cassava Biotechnology Network could thus be considered as a step in the right direction.

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WORKING GROUP REPORT: CASSAVA GENOME STUDIES

Cartagena de Indias, 25-28 August 1992

The objectives of the working group on Cassava Genome were to get an update on the projects already initiated and to establish links between the various institutions interested in this area of research. The meeting was chaired by Joe Tohmé from the Biotechnology Research Unit, CIAT. Robert Aseidu from the Root and Tuber Improvement Program at IITA acted as rapporteur.

1. Update on research projects related to Cassava genome characterization and organization

(a) CIAT-University of Georgia (Mapping)
(b) Washington University (Phylogeny)
(c) CENARGEN (Phylogeny)
(d) IITA (Cytogenetics)

Work is progressing in all projects and training opportunities are available with RF support.

The working group strongly recommends that IITA continue its support for the cytological characterization of wild Manihot species and cassava x wild Manihot hybrids, through the expertise already available in the IITA TRIP. Floral buds from crosses made at CIAT, in Brazil, or elsewhere, with species not available at IITA can be shipped to IITA in vials of preservative for cytological examination at this central location.

2. Mapping populations, genetic stock and priorities for gene tagging.

Intra-and Interspecific populations available at CIAT will be boosted with additional controlled hybridization. The need to get on with the mapping using an existing population to produce a quick framework map was emphasized. It will be useful if the population selected had some specific agronomic traits to be tagged but this should not restrict the choice of population. Integration of information from various characterization projects is required. CIAT and IITA will finalize strategy for making a suitable population(s) available for mapping.

3. Distribution of available DNA libraries and characterized genes

Existing libraries include those at CIAT and University of Newcastle upon Tyne. Others being developed at e.g. University of Georgia. A centralized location and distribution is recommended. Extra funds from the CBN to support this activity within the BRU of CIAT is recommended.

4. DNA Fingerprinting

Agreed that most laboratories will continue to experiment with different strategies.

5. Regular section in future CBN Newsletter for reports on genome characterization and organization

This was agreed but further action was recommended. An update of available libraries, mapping projects, trouble shooting, etc. will be circulated in 1993. Electronic mail contacts will be set up among members.

6. Training

Rockefeller Foundation grants for placement of postdocs in collaborating laboratories will be considered for African, Asian or Latin American scientists to be trained in advanced laboratories. Training of scientists in laboratories like that of M. Hughes (U.K.), CIAT, Washington University (St. Louis, USA), University of Georgia and Cornell University were discussed.

7. Genetic Erosion

The issue was raised at the Cassava Genetic Resources Network meeting. It was agreed that it is too much to take on the issue at the moment. The WG will wait for some initiative from IBPGR.

8. Non-radioactive labelling

CIMMYT has had some success on maize and beans as has IRRI for rice. CIAT will develop the technique for cassava, beans, rice, and tropical forages using systems like the Genius kit from Boehringer-Manheim. CIMMYT and CIAT are in contact with BRL to improve kits for the technique. It was recommended to pursue the discussions between CIAT and BRL, a company with major involvement in development of non-radioactive labelling systems.

9. Database

Needs to be built up from systems being generated mainly by the USDA, perhaps using Tanksley's format in the meantime.

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-recently, the presence of fungi and bacterial endophytes in in vitro cultures has been shown. It is a real concern as it is extremely easy to transform bacteria, although some groups claims to be able to differentiate between the two types of transformation.

-the latest generation of the helium particle gun is not damaging and easy to build. The plans can be provided to the people interested (from Dr. Sayre).

-bombardment of meristems in conjunction with multiple shooting protocols is being used as an alternative to embryos transformation.

-transformation by electroporation is being investigated in one or two groups.

RECOMMENDATIONS

- **Regeneration**

-though the somatic embryogenesis regeneration system is well established, it is recommended to improve it in terms of regeneration efficiency and to apply it to recalcitrant cultivars;

-alternatives to somatic embryogenesis regeneration should be encouraged;

-histological studies to provide conclusive evidence of the multi or monocellular origin of the primary and secondary embryos should be carried out.

- **Transient assays**

-a non-destructive marker to replace the existing GUS marker would be greatly appreciated for cassava transformation; however, this is not specific to cassava transformation and therefore should be considered as a general recommendation;

- **Promoters**

-studies leading to the isolation and characterization of cassava and tissue specific promoters should be recommended.

- **Transformation**

-the three techniques (*Agrobacterium*, particle gun and electroporation) have to be tested for cassava and consequently all their improvements should be encouraged until more information should be available.

