ANNUAL REPORT 2005
PROJECT IP-06

Tropical Fruits, a Delicious Way to Improve Well-being

CIAT
Centro Internacional de Agricultura Tropical
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CONTENTS

1. Introduction

The Tropical Fruit program is under new management since June 2005. Funding for the program is still highly dependent on special projects, a funding strategy that is foreseen to continue in the near future. Current funding for the project is based on special projects financed by the Rockefeller Foundation and the Colombian Ministry of Agriculture (MADR), and a very small proportion of core funding. Money from MADR was successfully assigned to projects lead by CIAT under a competitive grant scheme. The current research projects respond to needs identified by the fruit industry and will deliver solutions to production problems affecting the industries of Avocado (Persea americana), Lulo (Solanum quitoense), Mango (Manguifera indica) and Plantain (*Musa paradisiaca*).

The program's mission continues to be to: *"to use science, technology and modern information technology to provide information and support to partners in the public and private sector that promote production, processing and marketing of tropical fruits by rural communities which leads to increased wealth and improved welfare for present and future generations in the countryside."*

The strategy to follow in the near future is to develop case studies where the principles of participatory variety selection, application of biotechnology for improving planting material, strategies for sustainable crop and disease management, and access to markets are considered and the impact generated by these projects be evaluated.

The program will continue to focus its efforts on:

Developing case studies to demonstrate the proof of concept for using Homologue, a software that will assist in targeting which crops or cultivars will grow well in particular conditions

Selection and propagation of elite materials already grown by farmers (different species which has strong commercial value in the local markets, and has potential export market; i.e members of the solanacea family)

Developing alternative pest and disease management useful to small growers (IPM, biofungicides, biopesticides)

2. In vitro Propagation and Regeneration of *Solanum quitoense* (Lulo) Plants and their Use as Elite Clones by Resource Farmers

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2.1 Introduction

Solanum quitoense, also known as lulo in Colombia and naranjilla in other countries, has great potential to become a premium product for local and export markets. Recently, in Colombia, lulo evolved from being a fruit for local fresh consumption to become an industrial high value crop as ingredient of juices, yogurt, flavoring and processed food increasing its market value. Various diseases and pests affect its production, and plant breeding is at a young stage. A major constraint for the rapid adoption of lulo by the local farmers is the limited availability of elite clonally propagated germplasm free of pathogens. Rapid clonal multiplication of high-quality planting materials is of paramount importance to obtain uniform elite plants. Genetic transformation could also facilitate splicing in genes for traits of interest. The objective of this work is to develop *in vitro* protocols that facilitate *(a)* the conservation of germplasm, *(b)* the multiplication and distribution of healthy elite clones selected by farmers, *(c)* the evaluation of *in vitro* propagated plants vs. sexual seeds propagated plants in farmer's fields and *d)* the genetic transformation of this crop. This year report summarizes the progress attained in the field evaluation of *in vitro* propagated elite clones selected by experienced farmers in two commercial regions of Colombia using participatory research approaches; with the aim of comparing in the field the performance of the *in vitro* plants with those conventionally propagated materials through seeds. The current report also describes enhancement of plant regeneration protocol suitable for high efficiency genetic transformation.

2.2 Materials and methods

2.2.1 Plant Evaluation by Farmers in the field.

The *in vitro* propagated plants, from original elite clones selected by farmers in their fields, were established from greenhouse grown materials. *In vitro* plants were sub-cultured in order to sufficient material to conduct replicated trials. Conditions were standardized in order to have *in vitro* plants in a similar stage of development for the field trials. Thus, *in vitro* plants were grown for 30 to 45 days prior transferring them to the greenhouse for a stepwise process of acclimatizing for about 15 to 20 days, after which were taken to the field experimental sites for a final acclimatization period of about a week before planting them in the field. On each experimental field site, 26 *in vitro* propagated plants and 15 seed-derived plants of each originally collected clone were planted following a randomized split-plot design. Farmers from two commercial production areas of Colombia (Cauca and Huila) evaluated the plants performance from planting to fruit harvest. Each field-testing orchard was considered as a block and the global test had a split-plot design. Under each experimental site, the propagation systems (*in vitro* and seed) and the genotypes constitute the split-plots or sources of variation. This design allows farmers to compare the genotype and propagation system in a more

objective form. The traits evaluated included plant survival upon transplanting in the field, days to flowering and fructification, as well as fruit productivity and quality.

2.2.2 Plant Regeneration.

Experiments were performed using *in vitro* propagated plants. Shoots were obtained from plants of a clone selected in the experimental field; this particular clone has shown good response to plant regeneration (Ruiz *et al.*, in SB2 Annual Report 2003). Petioles were identified as the most responsive explant for plant regeneration as compared to leaves (Segovia, 2002). Petiole explants were cultured, without and with a sonication treatment for 1 minute, using a Branson 450 Ultrasonic Corporation apparatus, followed by vacuum at 500 mmHg for 15 minutes. Eight petioles explants were cultured per Petri dish. A total of 120 explants were sonicated and 120 were cultured without sonication per experiment. Four experiments were conducted. Explants were placed axial side down on solid regeneration media (Ultzen, *et al.*, 1995).

2.3 Results and discussion

With the participation of farmers a process was initiated to evaluate the advantages of using *in vitro* propagated plants as planting materials to establish new crops. The potential advantage of the *in vitro* source is the supply of pathogen-free, homogenous plants, maintaining the selected traits of the elite materials. During the first month, 100% of plant material (*in vitro* and seed) was grown successfully in 3 of 4 testing orchards at the farms. In this report, we have available information from two different locations in Pescador, Cauca that were selected to conduct this study (Ruiz *et al.*, in SB2 Annual Report 2004). According to the farmers, the *in vitro* generated plants showed higher vigor, earlier development and rooting respect to seed propagated plants. These observations were corroborated by the statistical analyses. Significant differences in plant development and performance were found between the *in vitro* and conventionally propagated plants. *In vitro* propagated plants initiated flowering and fructification earlier than seed-derived plants (Figure 2.1). Crop productivity was affected by water stress during the dry season period between July to October 2005, however, we have available production dates of 10 *in vitro* clones and 5 seed-derived clones (Figures 2.2 and 2.3). There are not statistically significant differences between production and number of fruit, per plant. It was also found that the *in vitro* clone JY-E1 showed the highest productivity. This clone was selected by farmers from Tierradentro-Cauca and then was evaluated in a different location (Pescador-Cauca). Farmers from Pescador-Cauca are very interested in having *in vitro* propagated material in their fields and they think that with the appropriated weather conditions, they will have a good development.

Earlier work by our group reported increased plant regeneration when only using sonicated petioles from clones with thorns in contrast to leaf segments. Field evaluation of regenerated plants indicated that lulo plant growth and development appears not to be affected by the organogenesis process (Segovia, 2002). Recent results indicate that it is possible to increase the efficiency without sonication. These results seem to indicate that the physiological condition of the donor plant has major influences on the explant response to plant regeneration. A high efficiency of plant regeneration was obtained

when juvenile material was used increasing the response near to 80% without sonication (Figures 2.4 and 2.5). Not statistically significant differences in the number neither of explant response nor in the number of plants regenerated are seen with or without sonication. (Figure 2.6). Approximately 50% of explants regenerated after 4 weeks of culture, and the highest regeneration rate from petioles was observed at 6 weeks (Figure 2.5). It was also found that petioles are the best explants. Results suggest that it is possible to use this protocol without sonication for genetic transformation avoiding the physical damage that sonication causes to the explants (Segovia, 2002).

2.4 Conclusions

A dedicated, enthusiastic and self-motivated group of farmers are very interested to grow *in vitro* propagated plants in their fields. The application of *in vitro* propagation technology reduced flowering and fructification time in about 1 month with respect to plants propagated through botanicals seeds used by farmers without affecting crop productivity and fruit quality. An optimum direct plant regeneration procedure via organogenesis using petiole explants without sonication was established. This protocol is suitable for the development of gene transfer technologies for this species, and is being considered on the future development of an *Agrobacterium*-mediated transformation protocol for lulo.

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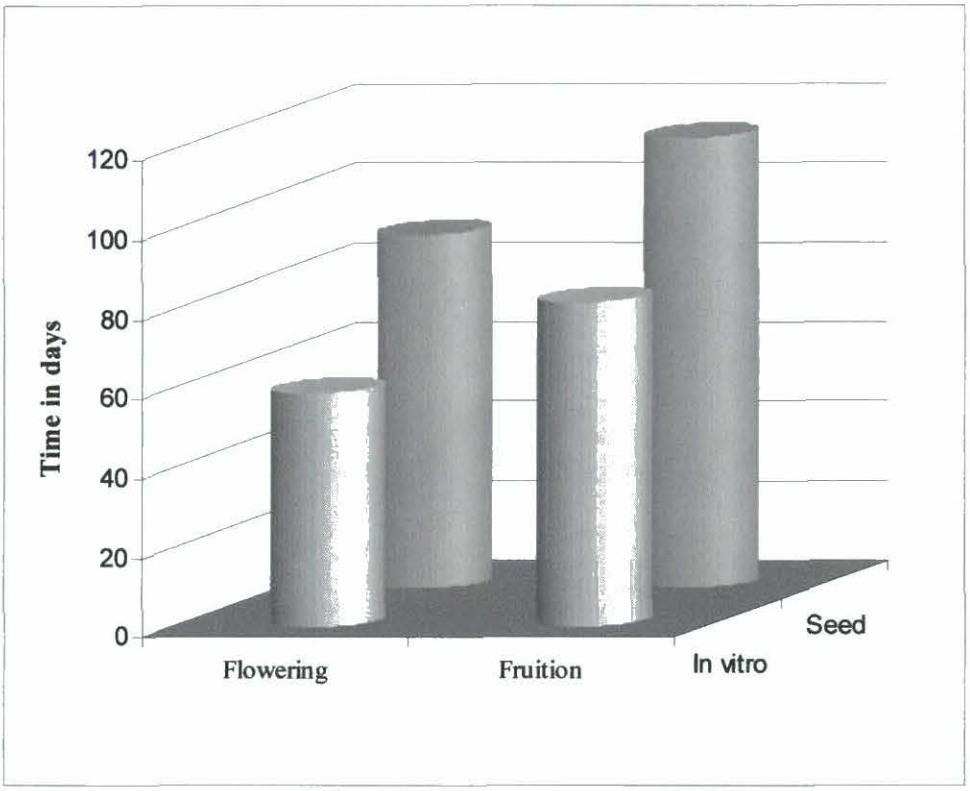

Figure 2.1. Days to flowering and fructification in the field (Pescador, Cauca) of plants propagated *in vitro* or from botanical seeds

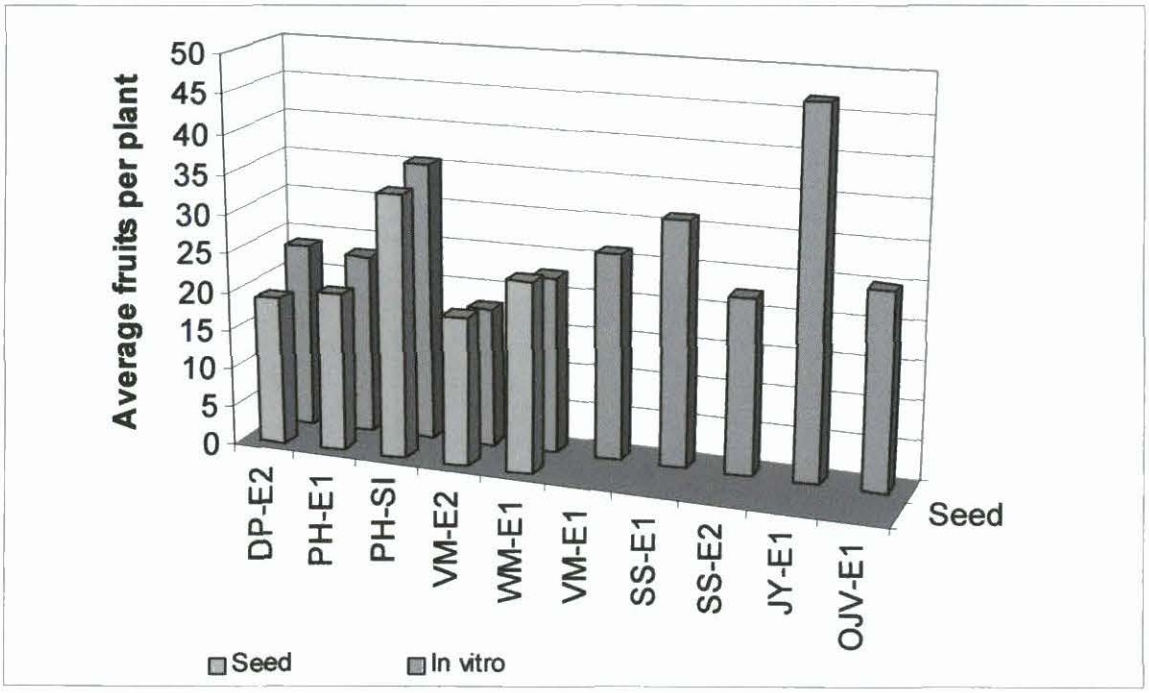

Figure 2.2. Average of number of fruits per plant from *in vitro* propagated plants and seed derived plants (Pescador, Cauca)

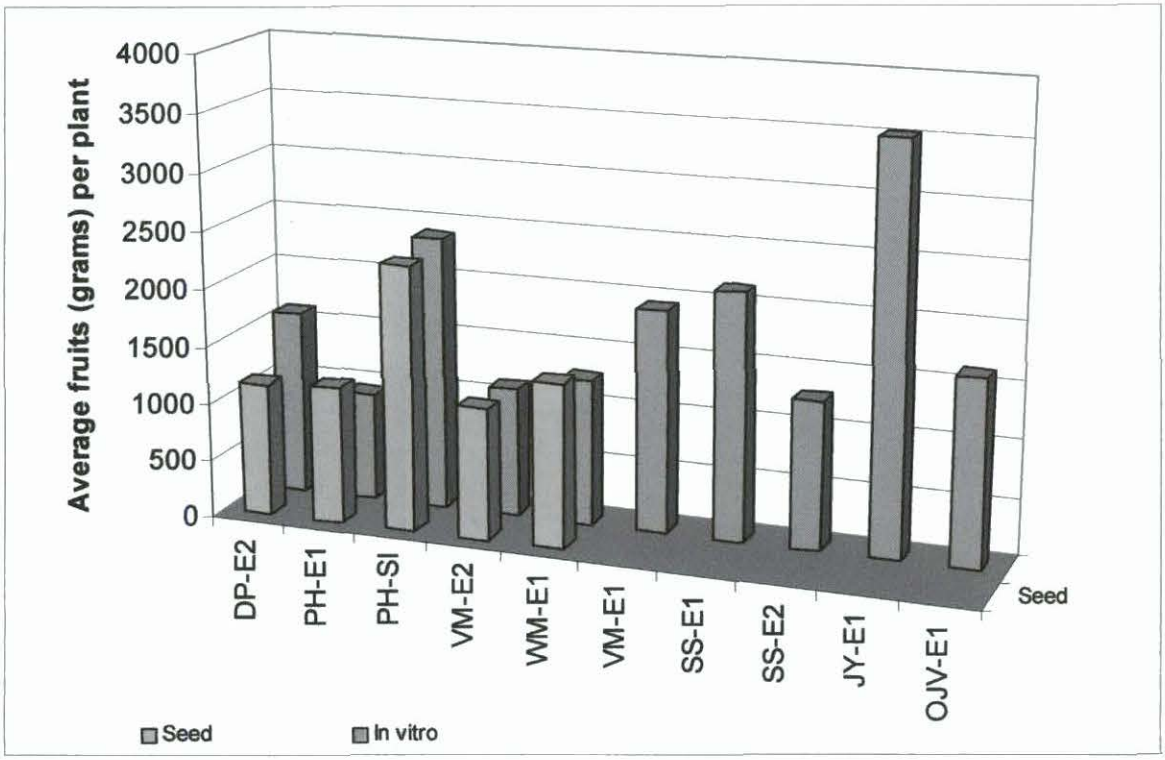

Figure 2.3. Average of production per plant from *in vitro* propagated plants and seed derived plants (Pescador, Cauca).

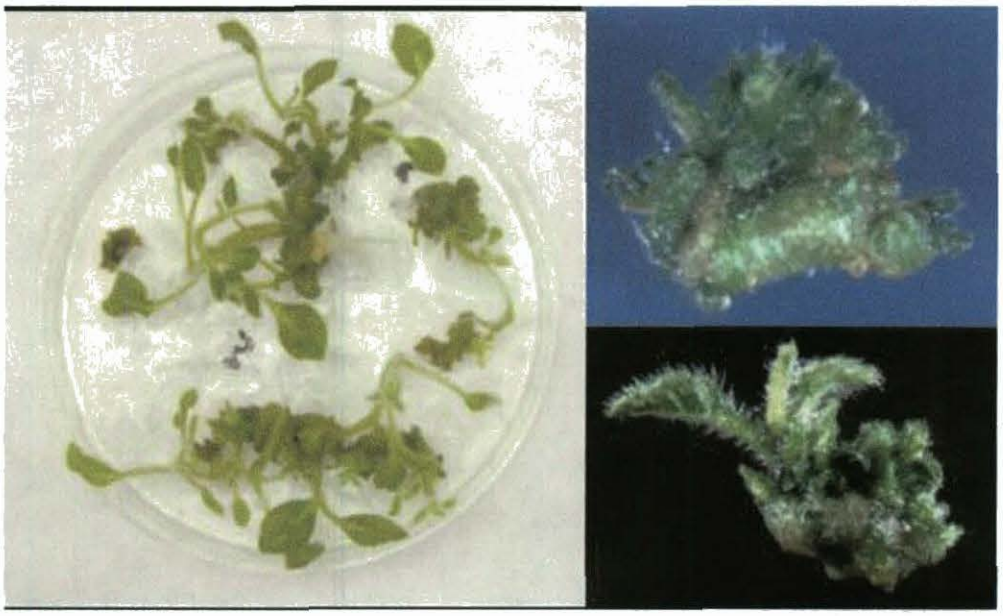

Figure 2.4. Plant regeneration from petioles without sonication. Multiple shoots formation throughout the explants.

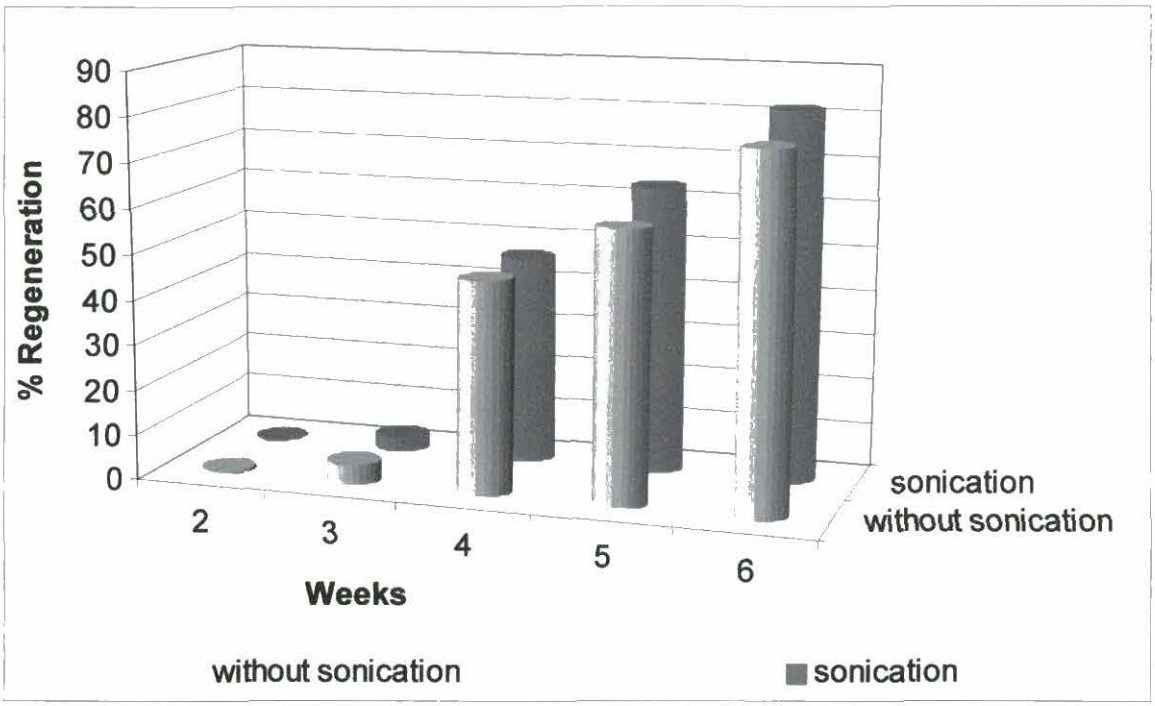

Figure 2.5. Plant regeneration from petioles of lulo (*Solanum quitoense*) with and without sonication

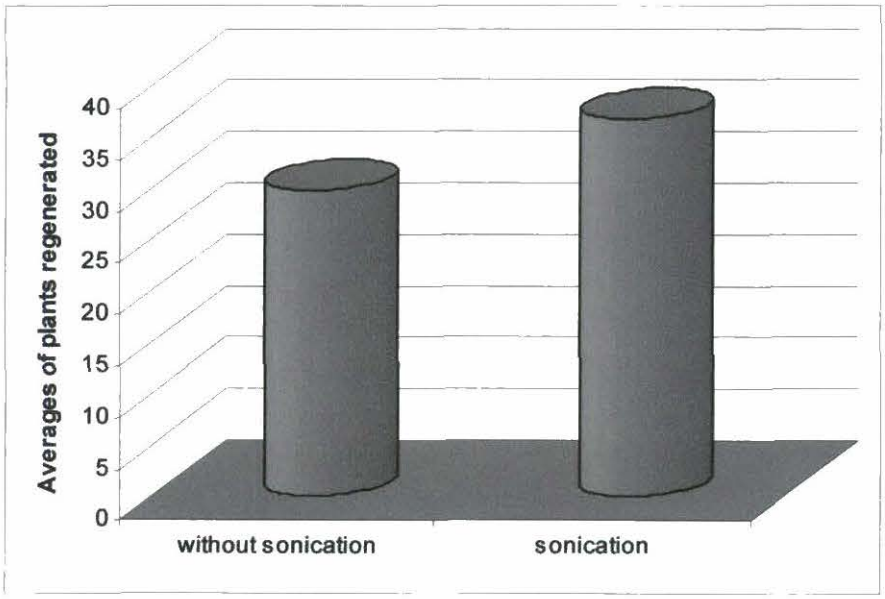

Figure 2.6. Plants regenerated from petioles of lulo (*Solanum quitoense*) with and without sonication

3. Somatic Embryogenesis and Plantlet Regeneration of Mango (*Mangifera indica L.*).

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3.1 Introduction

In the last 15 years there has been a tremendous increase in areas dedicated to mango production in the tropics and subtropics. Mango production is appealing because the fruit is nutritionally important and constitutes an attractive option to increase income and reduce poverty in the rural sector of developing countries.

Despite its importance and worldwide distribution, mango suffers from a long juvenile period, erratic flowering and alternate bearing habits. In fruit crops, control of flowering is a critical aspect in the production system, since it determines the seasonality of fruit supply to the market. One of the great advantages of the tropics is the possibility of producing during the whole year, nevertheless fruit producers and markets face major challenges to supply fruits of high quality throughout the year. Biotechnology can potentially be used to manipulate existing cultivars by targeting specific genetic traits, such as flowering behavior. If farmers were to gain the ability to control flowering in fruits, they could better target their produce to markets with narrow windows of opportunity.

This research aims to manipulate the expression of a target set of developmental genes known to modulate flowering. An efficient protocol for genetic transformation is needed in order to splice in genes for flowering control and other valuable traits of interest. However, to apply these tools, highly efficient embryogenesis and regeneration protocols are required.

3.2 Materials and methods

As preliminary work, published protocols for somatic embryogenesis induction, proliferation of proembryogenic masses, embryo maturation and plantlet regeneration were tested (DeWald et al., 1989a-b; Pateña et al., 2002; Rivera-Dominguez et al., 2004 and Xiao et al., 2004). Immature fruits (40-50 days after anthesis) were colleted from commercially grown mango plots from various regions in Colombia. Four monoembryonic Florida cultivars (Keitt, Tommy Atkins, Kent and Irwin), and nine polyembryonic Colombian cultivars (Magdalena River, Yulima, 505, Jobo, Azúcar, Arauca, Manzano Vallenato and Sufaida) were chosen. The Florida cultivars were selected because are commercially important in most developing countries including those in Africa, the main target region of this project.

Immature fruits were washed and disinfected with commercial bleach 20% (v/v) for 20 min, supplement with 3 drops of Tween 20, then washed three times with sterile, distilled water. The length of each immature fruits was determined before dissection, and the length of the zygotic embryo within each ovule was determined prior to its removal.

Ovules were bisected symmetrically and the nucellus was separated from each ovule and transferred to the induction media. The embryogenic response was evaluated as number of nucellus producing proembryogenic masses or somatic embryos directly.

3.3 Results and Discussion

Nucellar tissues cultivated on Pateña et al., 2002's formulation exhibited the best embryogenic response. All of the cultivars tested showed some level of somatic embryogenesis induction. Thus apparently the response was independently of cultivar used, in contrast to previous studies (Litz et al., 1998; Rivera-Dominguez et al., 2004).

The highest embryogenic response was observed in Keitt (48%), followed by local cultivars 505 (34%), Jobo (31%) and Magdalena River (20%), and Kent (12%). On Keitt, proembryogenic masses were observed on about 2-3 weeks after culture (Fig. 3.1 k). Polyembryonic cultivars, such as Magdalena River, induced somatic embryos directly from the nucellar tissue approximately four weeks after culture on induction medium (Fig. 3.1 f). Somatic embryos at different developmental stages of Magdalena River, Yulima, Keitt and cultivar 505 have been obtained (Fig. 3.1). Somatic embryos of Magdalena River reached cotyledonary stage in about three months (Fig. 3.1 h). At present, some somatic embryos developed roots (germinated) (Fig.3.1 j). Embryogenic cell suspension cultures of Keitt and Magdalena River are being established.

3.4 Future Works

- To establish protocols to improve plantlet acclimatization of mango developed *in vitro*.
- To initiate genetic transformation tests.