- **Selection**

-because selection is a major issue in the regeneration of fully transformed plantlets and because the production of chimeric plants is not considered as a suitable solution, it is

strongly recommended to develop the utilization for cassava of new selectable markers. In this respect a non-destructive marker gene would become a specific recommendation for cassava.

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WORKING GROUP: POST-HARVEST DETERIORATION OF CASSAVA

Cartagena de Indias, 25-28 August 1992

An interdisciplinary strategy to tackle the problem of PHD was presented. The elaboration of this strategy followed an initiative by FAO, and is based on the conclusions of a discussion group which met in Rome in December '91. The reasons for the selection of this integrated approach which includes molecular biology, physiology and biochemistry, were presented. These include the limited genetic variability found so far, though intensive screening for this trait has not been performed. Varieties which do not deteriorate after eight days have been found, as compared to others that start deteriorating after 24 hours. This is not a consistent trait however, it will vary with growth season and location.

It is not known how many genes are involved in this trait. A breeding approach would most probably take a long time, considering the highly heterogeneous genetic background of cassava. Additionally, introgression of the trait would have to be conducted for every cultivar developed by the breeding program, thus most likely limiting the introduction of the trait to only some widely used cultivars, due to the dimensions of such a task.

The biochemical basis of PHD offers several points for experimentation. It is recognized that we have got the tools to manipulate the different pathways leading to the biosynthesis of the compounds that lead to deterioration. Biosynthesis of coumarins, like scopoletin, and other phenolics are involved in the normal wound healing processes as well as in the development of physiological deterioration. Also the flavonoids, which are a constituent of the polymers that appear at the later stages of physiological deterioration, are derived from the same biosynthetic pathway, namely the phenylpropanoid pathway. Here we recognize well defined branching points for intervention.

Cassava roots are capable of producing a localized wound response under high relative humidity conditions. This response could be manipulated to take place under normal harvest conditions and its speed increased, to protect the tissue from microbial deterioration.

Discussions centered mainly around the desired shelf life and the relative priority of this issue as compared to other constraints, in the light of already existing traditional ways of handling fresh cassava, e.g. burying, pruning, and processing of cassava. These alternatives are obviously not suitable for transportation of fresh cassava to markets from remote production areas into the cities.

The solution to this problem is aiming at dramatically changing existing practices. A longer shelf life will open new markets for producers, relieving them from pressures imposed by the deterioration process. We also should start thinking about new avenues of how to respond to the pressure of an ever growing urban population. A longer shelf life will offer a higher degree of flexibility to the small producer as well as to the

processing plants, lowering the risk imposed by PHD. The study of the potential impact of increasing the shelf life of cassava should involve the input of socio-economists, who as proposed in the interdisciplinary approach, will be integrated into a feedback loop so as to redirect the priorities of the project where necessary.

The discussion showed that the ex ante analysis is not an easy one, as we are talking about the opening of completely new perspectives and new markets, on top of facilitating existing markets. The potential seems great, but will depend on a great amount of extension work as soon as the scientific goal is achieved.

The feasibility of such a goal was discussed with regard to the example of tomato, a highly perishable crop, whose shelf life could be extended to several months by manipulation of ethylene biosynthesis utilizing several diverse genetic engineering approaches. Cassava is not a climacteric fruit that can be manipulated using this approach, but what the tomato example shows, is that biosynthetic pathways can now be manipulated in a very specific manner. Moreover, the transgenic approach could provide solutions that would be universal and robust, i.e., less affected by environmental variation that is the natural variance of PHD.

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WORKING GROUP REPORT: INTEGRATION OF END-USER PERSPECTIVES IN THE CBN RESEARCH AGENDA

Cartagena de Indias, 25-28 August 1992

There is general consensus within CBN that participation of end-users in the R & D process is critical to an efficient use of research resources, to successful research design, and to adoption and desired impact of technologies. The goal of the Working Group was to move forward from this point of agreement through assessment of available and appropriate methodologies for end-user integration; current end-user integration in the CBN research agenda; and additional strategies for end-user integration within the CBN agenda.

OBJECTIVES

The organizers of the Working Group proposed three questions or tasks to the participants:

1. Does all biotechnology research require a high level of end-user integration? Assess the "level" of end-user integration, by technology.