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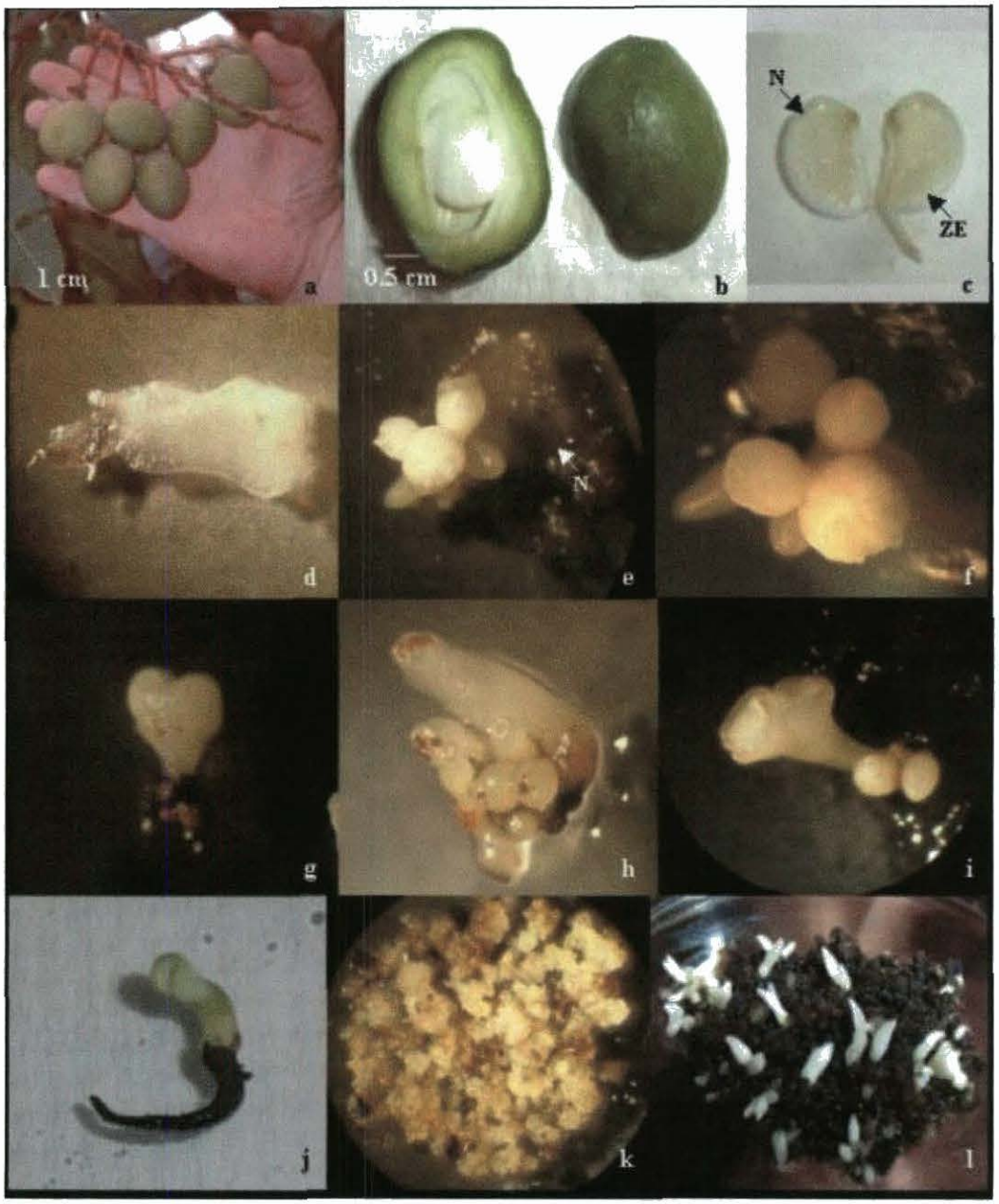

Figure 3.1 Somatic embryogenesis from nucellar tissues of Keitt and Magdalena River cultivars of mango. *(a-j)* Somatic embryogenesis of Magdalena River Mango. *(a)* Immature Fruits, source of nucellar explants; *(b)* immature fruits cut longitudinally; *(c)* ovules cut open lengthwise showing nucellus and zygotic embryo; *(d)* nucellar tissue isolated; *(e-f)* globular somatic embryos; *(g)* somatic embryo at heart stage; *(h)* torpedo somatic embryos; *(i)* cotyledonary somatic embryo; *(j)* germinated somatic embryo *(k)* proembryogenic masses from nucellar tissue of Keitt; (l) somatic embryos of Keitt at different developmental stages. N: nucellus; ZE: zygotic embryo(s).

4. Integrated Pest and Disease Management

4.1 Colombia's campaign to save plantain from moko Farmers and scientists join forces to counter bacterial wilt epidemic sweeping number 2 crop

By Gerry Toomey, science writer, Green Ink Publishing Services Ltd., Dec. 2004, e-mail g.toomey@cgnet.com; tel.1-819-827-1426

Smallholder farmers in Colombia have been working side by side with international and national agricultural scientists and extension agents for the past 3 years to save their plantain stands from bacterial wilt. Their collaborative campaign against *bacterial wilt*, as this highly destructive disease is known in Latin America, has fortunately begun to pay off – but none too soon.

Among the more promising weapons in the emerging *bacterial wilt*-management arsenal is a biopesticide that does double duty as an organic fertilizer. The liquid, called a "lixivium," is produced inexpensively on- farm by composting plantain residues, specifically the hanging, spine- like shafts called rachises from which the flowers and fruit protrude. This is the part of the plant that farmers routinely discard after harvest. Its systematic decomposition into a useable lixivium biopesticide takes about 75 days.

Typical symptoms of *bacterial wilt* in plantain.

More environment-friendly than conventional chemical treatments, the lixivium contains microorganisms that kill *Ralstonia solanacearum*, the bacterium responsible for bacterial wilt. It is also effective against black sigatoka, a fungal disease that attacks plantain and banana, and against powdery mildew in roses. As such, the biopesticide shows significant commercial promise, well beyond its current use by small-scale plantain growers.

Once the lixivium is collected from the composter, all that remains is a small quantity of solid organic material. This final solid residue is not used as a fertilizer or biopesticide as it may still harbor *bacterial wilt*-causing bacteria. Rather, it is left in the composter to maintain the decomposition process as new crop residues are added.

"We wanted to give the farmers simple, easy-to-use solutions because they don't like complex technology," says Silverio González, director of FEDEPLATANO, the National Federation of Plantain Producers of Colombia and the chief designer of the composting system. "Our members prefer to solve problems using their own local resources, so they don't have to spend too much money." González, himself a farmer, notes that the use of certain "green manures," as well as fertilizer rich in calcium and phosphorus, have also been shown to suppress *bacterial wilt*-causing bacteria in the soil. Green manure is any fresh, leafy vegetable matter, even whole plants, purposely incorporated into the soil to improve its fertility or other properties.

4.2 Club del Moko

FEDEPLATANO is just one of several public and private stakeholder groups that form the *Club del Moko*, a broad alliance working on *R. solanacearum* diagnostics and the design and testing of disease-control measures. Other key alliance members include the International Center for Tropical Agriculture (CIAT), the Colombian Institute for Agriculture and Livestock (Instituto Colombiano Agropecuario, ICA), the Colombian Corporation for Agricultural Research (Corporación Colombiana de Investigación Agropecuaria, CORPOICA), and the Internacional Network for the Improvement of Banana and Plantain (INIBAP).

A field day with the *Club del Bacterial wilt.*

The rationale for creating this forum for cooperation was simple and compelling: *bacterial wilt* was and is a national agricultural emergency. The disease, which sometimes causes total crop losses, now affects most or all plantain-growing regions of Colombia, including the highly productive central coffee zone. Annual plantain losses have been estimated at US$5.8 million. In 2003, CORPOICA estimated that 68 percent of

a plantain- growing area it had investigated in Meta Department was infected. It is clear, say researchers, that in the absence of concerted intervention, the spread of *bacterial wilt* could easily halt plantain production in many parts of Colombia in just a few years.

Several years ago, as the *bacterial wilt* problem steadily worsened, farme rs approached ICA and other institutions for help. A loose association of farmers, government and university researchers, and private agrochemical companies grew out of that contact. But unfortunately, they couldn't solve the problem.

By this point, producers were also quite worried about the ill effects, on human health and the environment, of continuously applying Formol (formaldehyde), one of the recommended pesticides for killing bacteria in the soil. The economic repercussions also concerned them since Formol's high toxicity rendered the soil lifeless, thus undermining production of other crops such as coffee, maize, and cassava.

All the while, the researchers and farmers were tracking the expansion of the *bacterial wilt* epidemic. Their projections were alarming. "We suspected it would take only 2 years to destroy 27,000 hectares of plantain in Quindío Department alone," recalls González.

4.3 A role for CIAT

So it was that sheer economic necessity once again found itself to be the motherhood of invention. With FEDEPLATANO and CIAT serving as institutional midwives, the *Club del Moko* was born in February 1998 Elizabeth Alvarez, a CIAT plant pathologist with long experience in farmer participatory research, sketches the background of her Center's involvement. "In the hope of finding a solution to the *bacterial wilt* epidemic, the farmers decided to approach CIAT for help. The reason they didn't come to us earlier was that they thought we worked only on beans, cassava, rice, and forages.

That was true a long time ago; my mandate, for example, was cassava diseases. But then we ended up helping out a group of flower growers who had a mildew problem. So the directors of CIAT allowed us to begin helping clients outside our traditional set of crops, on a demand-driven basis."

The collaborative project, operating under the *Club del Moko* umbrella, has covered a lot of R&D ground:

• A survey of 21 farmers in several municipalities in Quindío Department provided the scientists with a snapshot of the *bacterial wilt* problem in one area where disease pressure is growing. It showed that 11 percent of the plantain production area had become infected with the bacteria – a disturbing 43 percent increase in just 5 years. In addition, nearly all the respondent farmers reported problems with *bacterial wilt*, and most were using recommended control methods but with little positive effect.

• Later, back at the CIAT laboratory, Alvarez and her colleagues identified 68 strains of *R. solanacearum* bacteria using molecular markers. These were isolated from samples of

4.4 Three's a crowd: coffee, plantain ... and coca

Plantain, like coffee, occupies a special place in Colombian rural life, notes González. This crop is important even for our small farmers who grow it on a single hectare. It gives people the extra money they need to buy basic goods – like cooking oil and salt, and even books for their children's education. Plantain is an essential part of our people's daily diet, along with cassava and potatoes. In the Amazon region in the south, it's actually the number-one food crop, more important than cassava." Indigenous people there, says González, cultivate plantain almost exclusively for home consumption; but elsewhere in the country it is mostly a dual-purpose crop, for both food and income, often intercropped with coffee. Plantain is, after coffee, the country's most important crop. It is grown on about 125,000 farms and covers some 450,000 hectares. Among the traditional advantages of this starchy staple are its low production costs by weight – in comparison with rice and maize, for example – and the fact that it can be grown year-round in a diversity of environments. Around 14 percent of the 2.5 million tonnes of plantain grown annually in Colombia is exported. Colombia's biggest foreign customer is the USA, whose appetite for plantain is on the rise.

Apart from its role as an importer of plantain from Colombia, the USA also actively encourages its production there – as an alternative to coca. According to a 2001 fact sheet prepared by the US State Department, Colombia is the world's top coca producer and cocaine exporter and "90 percent of the cocaine and most of the heroin [made from poppies] in the US market now comes from Colombia."

US support of plantain production is part of its $1.3 billion contribution to Plan Colombia, a national program launched by former Colombian President Andrés Pastrana, aimed at promoting peace, security, democracy, and economic growth in that country. Among the Plan's key aims is a halt to the cultivation of coca and opium poppies – in part by encouraging alternative crops with income-earning potential. US involvement in Plan Colombia has been highly controversial, however, because a major thrust of the antidrug campaign is the eradication of coca fields by military force, via aerial spraying of chemical defoliants launched from helicopters. This strategy, critics argue, is counterproductive, causing social, economic, and environmental damage.

Besides plantain, the commodities being promoted as substitutes for illicit crops include oil palm, rubber, and cacao (the main ingredient of chocolate). But for most farmers these options are less attractive than plantain because they have longer payback periods. *Bacterial wilt* disease in plantain and banana was first reported in Colombia in 1954, in Tolima Department. By 1968 it was affecting banana production in Urabá in northern Colombia. In the ensuing years, its emergence as an economically significant disease prompted many poor farmers, particularly those in remote areas, to switch to coca production for the lucrative narcotics trade.

Over the past decade or so, following the price-related crisis in the coffee sector that began in 1993-94, many Colombian coffee growers have come under heavy economic

pressure to abandon production or at least find complementary sources of income. This partially accounts for the surge in production of coca during the 1990s. But now, with a well- financed war on illicit crops being waged by the government, that option is not as attractive as it once was, and plantain is once again seen as a viable option – a complement to coffee or a substitute for coca. Indeed, the rapid spread of bacterial wilt is undoubtedly linked to the resurgence of plantain as a smallholder crop, particularly the exchange of uncertified, *bacterial wilt*-infected materials between farms.

The coffee crisis forced many farmers to choose one of two options: abandon coffee production altogether and switch to cattle production, or keep the coffee and intercrop it with plantain. Industrious farmers, says González, chose the latter option, in part because plantain is more labor- intensive than livestock, thus providing rural jobs. "We also wanted to maintain our way of life related to coffee production." Gonzalez's faith in the economic complementarity of coffee and plantain for Colombian smallholders should come as no surprise. He is not only the current leader of FEDEPLATANO, but also its founder. And in the late 1920s his great-grandfather, Carlos E. Restrepo, who was President of the Republic from 1910 to 1914, founded the now famous National Federation of Coffee Growers of Colombia.

Silverio González, director of FEDEPLATANO, the National Federation of Plantain Producers of Colombia

4.5 Local benefits, global recognition

To date, the activities of the *Club del Moko* have provided four major benefits to farmers. First, the application of selected disease-control methods at test sites covering 4,000 hectares in Quindío has dramatically reduced disease incidence and therefore crop losses.

Second, local plantain growers have made the rural environment safer by eliminating the use of Formol (formaldehyde) and other chemical pesticides. Formol, although highly toxic to people, had been routinely used to disinfect soil. Three applications per year of farmer-produced plantain lixivium, diluted in water and then applied to the foliage, now replaces as many as 12 applications of commercial chemical pesticides to control black sigatoka. Third, reduced reliance on agrochemicals has helped farmers cut their production costs. Finally, the Club has enhanced local capacity for rural learning and innovation, mostly in Quindío, through on-farm experimentation. So far, more than 1,000 farmers have participated in the research and technology validation work. Several thousand more farmers and agricultural technicians were also trained in *bacterial wilt* control in 2003 and 2004 at demonstration sites and at CIAT headquarters in southwest Colombia.

4.6 Future prospects

So where should joint R&D by researchers and producers go from here? Alvarez and González see eye to eye on priorities for the *Club del Moko*. Rapid-diagnosis kits must be designed for farmers. *Bacterial wilt* control techno logies, including production and use of rachis-compost lixivium, should be further improved and widely disseminated. And communities should be assisted in producing disease- free planting materials. But can the momentum of better *bacterial wilt* management be sustained? And, just as important, how can the benefits of improved control technologies be expanded to other communities across Colombia?

In many Latin American countries, local agricultural research committees (called CIALs in Spanish) are an established mecha nism for boosting crop production, conserving natural resources, and fostering rural innovation. Encouraging the creation of new committees of this type, to focus specifically on plantain, may be a sound strategy, says Alvarez. It has worked for other commodities and inputs such as maize, cassava, soybean, and organic fertilizers. Why not for plantain?

Such a grass-roots effort in support of plantain would see diverse groups of small farmers build on the modest yet significant gains to date by the *Club del Moko.* It might also provide them welcome respite from the continuing stress of low coffee prices and, who knows, from the roar of low- flying helicopters defoliating the jungle.

5. Identifying and Characterizing Strains of *Ralstonia solanacearum* Race 2, Causal Agent of *Moko* of Plantain in Colombia.

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5.1 Introduction

Moko, *maduraviche*, or *ereke* is a bacterial wilt of plantain and banana caused by *Ralstonia solanacearum* E.F. Smith race 2 (Yabucchi et al. 1995, cited by Ito et al. 1998). It is the most important bacterial disease of these crops in Colombia, affecting possibly 125,000 families who depend directly on them for their livelihoods. Currently, despite dissemination of preventive measures and disease management, the disease is spreading, to the point where 95% of plantain fields have at the least one plant with *moko* (personal communication, Galindo 2004, ICA, Bogotá).

The BIO-PCR technique developed by Schaad et al. (1995) improves efficiency in detecting viable cells of this pathogen, especially in soil. This technique consists of isolating colonies in semi-selective medium, South Africa (SMSA) (Denny and Hayward 2001; Englebrecht 1994; Martins 2000). It shows higher sensitivity and specificity than does triphenyltetrazonium chloride (TTC) (Kelman 1954) with later amplification by the polymerase chain reaction (PCR).

Ralstonia solanacearum is variable in the range of hosts it attacks, geographical distribution, pathogenicity, epidemiological relations, and physiological properties. Hence, in the last three decades, races and biovars have been used informally to classify the pathogen at the infra-subspecific level (not governed by the *International Code of Nomenclature of Bacteria*). Race 1 (biovars 1, 3, or 4) attacks many plants, including sweetpotato, tomato, and solanums in general. Race 2 (biovars 1 or 3) affects banana, plantain, and heliconias. Race 3 (biovar 2) is considered specific to sweetpotato and is associated with some solanums. Race 4 (biovar 4) attacks ginger; and race 5, blackberry (Hayward 1991).

This study aimed to isolate *R. solanacearum* from soil and from infected plant tissue, using BIO-PCR, culture medium SMSA, specific primer OLI 1, and nonspecific primer Y2. We also identified strains belonging to race 2 by inoculating plantain plants, and by observing the hypersensitivity reaction they induce in tobacco. We then evaluated the strains' pathogenicity levels. Biovars were determined through biochemical characterization.

5.2 Materials and Methods

5.2.1 Sample sources

We processed 134 samples of infected plant tissue from pseudostems, rachis, fruits, and rhizomes of selected plantain, banana, and heliconia plants that had presented typical symptoms of the disease. We also processed soil samples from farms affected by *moko* and located in the production areas of the Departments of Valle del Cauca, Quindío, Antioquia, Caquetá, Meta, and Magdalena in Colombia (Table 5.1).

Table 5.1. Samples of plant tissue used to isolate *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt) of plantain, according to origin by department, crop, and source of isolate.

Sample No	Origin			Sample No	Origin		
	Department/ Locality	Crop	Source		Department/ Locality	Crop	Source
1	Quindío	Plantain	Rachis	79	Montenegro (Quindío)	Plantain	Rhizome
2	Quindío	Plantain	Petiole	80	Montenegro (Quindío)	Plantain	Pseudostem
3	Quindío	Plantain	Petiole	81	Montenegro (Quindío)	Plantain	Fruit
4	Urabá (Antioquia)	Banana	Pseudostem	83	Quindío	Plantain	Fruit
5	Urabá (Antioquia)	Banana	Rhizome	84	Quindío	Plantain	Pseudostem
6	Urabá (Antioquia)	Banana	Fruit	85	Quindío	Plantain	Sucker
7	Urabá (Antioquia)	Banana	Fruit	86	Calarcá (Quindío)	Plantain	Rachis
15	Quindío	Plantain	Soil	88	La Tebaida (Quindío)	Plantain	Rhizome
17	Jamundí (Valle)	Plantain	Soil	89	La Tebaida (Quindío)	Plantain	Pseudostem
18	Jamundí (Valle)	Plantain	Sucker	90	Montenegro (Quindío)	Plantain	Petiole
32	Caquetá	Plantain	Pseudostem	91	Montenegro (Quindío)	Plantain	Rachis
33	Caquetá	Plantain	Pseudostem	92	Quimbaya (Quindío)	Plantain	Petiole
34	Caquetá	Plantain	Rachis	94	Quimbaya (Quindío)	Plantain	Rachis
38	Quindío	Plantain	Soil	95	Quimbaya (Quindío)	Plantain	Rhizome
39	Quindío	Plantain	Soil	96	Quimbaya (Quindío)	Plantain	Pseudostem
40	Quimbaya (Quindío)	Plantain	Soil	97	Quimbaya (Quindío)	Plantain	Rhizome
41	Quimbaya (Quindío)	Plantain	Soil	98	Quimbaya (Quindío)	Plantain	Rachis
42	Fuente de Oro (Meta)	Plantain	Pseudostem	99	Quimbaya (Quindío)	Plantain	Pseudostem
43	Fuente de Oro (Meta)	Plantain	Pseudostem	100	Armenia (Quindío)	Plantain	Sucker
48	Armenia (Quindío)	Plantain	Fruit	101	Armenia (Quindío)	Plantain	Pseudostem
54	Fuente de Oro (Meta)	Plantain	Pseudostem	102	Quimbaya (Quindío)	Plantain	Petiole
55	Fuente de oro (Meta)	Plantain	Pseudostem	104	Armenia (Quindío)	Plantain	Fruit

Continue...							
57	Fuente de oro (Meta)	Plantain	Pseudostem	106	Armenia (Quindío)	Plantain	Pseudostem
58	Fuente de oro (Meta)	Plantain	Pseudostem	107	Armenia (Quindío)	Plantain	Fruit
59	Fuente de oro (Meta)	Plantain	Pseudostem	109	Armenia (Quindío)	Plantain	Petiole
60	Fuente de oro (Meta)	Plantain	Pseudostem	110	Magdalena	Banana	Pseudostem
63	Granada (Meta)	Plantain	Pseudostem	111	Magdalena	Banana	Rhizome
64	Granada (Meta)	Plantain	Pseudostem	112	Magdalena	Banana	Sucker
65	Granada (Meta)	Plantain	Pseudostem	113	Palmira (Valle)	Heliconia	Pseudostem
66	Granada (Meta)	Plantain	Pseudostem	114	Palmira (Valle)	Heliconia	Rhizome
67	Fuente de oro (Meta)	Plantain	Pseudostem	115	Palmira (Valle)	Heliconia	Rhizome
69	Granada (Meta)	Plantain	Pseudostem	160	Quindío	Plantain	Soil
70	Granada (Meta)	Plantain	Pseudostem	161	Quindío	Plantain	Soil
71	Urabá (Antioquia)	Plantain	Rhizome	588	Fuente de Oro (Meta)	Plantain	Petiole
72	Urabá (Antioquia)	Plantain	Pseudostem	CIAT 1008[4]	Ibagué (Tolima)	Plantain	Sin Inf.
73	Urabá (Antioquia)	Plantain	Pseudostem				
76	Montenegro (Quindío)	Plantain	Pseudostem				
78	Montenegro (Quindío)	Plantain	Rachis				

5.2.2 Processing plant-tissue samples

The bacterium was extracted from selected infected tissue fragments, which had been washed, disinfected, and macerated in a mortar containing a buffer solution of 10 mM Tris-HCl and 1 mM EDTA at a pH = 7.6. This suspension was cultured, using a sterilized micro-spade, in petri dishes containing the semi-selective culture medium, South Africa (SMSA), a modification of the medium triphenyltetrazonium chloride (TTC).

The SMSA medium contained 10 g/L peptone, 5 mL/L glycerol, 1 g/L casamino acids, 18 g/L agar, antibiotics (100 mg/L, i.e., 600,000 U polymyxin β sulfate; 25 mg/L bacitracin; 0.5 mg/L, i.e., 82.5 U penicillin, and 5 mg/L chloramphenicol), 50 mg/L of 2,3,5-TTC, and 5 mg/L crystal violet (Denny and Hayward 2001; Englebrecht 1994). The dishes cultured with the suspension were incubated at a temperature of 28 °C for 3 to 5 days, depending on when the colonies appeared.

5.2.3 Processing soil samples

With soil taken from around plantain plants infected by the bacterium, suspensions were prepared by adding 3.3 g soil to 30 mL TE buffer at pH = 7.6. Serial dilutions were

carried out in TE buffer. We then took 100 µL of each of the dilutions 10^{-1}, 10^{-2}, and 10^{-3}, culturing into petri dishes containing SMSA medium. The dishes were incubated at 28 °C for 3 to 5 days, depending on when the colonies first appeared.

5.2.4 Isolating and testing the bacterium

5.2.4.1 Isolation. From the samples, we selected bacterial colonies that showed similar growth patterns to those of the *R. solanacearum* control strain CIAT 1008. The control strain came from Ibagué (Tolima, Colombia) and was held at the strain bank in the Cassava Pathology Laboratory, CIAT.

5.2.4.2 KOH test. Purified bacterial colonies belonging to the Gram-negative group of bacteria were selected, using the KOH (3%) test. A drop of this reagent was placed on a glass slide and a colony from a pure and metabolically active culture with a 24-h growth then dissolved in it. The reaction was considered positive when a mucous thread could be seen on lifting the micro-spade from the bacterial suspension.

5.2.4.3 Oxidase test. The oxidase test was carried out by placing two drops of a 1% aqueous solution of dichlorohydrate of tetra-methyl-p-phenylenediamine on a strip of filter paper, which was then rubbed over a colony. The reaction was considered positive when the solution in the paper turned from colorless to dark purple within the next 30 to 60 s (Goszczynska et al. 2000).

The strain CIAT 1008 was included as check for both tests.

5.2.5 Extracting DNA and conducting PCR

Genomic DNA was extracted from pure colonies of selected strains with a 36-h growth in nutritive agar (Seal et al. 1999). Each colony was suspended in vials containing 100 µL of sterilized distilled water, and heated in a bain-marie at 96 °C for 5 min. The vials were then centrifuged at 12,000 rpm for 2 min and 2.5 µL of the supernatant taken as DNA mold for the polymerase chain reaction (PCR).

The volume of the cocktail for amplification was 9.98 µL, which contained a 1.25X buffer for *Taq* polymerase; 0.012 mM of each dNTP; 1.87 mM $MgCl_2$; 0.25 U *Taq* polymerase; and 0.16 µM of each of the primers OLI 1 (5'-GGGGGTAGCTTGCTACCTGCC-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3'), (Martins 2000; Seal et al. 1999).

The DNA was amplified in an MJ Research PTC-100 thermal cycler, using the following program: initial denaturation for 2 min at 96 °C; 50 denaturation cycles, each for 20 s at 94 °C; annealing for 20 s at 62 °C; extension for 30 s at 72 °C; and a final extension of 5 min at 72 °C (CIAT 2004; Seal et al. 1999). The PCR products were separated in 1.5% agarose gels, dyed with 0.001% ethidium bromide, and visualized under ultraviolet light.

Evaluations were based on the presence of a band, 287–288 base pairs long, from the 16S rRNA fragment generated by amplification with the specific primer OLI 1 and the nonspecific primer Y2 (Woese et al. 1983; Young et al. 1991, cited by Seal et al. 1999).