2. What are appropriate methodologies for end-user integration in the CBN's research prioritization process?

3. Assess current end-user integration by technology and institute and develop a matrix of current involvement

DISCUSSION SUMMARY

Two levels of "users" of biotechnology were identified, and two corresponding levels of end-user integration:

At the first user level are scientists who are generally users of biotechnological tools such as transformation systems or cryopreservation, and of intermediate products that are tools for further ends, such as gene constructs or genetic maps.

Needs of scientists in NARDS in developing countries may be different from needs of scientists in developed countries, due chiefly to differences in their working conditions.

At the second user level are not only farmers but also small-and large-scale cassava processors and rural and urban consumers. These groups use finished products

(whether direct from biotechnologists, or, more often, indirectly through agricultural researchers) such as crop varieties, useful microorganisms, or bio-control agents.

It was noted that CIAT and IITA have served the CBN since its founding in 1988 as conduits of information about end-user needs. The information provided by the two centers has been obtained through the work of socioeconomic staff and through knowledge gained in collaborative projects between cassava commodity program scientists and NARDS. Thus although end-user integration in formulation of the CBN research agenda will be conducted in a new mode, incorporation of end-user needs in CBN priorities has been an innovation and strength of the network approach since its beginning.

Most participants considered that the CBN is best advised to integrate the needs of the second user level in its research prioritizing process by increasing its active contacts with existing structures and networks, and particularly with NARS having strong farmer/processor contacts.

* Because collaboration with NARDS is its preferred mode of end-user integration, CBN should identify NARDS with strong farmer/processor contacts through which to work;

* Strong farmer contacts in any given NARDS may exist, or not, or exist but need strengthening

* CBN is not advised to set up its own independent structures for end-user contact and integration; this is too complicated for CBN and for NARDS, who are already dealing with a multitude of organizations

* The CBN Coordinator is responsible for verifying that NARDS with which CBN cooperates do have effective communication with their clients/beneficiaries/end-users

* Farmer/NARDS contact, or farmer/scientist communication of any kind, may be most deficient in the poorest countries, i.e., where it is most needed

* If the CBN detects gaps where NARDS/research end-user contacts are lacking but critically needed, it may consider specific limited direct action in those cases

MAJOR ISSUES RESOLVED

- The CBN should be involved with user integration at both levels: researchers and farmer/processors.

- The recommended mechanism for farmer/processor integration in the CBN research planning process is contact with NARDS having strong clientele interaction.

- In general, CBN is not advised to develop independent structures for end-user contact and integration. In a case in which a critical gap was identified, direct measures to obtain end-user input may be proposed.

- Current integration of end-users in cassava biotechnology research planning was not systematically assessed; however, it was noted that a beginning was made at CIAT and IITA as early as 1988 and before.

MAJOR ISSUES REQUIRING FURTHER DISCUSSION

The following issues will need further discussion as the CBN proceeds to carry out the recommendations of the Working Group:

- How should CBN establish a dialogue with NARDS on both levels of integration?

- What mechanisms should CBN use to permit feedback from both levels to set its priorities?

- What kind of monitoring and evaluation system is needed for new biotechnology research?

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CBN STEERING COMMITTEE MEETING REPORT: HIGHLIGHTS OF THE CBN SCIENTIFIC MEETING

Cartagena de Indias, 25-28 August 1992

This first meeting of the CBN is undoubtedly a success. The number of participants (125) and the 75 communications and posters show a growing interest for cassava biotechnology in the international community, reenforcing the need for a network dedicated to the biotechnology of cassava.

Interestingly, not all papers presented strictly biotechnological data. There was a good balance of presentations on several disciplines and a much greater number than in 1988.

It is obvious that biotechnology will play an increasing role in cassava germplasm activities, especially in conservation and characterization. In the past, biochemical markers helped to identify duplicates in the cassava collection. DNA marker techniques have developed greatly since 1988, to allow further characterization and reduction in the number of accessions required. Cryoconservation has been developed and seems very promising although long term experiments will be necessary before routine applications will be made. For seed and pollen conservation of cassava, further studies on flowering are envisaged.

There is an increasing interest in Manihot wild species for breeding purposes and also as a source of new genes for biotechnological manipulation. Studies on molecular genetic markers for a better understanding of the evolution of the different Manihot species have been initiated and will be reinforced in the coming years. What has been done already shows the occurrence of large polymorphisms, both intra- and interspecific. The evaluation of the molecular and biological variability of pest and diseases of cassava will be absolutely necessary for the application of biotechnology. Several studies have been initiated in this respect but efforts will have to be expanded.