5.2.6 Biovar determination

Ralstonia solanacearum strains can be classified into different biovars according to Hayward (1964) by their production of acids from the disaccharides cellobiose, lactose, and maltose and by their oxidation of the hexose alcohols sorbitol, dulcitol, and mannitol in base medium (Denny and Hayward 2001).

The base medium contained (per liter) 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1.0 g Bacto™ Peptone, 3.0 g agar, and 80.0 mg bromothymol blue with a pH between 7.0 and 7.1, becoming green olive. The medium was then sterilized by autoclaving at 121 °C, with a 20-lb pressure, for 20 to 30 min (Denny and Hayward 2001).

We also prepared 10% aqueous solutions of each of the test carbohydrates and sterilized them by filtration, using Millipore® filters with a pore size of 0.22 µm. These solutions of carbohydrates were added to the sterilized base medium when the temperature was between 55 and 60 °C, obtaining a final concentration of 1%. After mixing the base medium with each sugar, about 5 mL of the liquid medium placed within tubes containing sterilized cultures (Denny and Hayward 2001).

The medium was inoculated with the bacterium by means of puncturing as deep as three quarters of the medium, using colonies with a 24-h growth in nutritive agar. Reactions were assessed after 1, 3, 7, 14, and 28 days of incubation at 28 °C. The color changed from green olive to yellow as acids were produced from the disaccharides and the hexose alcohols were oxidized (Denny and Hayward 2001).

5.2.7 Testing for pathogenicity in plantain

The strains identified by PCR as being *R. solanacearum* were inoculated into plantain plants of 'Africa' (*Musa* cv. AAB) derived from in vitro meristem culture. At 15 days old, the plantlets were transplanted to plastic bags carrying 1 kg of sterilized sand and soil mixed in a 3:2 ratio. For the next 15 days, the plants were continuously humidified to guarantee their optimal development.

The plants were not watered for 24 h before inoculation. For each strain of *R. solanacearum*, four plantain plants were inoculated at about 6 weeks old by injection of the pseudostem, using sterilized 1-mL syringes with needle size 27G × 1/2". Injection was to the center of the pseudostem at 2 cm above the soil surface.

The substance injected comprised 0.5 mL of bacterial suspension made with pure bacterial cultures with a 24-h growth in nutritive agar that were suspended in sterilized deionized water. The concentration of the suspension was determined by absorbance

readings in a spectrophotometer and adjusted to 0.1 with a wavelength of 600 nm. This corresponded to about 1×10^{-8} cfu · mL^{-1} (He et al. 1983).

As positive check, the pathogenic strain *R. solanacearum* CIAT 1008 was used. The negative check was inoculated sterilized water. The inoculated plants remained under controlled conditions of temperature between 24 and 29 °C, about 13 h of light, and relative humidity from 80% to 91% for the first 4 days, with humidification being later reduced to 1 h per day.

Severity of symptoms were evaluated in terms of wilt, using a visual scale of 1 to 5, where 1 referred to a plant with 1 wilted leaf and 5 to a plant with five wilted leaves. Daily evaluations were made over 18 days, starting from the fourth day after inoculation, for symptoms such as flaccid leaves, wilting, and stunting. With this information, the area under the disease progress curve (AUDPC) was calculated. In preliminary research (unreported), CIAT had found that, from day 5, plants can show disease symptoms such as flaccid and/or wilting leaves.

5.2.8 Hypersensitivity test

The capacity of the strains to induce a hypersensitivity reaction was tested in leaves of tobacco (*Nicotiana tabacum*). From pure cultures, a suspension was prepared in sterilized deionized water, using colonies with 24 h of incubation in nutritive agar and an absorbance of 0.1 with a 600-nm wavelength, thus corresponding to a concentration of about 1×10^{8} cfu · mL^{-1} (He et al. 1983).

In this test, 8-week-old tobacco plants were used. These were inoculated by infiltration of the bacterial suspension, injecting with a 1-mL syringe into the veins on the lower side of leaves, permitting distribution of the suspension in the palisade layer of the parenchyma. Two leaves per plant and two plants per strain were inoculated.

The reaction was evaluated, beginning 16 h after inoculation, for symptoms corresponding to hypersensitivity to race 2 of *R. solanacearum*. These are chlorosis in infected cells of the parenchyma; wet tissue limited by a defined margin of uninoculated tissue; the area of infiltrated leaf becoming, between 36 and 60 h later, necrotic and dry from water loss; and, finally, the affected area becoming thin, white, and translucent (Lozano and Sequeira 1970).

5.2.9 Data analysis

An analysis of variance was carried out for the variable AUDPC. A test for the separation of means by minimum significant difference (MSD; α = 5%) was also conducted to separate the strains into groups according to their levels of pathogenicity (Table5.2).

Table 5.2. Origin and pathogenicity of 72 strains of *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt), isolated from banana, plantain, and heliconias.

Strain No	Origin			Path'y			Strain no.	Origin			Path'y		
	Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	Hypers.[4]		Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	Hypers.[4]
1	Quindío	Pl.	R.	18,00	2	+	79	Montenegro (Quindío)	Pl.	Rh.	66,88	1	+
2	Quindío	Pl.	Pe.	69,38	1	+	80	Montenegro (Quindío)	Pl.	Ps.	67,88	1	+
3	Quindío	Pl.	Pe.	49,00	1	+	81	Montenegro (Quindío)	Pl.	F.	10,88	3	+
4	Urabá (Antioquia)	B.	Ps.	43,13	2	-	83	Quindío	Pl.	F.	55,00	1	+
5	Urabá (Antioquia)	B.	Rh	38,75	2	-	84	Quindío	Pl.	Ps.	61,00	1	+
6	Urabá (Antioquia)	B.	F.	31,83	2	-	85	Quindío	Pl.	Su.	68,38	1	+
7	Urabá (Antioquia)	B.	F.	62,17	1	-	86	Calarcá (Quindío)	Pl.	R.	59,75	1	+
15	Quindío	Pl.	S.	37,63	2	+	88	La Tebaida (Quindío)	Pl.	Rh.	61,75	1	+
17	Jamundí (Valle)	Pl.	S.	69,50	1	+	89	La Tebaida (Quindío)	Pl.	Ps.	60,38	1	+
18	Jamundí (Valle)	Pl.	Su.	42,50	2	+	90	Montenegro (Quindío)	Pl.	Pe.	19,25	2	-
32	Caquetá	Pl.	Ps.	33,88	2	+	91	Montenegro (Quindío)	Pl.	R.	19,38	2	+
33	Caquetá	Pl.	Ps.	40,38	2	+	92	Quimbaya (Quindío)	Pl.	Pe.	63,13	1	+
34	Caquetá	Pl.	R.	27,63	2	+	94	Quimbaya (Quindío)	Pl.	R.	47,25	1	+
38	Quindío	Pl.	S.	59,50	1	+	95	Quimbaya (Quindío)	Pl.	Rh.	65,63	1	+
39	Quindío	Pl.	S	62,00	1	+	96	Quimbaya (Quindío)	Pl.	Ps.	33,00	2	+
40	Quimbaya (Quindío)	Pl.	S.	15,75	3	+	97	Quimbaya (Quindío)	Pl.	Rh.	28,63	2	+
41	Quimbaya (Quindío)	Pl.	S.	56,25	1	+	98	Quimbaya (Quindío)	Pl.	R.	59,50	1	+
42	Fuente de Oro (Meta)	Pl.	Ps.	28,00	2	+	99	Quimbaya (Quindío)	Pl.	Ps.	40,25	2	+
43	Fuente de Oro (Meta)	Pl.	Ps.	20,75	2	+	100	Armenia (Quindío)	Pl.	Su.	71,88	1	+
48	Armenia (Quindío)	Pl.	F.	37,13	2	+	101	Armenia (Quindío)	Pl.	Ps.	58,50	1	+
54	Fuente de Oro (Meta)	Pl.	Ps.	36,25	2	+	102	Quimbaya (Quindío)	Pl.	Pe.	1,38	3	+
55	Fuente de oro (Meta)	Pl.	Ps.	55,25	1	+	104	Armenia (Quindío)	Pl.	F.	41,25	2	+
57	Fuente de	Pl.	Ps.	45,63	1	+	106	Armenia	Pl.	Ps.	24,75	2	+

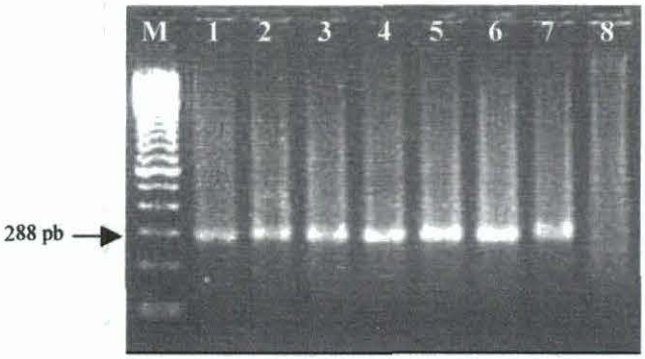

Figure 5.1. The figure shows the characteristic band of the bacterium *Ralstonia solanacearum*, causal agent of *moko* (bacterial wilt) in plantain. The product, measuring 288 bp, was amplified with primers OLI 1 and Y2 in the 16S rRNA region. M = 100-bp marker; lanes 1 to 3 = strains from Quindío, Colombia; lanes 4 to 6 = strains from Urabá, Colombia; lane 7 = CIAT 1008; lane 8 = negative control.

5.3.3 Determining biovars

The reaction of each of 72 strains (8 from soil and 64 from plant tissue) to sugars and alcohols indicated that all the strains characterized belonged to biovar 1. They had not used any of the three sugars, nor oxidized the three hexose alcohols used in the biochemical tests.

5.3.4 Pathogenicity test and confirmation of race 2

The reaction of hypersensitivity obtained in tobacco leaves 48 h after inoculation indicated that 63 of the 72 strains caused a typical hypersensitivity reaction in tobacco leaves (Figure 5.2). The remaining 9 strains induced yellowing, an atypical reaction of hypersensitivity for this race. Eight of these strains came from the Colombian Atlantic Coast (Urabá).

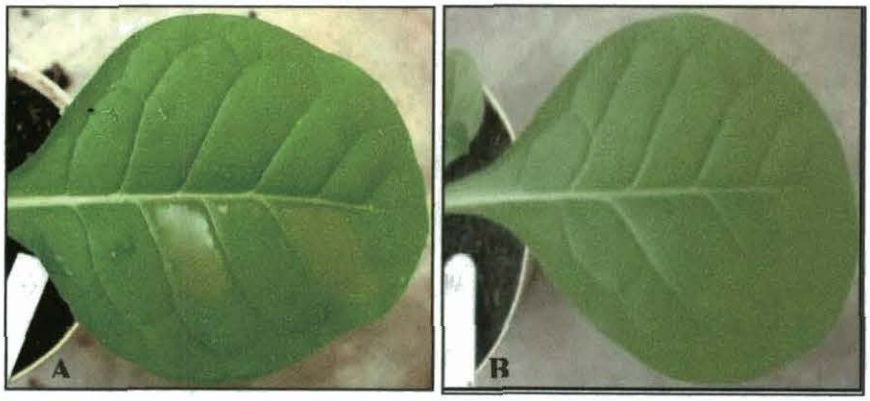

Figure 5.2. (A) Typical reaction of hypersensitivity in tobacco leaf, 48 h after inoculation with strain 79 of *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt) in plantain. (B) Control inoculated with sterilized distilled water.

Seventy-one strains were pathogenic when inoculated into plantain plants, confirming that they belonged to race 2. Only one strain, which came from Urabá, was not pathogenic (Figure 5.3). The separation-of-means test, estimated through MSD ($\alpha = 5\%$), led to grouping the strains in three categories according to their pathogenicity (AUDPC). The highly pathogenic strains had AUDPC values between 45.13 and 73.38; the strains with moderate pathogenicity showed values between 18.00 and 43.13; and the strains with low pathogenicity had values between 0 and 15.75 (Table 5.2).

Figure 5.3. Plantain inoculated the center of the pseudostem at 2 cm above the soil surface, under greenhouse conditions: (A) Control with water. (B) Wilt and yellowing of leaves caused by strain 85 of *R. solanacearum* race 2.

Of seven strains isolated from banana, five were moderately and two were highly pathogenic. Three strains from heliconias showed moderate levels. Differences in pathogenicity were found among strains isolated from soil or various plant tissues, for example, those from rhizomes and rachis were more highly pathogenic than those from other tissues. No relationship was found between pathogenicity and geographical origin (Table 5.2).