Four years ago, it was recommended that molecular maps of cassava should be developed and some progress has been made. The techniques involved are changing rapidly, becoming quicker and cheaper so that maps can be generated in increasingly shorter times. Because the quality of a map greatly depends on the genetic stocks used, and the range of molecular markers available, collaborative interchanges of plant material, molecular tools, and information were agreed to by workers in this field. This interchange represents a major contribution to the future development of this technology in cassava.

Concerning cassava regeneration, development of new routes of regeneration other than somatic embryogenesis has not been achieved. A research program on protoplast regeneration started a few months ago but the results are not yet available. However, in the area of somatic embryogenesis significant progress has been made;

recalcitrant cassava cultivars are now producing embryos with satisfactory efficiency and improvements have been realized in the mass production of embryos. Embryo suspension studies are promising and should provide new possibilities for transformation in the near future, but other systems such as cell suspensions and organogenesis should be developed.

Transformation, strongly recommended in 1988, is the bottleneck for the use of genetic engineering to improve many traits in cassava. Although transgenic cassava plants are not yet available, there is now a critical mass of researchers dedicated to this task and progress has been made in this research area. Transformation of cassava cells has been proven and chimeric somatic embryos are regularly obtained for several cultivars. A range of transformation methods is currently used on cassava and, together with the improvement of regeneration, should produce transgenic plants in a reasonable time frame.

Virus control by the coat protein strategy has been established or is under improvement on tobacco. Gene expression in cassava cells has been evaluated and direct application will follow when transformation is available.

Concerning quality of cassava roots, there has been significant progress in the acquisition of biochemical knowledge. We now know much more about cyanogenic compounds, protein quality, and starch content. The biochemical pathway for cyanogenesis has been described and genes coding for several key enzymes have been cloned. The desirability of protein content increase with regard to human nutritional value needs further consideration with regard to socioeconomic and acceptability criteria. Modifications in the protein type and content are more likely to have an immediate impact with regard to crop protection and root quality changes. There are a number of tuber storage protein genes and starch enzyme genes which could be used for cassava as soon as the transformation protocols become available.

Post-harvest root deterioration was considered four years ago to be of only moderate priority with regard to biotechnological approaches. This reflected a lack of knowledge about the basic biochemistry of the deterioration process together with the success of empirical approaches for delaying this deterioration. A multi-disciplinary project has been proposed to further investigate the basic biochemistry of deterioration utilizing recent advances in genetic methodologies.

Cassava production has increased substantially in recent years in Africa, where cassava is usually eaten following processing, of which a wide variety of procedures exist. R & D projects are required to further characterize these diverse processing procedures with regard to optimizing quality, acceptability, and nutritional value. Several participants stated that more emphasis should be put on downstream research in cassava biotechnology as related to processing.

A key event at this first CBN meeting was the presentation on the importance and impact of cyanoglucosides upon human health. Cassava cyanide usually only causes health problems in crisis circumstances or changed socio-economic circumstances. For example, droughts and famines or rapid socio-economic developments lead to modifications in cassava utilization patters reflected in enhanced residual cyanide levels. These conditions are often accompanied by other dietary stresses (commonly, reduced protein levels). This combination of factors promotes the health problems reported such as konzo. These results emphasized the need for further R & D to characterize cassava storage and processing procedures and their effects on residual cyanide. The priority associated with the study of cassava cyanogenesis at the 1988 meeting was concerned with the nutritional impact of cassava use in Africa. This priority now relates to more general concerns of cassava physiology and the role of cyanide in pest management.

This re-evaluation of the basis for priority setting illustrates the need for multidisciplinary research in order to target appropriate R & D projects. Studies are also necessary to understand the reasons for the success and failure or previous biotechnological projects. Scientists should adopt more a holistic view enabling them to participate in policy dialogue. Socio-economists have an important role to play in priority setting or R & D programmes.

Concerning priorities, within six months the Technical Advisory Committee of the CBN will take into account the recommendations made during this scientific meeting to reconsider the priorities established four years ago. This committee will be pleased to receive additional comments. In the future priorities will be reconsidered at each scientific meeting and reevaluated according to new information and knowledge.

There was also a consensus about the fact that the CBN should not duplicate existing structures and networks. The CBN should be an interface between biotechnology and other sciences and therefore should build and interact upon the existing structures.

The discussions of the week, sometimes passionate, proved that the CBN is already a success. Such meetings allow the possibility to acquire information from different fields of expertise and consequently allow progress and growth.

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