5.4 Conclusions

The BIO-PCR technique facilitated detection of the pathogen in soil and plants. All the *R. solanacearum* strains isolated corresponded to biovar 1.The strains showed variation in pathogenicity according to the crop and tissue from which they were isolated.

	oro (Meta)							(Quindío)					
58	Fuente de oro (Meta)	Pl.	Ps.	56,63	1	+	107	Armenia (Quindío)	Pl.	F.	68,25	1	+
59	Fuente de oro (Meta)	Pl.	Ps.	0,00	3	+	109	Armenia (Quindío)	Pl.	Pe.	45,13	1	+
60	Fuente de oro (Meta)	Pl.	Ps.	47,25	1	+	110	Magdalena	B.	Ps.	63,25	1	+
63	Granada (Meta)	Pl.	Ps.	50,69	1	+	111	Magdalena	B.	Rh.	34,38	2	+
64	Granada (Meta)	Pl.	Ps.	46,13	1	+	112	Magdalena	B.	Su.	29,50	2	-
65	Granada (Meta)	Pl.	Ps.	47,63	1	+	113	Palmira (Valle)	H.	Ps.	40,50	2	+
66	Granada (Meta)	Pl.	Ps.	69,75	1	+	114	Palmira (Valle)	H.	Rh.	40,38	2	+
67	Fuente de oro (Meta)	Pl.	Ps.	41,63	2	+	115	Palmira (Valle)	H.	Rh.	33,63	2	+
69	Granada (Meta)	Pl.	Ps.	27,00	2	+	160	Quindío	Pl.	S.	12,38	3	+
70	Granada (Meta)	Pl.	Ps.	5,75	3	+	161	Quindío	Pl.	S.	1,75	3	+
71	Urabá (Antioquia)	Pl.	Rh.	21,25	2	-	588	Fuente de Oro (Meta)	Pl.	Pe.	71,25	1	+
72	Urabá (Antioquia)	Pl.	Ps.	10,75	3	-	CIAT 1008[5]	Ibagué (Tolima)	Pl.	No date.	65,13	1	+
73	Urabá (Antioquia)	Pl.	Ps.	10,75	3	-							
							DMS α= 5%, 28,33						
76	Montenegro (Quindío)	Pl.	Ps.	61,88	1	+							
78	Montenegro (Quindío)	Pl.	R.	73,38	1	+							

1. Pl = plantain; B = banana; H = heliconia; R = rachis; Pe = petiole; Ps = pseudostem; Rh = rhizome; Su = sucker; F = fruit; S = soil.
2. AUDPC = area under the disease progress curve.
3. Group = pathogenicity group, where 1 = high pathogenicity; 2 = moderate pathogenicity; 3 = low pathogenicity.
4. Hypers. = hypersensitivity, where + = typical hypersensitive reaction; - = atypical yellowing reaction.
5. Check strain from the CIAT collection,

5.3 Results and Discussion

5.3.1 Isolating Ralstonia solanacearum

Samples from six regions in Colombia were taken from soil in plantain crops affected by *moko* and from plant tissues of infected plantain, banana, and heliconias. From these samples, 189 strains of the bacterium were initially selected for their growth in SMSA medium. This growth was similar to that of the *R. solanacearum* control strain CIAT 1008 when observed 48 h after incubation at 28 °C. This medium reduced the growth of saprophytic bacteria.

5.3.2 Analysis through the polymerase chain reaction (PCR)

In a 1.5% agarose gel, a band with a molecular weight of 288 bp was detected. For 106 of the 189 strains obtained, the fragment was located in gene 16S rRNA, which enabled us to identify them as *R. solanacearum* (Figure 5.1).

5.5 Acknowledgements

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6. Determining the minimum concentrations of different products that inhibit *Ralstonia solanacearum* race 2

Elizabeth Alvarez (PE-1, IP-6)

6.1 Objective

To determine the minimum concentrations of different products that inhibit *Ralstonia solanacearum* in vitro

6.2 Methodology

In 50-mL glass tubes with lids were placed 5 mL of sterilized liquid tetrazolium chloride (TZC) medium (10 g dextrose, 10 g peptone, and 1 g casamino acids per liter of water) with 1% TZC added at 25 µL per tube. Table 1 lists the products that were evaluated:

Table 6.1. The minimum concentrations of several natural and commercial products that inhibit *Ralstonia solanacearum*.

Product	Inhibitory minimum concentration (%)[a]	
	Not filtered	Filtered
• Lixiviate of compost (obtained from "La Guaira" Farm, Quindío)	3.5	3.5
• Lixiviate of compost (obtained from "La Manigua" Farm, Quindío)	–	25.0
• Potassium phosphite (phosphorous acid neutralized with potassium hydroxide and potassium citrate)	3.5	9.4
• Phosphorous acid (60% P_2O_5)	0.5	0.5
• Cumbre® (antibiotics: 10% gentamicin + 30% oxytetracycline)	–	0.5
• Kasumin® (antibiotic: kasugamycin)	–	0.5
• Dolomite lime	25.0	–
• Slaked lime ($CaCO_3$)	1.2	–
• Phosphoric rock (29% P_2O_5)	–	1.3
• Calfos (slag, Thomas)	12.6	–
• Calfomag (25% calcium, 5.2% phosphorus, 6% magnesium, 1% sulfur)	16.0	–
• EcoSwing® (extract of *Swinglea glutinosa*)	1.3	0.5
• *Tagetes patula* (aqueous extract)	0.5	9.4
• Hydrolate of *T. patula*	–	Does not inhibit
• Hydrolate of *S. glutinosa*	–	Does not inhibit

a. – = not determined

Dilutions were made, starting with a base concentration of 25% of each product to be evaluated and transferring 3 mL to another tube, vortexing, and transferring 3 mL to another tube, and so on for 4 tubes, so that the concentrations obtained were 25%, 9.4%, 3.5%, 1.3%, and 0.5%. Each tube was then inoculated, at 125 µL per tube, with a bacterial suspension of 0.3 absorbance at 600 nm. The tubes used as controls for each treatment were 1 tube with a product but no bacterium, 1 tube with the bacterium but no product, and 1 tube with no bacterium and no product. The products based on extracts and lixiviate were filtered through a 0.22-µm mesh to reduce the presence of bacteria other than *R. solanacearum*.

6.3 Results

Table 1 indicates the minimum concentrations that inhibited the bacterium *in vitro*. The concentration of lixiviate of compost changed according to origin of product, as it is made on an artisanal basis and is not standardized. It is also affected by rain and the crop management practices prevalent in the area where the material was obtained for composting.

The products that inhibit the bacterium at lower concentrations are phosphorous acid, the antibiotics Cumbre® and Kasumin®, aqueous extract of *T. patula* without filtering, and EcoSwing®. Although the extracts of *T. patula* and *S. glutinosa* inhibit the bacterium, the hydrolates of the same species do not act, perhaps because the hydrolates are obtained by cooking the plant material, which could reduce or inactivate the metabolites that act against the bacterium.

7. Detecting *Ralstonia solanacearum* in lixiviate of compost of plantain tissue infected by bacterial wilt

Elizabeth Alvarez (PE-1, IP-6)

7.1 Objective

To determine the permanence of *Ralstonia solanacearum* in lixiviate of compost of plantain infected by bacterial wilt

7.2 Methodology

Under laboratory conditions, lixiviate was obtained from compost of plant material of plantain infected by bacterial wilt, moistening it with lixiviate produced by farmers in Quindío. The lixiviate was moistened every week with that obtained in the laboratory. Samples were collected from the moment of application and at 1, 2, 4, 6, 8, 10, 15, and 20 days after the lixiviate was applied. The collected samples were conserved for the experimental period at room temperature and cultured onto semi-selective medium, South Africa (SMSA) each time a new sample was taken. To isolate the bacterium, a tissue sample was also taken at the beginning and end of the trial. The samples were cultured onto SMSA medium.

Under field conditions, samples were collected of lixiviate made by farmers from compost of plantain rachises from racemes. At the time of sampling, we verified that 100% of a sample of fresh rachises taken at random from the composting site had symptoms of bacterial wilt. To detect the bacterium itself, samples were also collected from the high, middle, and low parts of the decomposing plant material and from fresh materials stacked onto the compost heap.

7.3 Results

The bacterium could be detected only in the fresh lixiviate on the first day it was obtained. After the second day, the bacterium could not be isolated. Taking into account that lixiviate can inhibit the bacterium at concentrations of 3.5% (Table 1, Activity 2), we conclude that the lixiviate inhibited the bacterium present in the infected tissue. By day 20 after applying the lixiviate, the tissue was decomposed and neither could the bacterium be isolated.

Of the samples collected from the composting site in the field, the bacterium could be isolated only from fresh rachises with disease symptoms.

8. Disinfecting tools to manage bacterial wilt (*Ralstonia solanacearum*) of plantain

8.1 Objective

To evaluate different chemical products for the reliable disinfection of machetes

8.2 Methodology

8.2.1 Evaluated products

We evaluated 18 products at different concentrations for disinfecting machetes, as listed in Table 8.1:

Table 8.1. List of 18 natural and commercial products used to disinfect machetes, as part of treating plantain crops infected with bacterial wilt.

Product	Concentration used (%)
• Water from Cali's drinking supplies	100
• Sodium hypochlorite, from 5.25% (Patojito®) to 50%	2.5
• Sodium hypochlorite, from 5.25% (Patojito®) to 20%	1.02
• Polyethoxy polypropoxy polyethoxy ethanol-iodine complex (Agrodyne®) at 100%	100
• Polyethoxy polypropoxy polyethoxy ethanol-iodine complex (Agrodyne®) at 50%	50
• Antiseptic alcohol at 70%	100
• Detergent (Fab®)	5
• Chlorated disinfectant detergent (Espuma Clorada®)	5.6
• Glutaraldehyde at 6.5% (ASAP® 65)	50
• Chlorated disinfectant detergent (Yodospar A®)	0.8
• Disinfectant detergent with quaternary ammonium (Metaquat®)	1.5
• Phenolaldehydic disinfectant (TB-Cide® 100)	1
• Quaternary ammonium at 80,000 ppm (Sani-T-10®)	3
• Quaternary ammonium at 160,000 ppm (Sani-T-10 A®)	0.8
• Propiconazole (TILT®) at 10 mL/L	1
• *Swinglea* extract	100
• Quaternary ammonium at 160,000 ppm (Sani-T-10 A®)	100
• Glyphosate (Roundup®) at 20%	20

8.2.2 Inoculum

A pathogenic strain of *Ralstonia solanacearum* from Quindío (strain 79) that had been cultured 24 h previously in nutrient agar (NA) medium was placed in petri dishes. Sterilized distilled water was then added to obtain a bacterial suspension of 1.5 absorbance. A plantain seedling was macerated to obtain 20 mL of sap to which was

added 2.2 mL of bacterial suspension, obtaining a final volume of 22.2 mL. The inoculum was distributed evenly over one side of a machete blade.

8.2.3 Procedures

We used 4 machetes, half of which were new and the other half used. Each machete was divided into 3 different sections for sampling at intervals of 0, 5, and 10 min, applying a randomized complete block design for sampling.

Each sample was obtained by swabbing with cotton tips on polypropylene supports that were resistant to autoclaving. To apply the product, 40 mL of each product were placed in a petri dish and, to disinfect the tool, a piece of gauze, 15 × 40 cm, was used, immersing it completely in the product.

8.2.4 Liquid medium used as indicator to collect the samples

The medium used to collect samples from the surface of the machete blade was liquid SMSA that, for 1 L, was made up with 1 g casamino acids, 10 g Bacto™ Peptone, and 5 mL glycerol. A total of 54 tubes were prepared with 20 mL of SMSA in each and autoclaved. Antibiotics were then added, for 1 L: 36 mg bacitracin, 74.2 mg polymyxin, 5 mg chloramphenicol, 0.5 mg penicillin, and 50 mg triphenyltetrazolium chloride (TTC).

8.3 Results

Treatments were classified as not effective when the bacterium was found to be still present on the machete at different intervals of time. Presence was determined by the liquid SMSA medium taking up a reddish staining. Tubes showing such staining were confirmed as having the bacterium by culturing 25 µL in a petri dish containing solid SMSA medium and incubating at 28 °C.

The products that controlled the pathogen were:

- Quaternary ammonium at 80,000 ppm (Sani-T-10®), 30 mL/L (priced at Col$12,500 per liter)
- Quaternary ammonium of 160,000 ppm (Sani-T-10 A®), 8 mL/L
- Sodium hypochlorite at 5.25% (Patojito®) at 50% (priced at Col$1000 per liter)
- Sodium hypochlorite at 5.25% (Patojito®) at 20%
- Polyethoxy polypropoxy polyethoxy ethanol-iodine complex (Agrodyne®) at 100% (priced at Col$26,000 per liter)
- Polyethoxy polypropoxy polyethoxy ethanol-iodine complex (Agrodyne®) at 50%
- Antiseptic alcohol at 70%
- Glutaraldehyde at 6.5% (ASAP® 65) at 500 mL/L
- Phenolaldehydic disinfectant (TB-Cide® 100) at 10 mL/L

The check products, which did not inhibit bacterial cells, were:

- Water from Cali's drinking supplies
- Detergent (Fab®) at 50 g/L

- Chlorated disinfectant detergent (Yodospar A) at 8 mL/L
- Disinfectant detergent with quaternary ammonium (Metaquat®) at 15 mL/L
- Propiconazole (TILT®) at 10 mL/L
- *Swinglea* extract
- Glyphosate (Roundup®) at 20%

8.4 Conclusions

Those products that presented reliable and effective results should be recommended to farmers so that they are incorporated into other farming practices. Those products that are economically viable and effective in disinfecting tools should also be recommended such as quaternary ammonium and sodium hypochlorite, although these have little acceptability in that they are corrosive and stain clothes.

New techniques for more precisely identifying the bacterium on the surface of tools should be evaluated such as LIVE/DEAD® kits, which would indicate more accurately the presence of live or dead cells of *Ralstonia solanacearum*, as disinfectants may inhibit the bacterium without killing it.

9. Inducing resistance to *Ralstonia solanacearum* with potassium phosphites and lixiviate of compost of plantain rachis

Elizabeth Alvarez (PE-1, IP-6)

Potassium phosphites derive from phosphorous acid and, being constructed with one molecule of oxygen less than the acid, are more mobile and soluble. They are rapidly absorbed and transported through plant membranes. They act as catalyst for resistance against *Phytophthora* spp. and *Fusarium oxysporum* f. sp. *cubense* (Davis et al. 1994; Smillie et al. 1989), and activate responses of resistance to phytoalexins.

9.1 Objective

To evaluate the effect potassium phosphites and lixiviate of compost of plantain rachis have in inducing resistance

9.2 Methodology

9.2.1 Trial 1

In the first trial, 5-week-old plantain plants, variety Africa, derived from meristems, were sprinkled weekly for 4 weeks with 0.5% potassium phosphite (10 cc/plant), 25% lixiviate (10 cc/plant), Kasumin® (kasugamycin at 0.75% of commercial product), and water. To improve the products' adherence, INEX-A® was added to each suspension as a dispersal agent.

Five weeks after the applications, the plants were inoculated by injection with 0.5 mL of a bacterial suspension of 0.1 absorbance at 600 nm wavelength, and incubated for 5 days with continuous wetting at 22 °C at night and 30 °C during the day, and 95% relative humidity. From the sixth day after incubation, wetting was carried out for 1 h per day.

9.2.1.1 Results

Ten days after the plants were inoculated, the check with water and the plants treated with Kasumin® wilted and died 1 week later. The plants treated with lixiviate remained healthy for 4 weeks, before beginning to wilt slowly, dying 2 to 3 weeks later. The plants treated with potassium phosphite did not develop the disease, although a gray band of 3 cm was observed rising from the inoculation point.

9.2.2 Trials 2 and 3

Two trials were established: in trial 2, the plants were inoculated by injection; and in trial 3, for half of the replications the soil was inoculated and for the other half, the plants were injected. Over 4 weeks, under greenhouse conditions, plantain plants 'Dominico Hartón', derived from meristems, were treated on a weekly basis with potassium phosphites, phosphorous acid, and lixiviate of compost of plantain rachis to induce resistance to the bacterium. Treatments were as follows:

1. NutriPhite 0-40-0 for soil (phosphorous acid): concentration at 0.5%, volume added at 30 cc/plant.
2. NutriPhite P+K foliar (phosphorous acid neutralized with KOH and potassium citrate): concentration at 0.5%; volume of 10 cc/plant. Inex® was used as a dispersal agent.
3. 100% lixiviate (obtained from "La Guaira" Farm, Quindío) spread on the soil at 20 cc/plant and 50% at 10 cc/plant on the foliage, totaling 30 cc/plant.
4. Check with water at 30 cc/plant (20 cc for the soil and 10 cc for the foliage).

9.2.2.1 Results

For trial 2, a randomized complete block design was used with 5 replications. For trial 3, split plots were used with 3 replications, the main plot for inoculation method and subplots for treatments to induce resistance.

Two days after applications were completed, the plants were inoculated with strain CIAT 1008 from the *R. solanacearum* collection held at CIAT. Three replications were inoculated by injection of 0.3 mL of a bacterial suspension with 0.1 absorbance at 600 nm, corresponding to about 1×10^8 cfu/mL. The other three replications were inoculated through the soil at 20 cc/plant of the same bacterial suspension. The inoculated plants were incubated for 5 days with continuous wetting at 22 °C at night and 30 °C during the day, and 95% relative humidity. Wetting then continued at 1 h/day.

One week after inoculation by injection, the plants began to show wilting, first in the oldest leaf, with the other leaves observed as flaccid and weak. The plants inoculated through the soil showed symptoms after 2 weeks. After 4 weeks of evaluation, no significant differences were found between treatments.

For variety Africa, the phosphites showed their effect as catalysts of resistance, but variety Dominico Hartón did not respond. This finding suggests a possible effect of variety. This is now being evaluated.

The effects of phosphites and lixiviate of compost are currently being validated on two farms in Montenegro (Quindío).

9.3 References

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10. Inducing symptoms of bacterial wilt of plantain with a strain of *Ralstonia solanacearum* isolated from tomato

Elizabeth Alvarez (PE-1, IP-6)

10.1 Objective

To determine the pathogenicity of a strain, isolated from tomato, in plantain seedlings

10.2 Methodology

Samples of leaves and stem bases were taken from an infected plant growing in a tomato crop with symptoms of wilt. The crop was located on a farm in the Municipality of Montenegro (Quindío) and was being cultivated beside a plantain crop that had foci of bacterial wilt. The samples were processed in the laboratory. They were first washed for 20 min under running water, disinfected with 1% sodium hypochlorite for 1 min and then in 50% alcohol for another minute. They were then rinsed twice with sterilized distilled water, and finally macerated in sterilized distilled water. The mash was cultured onto SMSA medium (*see* Activity 1) and incubated at 28 °C for 4 days.

The mucoid and reddish colonies grew in the medium, and were purified by droplet transfer to SMSA. After 3 days, they were transferred to nutrient agar (NA) medium, where the bacterium grew for 36 h. A bacterial suspension was then prepared with the bacterium grown on NA by scraping the dishes in sterilized distilled water and passing through a spectrophotometer at an absorbance of 0.1 at 600 nm wavelength ($\approx 1 \times 10^8$ cfu/mL). This suspension was injected at 0.5 mL/plant into 4 seedlings of plantain variety Africa. The control used was strain CIAT 1008, isolated from plantain. The inoculated plants were incubated for 5 days with continuous wetting at 22 °C at night and 30 °C during the day, and 95% relative humidity, after which they were moistened daily for 1 h.

Once the symptoms were reproduced in the plantain, the bacterium was re-isolated. The pathogenic strains were again inoculated into plantain and into 15-day-old tomato seedlings by injection of a bacterial suspension.

In another trial, the soil was inoculated with a bacterial suspension of strain CIAT 1008 at 0.1 absorbance at 600 nm wavelength in a 25-mL volume that was added to 1-kg flasks carrying 30-day-old tomato plants. In some flasks, perpendicular cuts were made into the soil, 5 cm from the stem thus causing root wounds. This treatment was compared with unwounded plants. Plants were also inoculated by injection of 0.5 mL of the bacterial suspension. The plants were incubated as described for plantain.

10.3 Results

Symptoms began appearing in plantain plants in the second week after inoculation. The plants showed wilting of leaves and later death in a manner very similar to that of the control (Figure 10.1). Before the plants totally wilted, the bacterium was re-isolated and

cultured onto SMSA, where the typical morphology of *R. solanacearum* was observed. The inoculated tomato plants showed wilting within 6 days and died 3 to 5 days later.

Figure 10.1. (A) Bacterial wilt in tomato cultivated in the plantain-growing area of Montenegro (Quindío, Colombia). (B) Symptoms of bacterial wilt are reproduced in plantain seedlings inoculated with a strain of *Ralstonia solanacearum* isolated from the tomato plants observed in A.

Plants that received the soil inoculation treatment causing root wounds showed typical symptoms of bacterial wilt 6 days after inoculation. The plants that were not wounded showed symptoms 4 days later (Figure 10.2).

Figure 10.2. Bacterial wilt in tomato caused by isolate CIAT 1008 of *Ralstonia solanacearum* obtained from plantain, 10 days after inoculation. (A) Plant with wounds to the roots. (B) Plant without wounds to the roots.

The results contradicted reports that suggested that race 2 is specific to the Musaceae and does not attack tomato. On the contrary, they confirmed Fegan and Prior's findings (2005, CIRAD), where they had identified several phylotypes of *R. solanacearum* attacking both plants. The strains we isolated from both plantain and tomato classified as phylotype II (Fegan and Prior 2005).

10.4 References

Fegan M; Prior P. 2005. How complex is the *Ralstonia solanacearum* species complex? In: Allen C; Prior P; Hayward AC, eds. Bacterial wilt disease and the *Ralstonia solanacearum* species complex. American Phytopathological Society (APS), pp 449–461.

11. Evaluating ecological practices of soil management in foci affected by bacterial wilt *(Ralstonia solanacearum)* in two plantain crops in the Department of Quindío

Elizabeth Alvarez (PE-1, IP-6)

11.1 Objective

To evaluate the effect of incorporating into the soil lixiviate of compost of plantain, phosphoric rock, and *Tagetes patula* on inhibiting *Ralstonia solanacearum* in the soil of two farms in the Department of Quindío

11.2 Methodology

11.2.1 Field

Three foci affected by bacterial wilt were chosen in two plantain crops on "La Guaira" and "Cataluña" Farms (Montenegro, Quindío). To evaluate their effects on the bacterium *R. solanacearum*, causal agent of bacterial wilt, the following treatments were applied:

- Phosphoric rock (29% P_2O_5), lixiviate, and entire *T. patula* plants were incorporated at rates of 25 kg, 24 L, and 4 kg, respectively, at each affected site
- Check with 50% formalin
- Check with no treatment

The treatments were distributed in a randomized complete block design, with 3 replications in "La Guaira" and 6 in "Cataluña". The experimental unit corresponded to an affected site (i.e., one plant with daughter and granddaughter).

Before applying treatments, samples were collected from soil at 20–25 cm deep and from affected plants in 25-cm-long pieces that encompassed both above and below ground parts, as well as tissues. The samples were used to detect the bacterium.

Once sick plants in the foci were identified, these plants were uprooted and chopped into pieces of about 30 to 40 cm long, stacking them on the affected site. Above the pieces the phosphoric rock was applied first, then the lixiviate, and finally the *T. patula* plants, cut into pieces on application to favor release of the plants' thiophenes, which are probably responsible for inhibiting the bacterium (Arenas et al. 2005). The mixture was covered with soil and with black polyethylene to prevent weeds growing.

To apply formalin to the affected site, the soil was perforated at five points with a ¼-inch-diameter rod to as deep as 60 cm. Two liters of 50% formalin were applied, filling up every orifice. The treated site was then covered with black polyethylene.

The check consisted of plant material that was chopped up and covered with black polyethylene, receiving no treatment.

Once the treatments were applied, a ditch was dug around each site to prevent contamination among sites.

Every 30 days, samples were taken of the soil at 20 and 50 cm deep in the same site where the affected plant was found.

Three months after the trial was established, the treatments were re-applied.

11.2.2 Isolation

From each soil sample, 3.3 g were taken and 30 mL of TE buffer (10 mM blend of TRIZMA® base and TRIZMA® HCl, and 1 mM of EDTA) at pH 7.6 were added and homogenized by vortexing. This suspension became the base solution.

From this base solution, dilutions at 10^{-1} to 10^{-3} strengths of TE buffer were prepared. Of each dilution, 100 µL were placed in petri dishes containing SMSA (1 liter of medium contained 10 g Bacto™ Peptone, 5 mL glycerol, 1 g casamino acids, 18 g Bacto™ Agar, 26 mg bacitracin, 100 mg polymyxin-β sulfate, 5 mg chloramphenicol, 0.5 mg penicillin, 5 mg crystal violet, and 50 mg 2,3,5 chlorotriphenyltetrazole). The samples cultured onto this medium were incubated at 28 °C for 7 days.

After 7 days, colonies that looked like *R. solanacearum* (reddish color, mucoid, and amorphous) were transferred to SMSA and incubated for 4 days. Colonies confirmed as possibly positive were transferred to nutrient agar (20 g/L), incubated at 28 °C for 24 h, and inoculated onto plants of plantain variety Africa that had derived from meristem culture.

11.2.3 Inoculation

For inoculation, a bacterial suspension of 0.1 absorbance at 600 nm was prepared and 0.5 mL injected into each plant. The inoculated plants were incubated for 10 days in a humid chamber at 29 °C during the day and 24 °C at night and relative humidity between 80% and 91%. From day 10 to day 30, records were made of those plants that produced typical symptoms of bacterial wilt. Detection by PCR was also carried out with specific primers, as described in Activity 2.

11.2.4 Indicator plants

Three methodologies were carried out for evaluations with indicator plants:

1. In one focus in "La Guaira" Farm, with two replications, 55 plantain plants were planted in an area of 4 × 4 m to act as indicators of the presence of *R. solanacearum* in the soil.

2. Because the bacterium was difficult to isolate from the soil, apparently because of its small population, 4 plantain plants were planted in each experimental unit 4 months after the trials were established.

3. Fegan and Prior (2005; CIRAD) reported phylotype II of *R. solanacearum* as being infectious in tomato and plantain. Although no other report is known in Colombia, a tomato growing in Montenegro (Quindío) was found to have symptoms of bacterial wilt, infected by the same phylotype of *R. solanacearum* pathogenic in plantain (CIAT 2005). Based on this report, tomato plants were also planted as indicators, on each site.

11.3 Results

In direct form, the soil samples were detected as having very low levels of the bacterium, even in the check with no treatment. Hence, 1 mL of each of the base solution and 10^{-1} to 10^{-3} dilutions were mixed with 1 mL of liquid broth medium to enrich the bacterial population before culturing onto SMSA medium. Of the samples collected before applying the treatments, the bacterium was detected in two sites in each of "La Guaira" and "Cataluña" Farms.

At 1 and 5 months of applied treatments, the bacterium was detected in a check established on the "Cataluña" Farm. The presence of the bacterium in these samples was verified by inoculating plantain and amplifying through PCR.

At 4 months after establishing the trial in "La Guaira", one of the indicator plants (planted in 4 m square) manifested symptoms of bacterial wilt. This plant was located on the margin of a ditch that separated a check treatment with no applications.

After 8 months of applying the treatments in "Cataluña", four indicator plantain plants, planted on the site, were recorded as infected. These plants corresponded to two treatments with formalin and two controls, indicating a possible re-inoculation of the bacterium. In "La Guaira", no new infected plants were detected.

11.4 References

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12. Determining the genetic variability of *Ralstonia solanacearum* of plantain, using microsatellite markers (RAMs)

Characterization and knowledge of the genetic structure of pathogen populations have direct applications in disease management. This study therefore aimed to obtain information on the genetic diversity of a population of *R. solanacearum* race 2 from Colombia, causal agent of bacterial wilt of plantain. For the first time for this pathogen, a technique based on microsatellites, known as random amplified microsatellites (RAMs), was used to help develop strategies to improve the acquisition of durable resistance to the said pathogen.

12.1 Objective

To determine the variability of *Ralstonia solanacearum* from Musaceae crops in different regions of Colombia, using random amplified microsatellites (RAMs)

12.2 Methodology

We used 59 strains of *R. solanacearum* from tissues of sick plants, infected by bacterial wilt and collected from plantain, banana, and heliconia crops growing in six departments of Colombia, and from soil of infected plantain crops in Quindío. We used, for controls, a strain from eggplant from Kenya and four strains from tobacco (two from Floridablanca, Santander, Colombia; one from Quency, Florida, USA; and one from Japan) (Table 12.1). The *R. solanacearum* strains were obtained from the collection held by the Cassava Pathology program at CIAT. This study thus became the most complete on the genetic variability of the causal agent of bacterial wilt of plantain in Colombia. The strains preserved in solution with 60% glycerol were reactivated in a semi-selective medium (SMSA) and then transferred to nutrient agar to obtain pure strains of 24-h growth for later DNA extraction.

Table 12.1. Description of Ralstonia solanacearum strains, causal agent of bacterial wilt of plantain, used for the study.

Crop and strain no.	Geographical origin	Source
Plantain		
1	Quindío	Rachis
3	Quindío	Petiole
15	Quindío	Soil
16b	Quindío	Soil
17	Jamundí (Valle del Cauca)	Soil
18	Jamundí (Valle del Cauca)	Sucker
32	Caquetá	Pseudostem
33	Caquetá	Pseudostem
81	Montenegro (Quindío)	Fruit
38	Quindío	Soil

Crop and strain no.	Geographical origin	Source
39	Quindío	Soil
40	Quimbaya (Quindío)	Soil
41	Quimbaya (Quindío)	Soil
42	Fuente de Oro (Meta)	Pseudostem
43	Fuente de Oro (Meta)	Pseudostem
48	Armenia (Quindío)	Fruit
54	Fuente de Oro (Meta)	Pseudostem
57	Fuente de Oro (Meta)	Pseudostem
58R	Fuente de Oro (Meta)	Petiole
59	Fuente de Oro (Meta)	Pseudostem
60	Fuente de Oro (Meta)	Pseudostem
63	Granada (Meta)	Pseudostem
64	Granada (Meta)	Pseudostem
65	Granada (Meta)	Pseudostem
66	Granada (Meta)	Pseudostem
67	Fuente de Oro (Meta)	Pseudostem
69	Granada (Meta)	Pseudostem
71	Urabá (Antioquia)	Rhizome
72	Urabá (Antioquia)	Pseudostem
73	Urabá (Antioquia)	Pseudostem
76	Montenegro (Quindío)	Pseudostem
78	Montenegro (Quindío)	Rachis
79	Montenegro (Quindío)	Rhizome
80	Montenegro (Quindío)	Pseudostem
83	Quindío	Fruit
84	Quindío	Pseudostem
85	Quindío	Sucker
86	Calarcá (Quindío)	Rachis
88	La Tebaida (Quindío)	Rhizome
89	La Tebaida (Quindío)	Pseudostem
97	Quimbaya (Quindío)	Rhizome
107	Armenia (Quindío)	Fruit
1008	Ibagué (Tolima)	CIAT collection
Banana		
5	Urabá (Antioquia)	Rhizome
6	Urabá (Antioquia)	Fruit
7	Urabá (Antioquia)	Fruit
110	Magdalena	Pseudostem
111	Magdalena	Rhizome
112	Magdalena	Sucker
Heliconia		
113	Palmira (Valle del Cauca)	Pseudostem
114	Palmira (Valle del Cauca)	Rhizome
115	Palmira (Valle del Cauca)	Rhizome

12.2.1 Extracting DNA

The protocol described by Boucher et al. (1985) was used to extract DNA from the 59 strains.

12.2.2 Analyzing RAMs

To determine the genetic variability of the *Ralstonia solanacearum* strains, random amplification of microsatellite primers (RAMs) was used. RAM primers are tandem repeats of sequences of two or three nucleotides with random bases in the 3' extreme. With these, the genetic variability of individuals belonging to very close gene pools can be estimated, thus permitting differentiation between species and even within a single species, according to patterns of amplification from the total DNA.

We evaluated polymorphism among five isolates of *R. solanacearum,* obtained with seven RAM primers: HVH $(TG)_7T$, DHB $(CGA)_5$, DYD $(CT)_7C$, DBD $A(CA)_7$, VHV $(GT)_5G$, HBH $(AG)_7A$, and DDB $(CCA)_5$, where H is (A,T,C); B is (G,T,C); V is (G,A,C); and D is (G,A,T). We then amplified the 59 strains with the most polymorphic primer (Hantula et al. 1996).

Every PCR-RAM reaction was carried out in volumes of 12.5 µL, made up of dATP, dCTP, dGTP, and dTTP in proportions of 0.2 mM each; 1.25 µL 10X *Taq* polymerase buffer solution; 1 mM $MgCl_2$; 0.008 U *Taq* polymerase (Promega); 2 µM primer; and 5 ng total DNA. Amplification was carried out in a MJ Research PTC-100 thermal cycler, programmed at 95 °C for 5 min; 37 cycles of denaturation at 95 °C for 30 s; annealing at 55 °C (TG primer), 61 °C (CGA), 41 °C (CT), 50 °C (CA and AG), and 58 °C (GT), all for 45 s, and 55 °C (CCA) for 50 s; extension at 72 °C for 2 min; and final extension at 72 °C for 7 min (Henríquez et al. 2002).

The amplified products were separated by electrophoresis in 2% agarose gels with 0.5X TBE buffer, dyed with 0.001% ethidium bromide, run for 2½ h at 140 volts, and visualized under ultraviolet light in a Stratagene Eagle Eye® II. The band patterns of each primer were compared according to the presence or absence of a fragment; same-sized fragments were determined as identical.

To estimate the genetic relationships among the isolates, a dendrogram was generated from data on the 59 strains, using the CGA primer. Each fragment generated with the RAM technique was analyzed as an independent character. The same-sized DNA fragments were assumed to represent the same genetic locus, which was evaluated as absent or present. For each individual band, the value of 1 was assigned for presence and 0 for absence. Similarity among individuals was estimated, using the Dice coefficient of similarity. The dendrogram was generated, using the unweighted pair group method with arithmetic mean (UPGMA) and the statistical packet NTSYS-PC, version 2.02.

12.3 Results

Of the seven primers evaluated, we standardized the CGA primer with 1.5 mM $MgCl_2$, managing to obtain patterns of reproducible bands for *R. solanacearum* and observing polymorphisms among the strains from different sites.

To read the bands, a range between 201 and 1327 bp was selected, observing characteristic bands for the strains from different crops and departments (Figures 12.1 to 12.3).

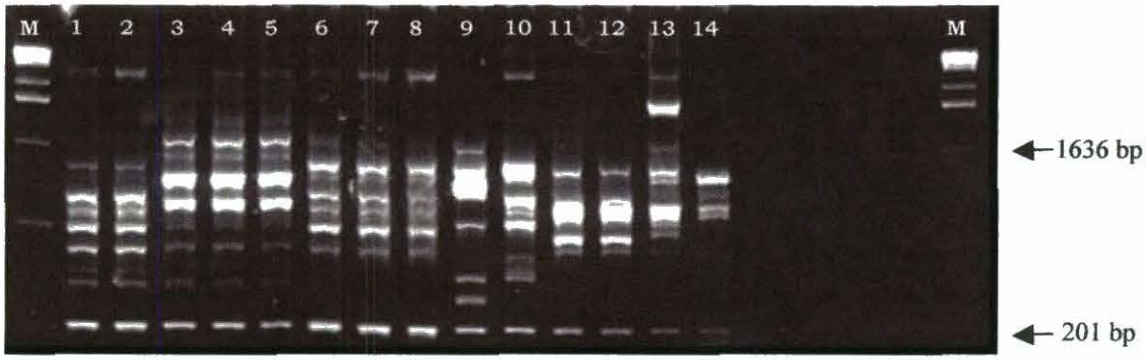

Figure 12.1. RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from different plant species. M = 1-kb marker; lanes 1 and 2 = strains from banana, Antioquia, Colombia; lanes 3–5 = strains isolated from banana, Magdalena, Colombia; lanes 6–8 = strains isolated from heliconias, Valle del Cauca, Colombia; lane 9 = strain from eggplant, Kenya; lane 10 = strain from tobacco, Japan; lanes 11 and 12 = strains from tobacco, Santander, Colombia; lane 13 = strain from tobacco, Florida, USA.

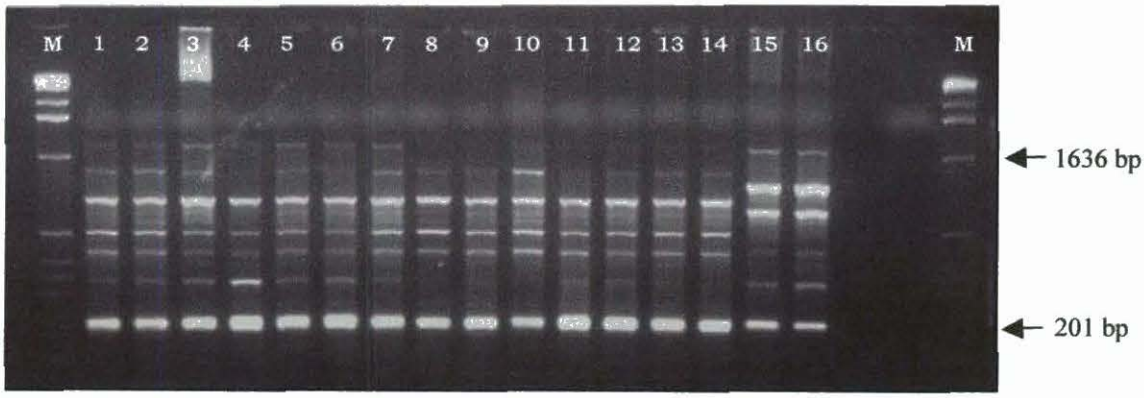

Figure 12.2. RAM patterns obtained with the CGA primer for Ralstonia solanacearum strains isolated from two different sites in Colombia. M = 1-kb marker; lanes 1–14 = strains isolated from plantain, Quindío; lanes 15 and 16 = strains isolated from banana, Magdalena.

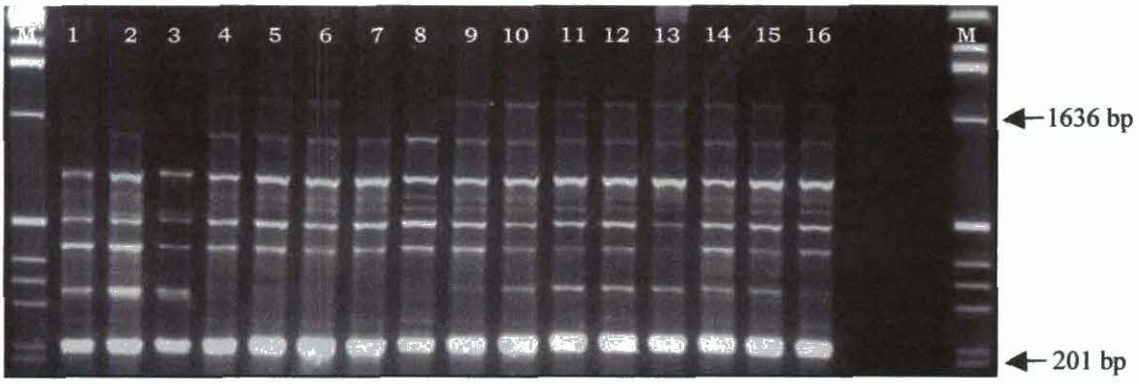

Figure 12. 3. RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from plantain in three different sites in Colombia. M = 1-kb marker; lanes 1–3 = strains from Antioquia; lane 4 = strain from Caquetá; lanes 5–16 = strains from Meta.

By analysis in NTSYS, a dendrogram (Figure 12.4) was prepared that generated 12 clusters, having a coefficient of similarity of 0.87 and differentiating according to crop type and geographic location. These were:

- Two of the three strains isolated from plantain in Antioquia, forming a cluster and showing 83% similarity with strains isolated from banana in Antioquia.

- The strains isolated from heliconia from Rozo (Valle del Cauca) showed 85% similarity with the strains isolated from the rhizosphere and plantain tissue from Jamundí (Valle del Cauca).

- The strains isolated from plantain in different municipalities of Quindío showed close similarity (82% to 100%) with strains from different municipalities of Meta.

- The Meta cluster showed moderate similarity (60% to 80%) with strains from plantain in Antioquia, indicating that no apparent separation of plantain strains exists, even though some clustered independently of others.

- Highest variation was observed between the strains from banana in Antioquia and those from banana in Magdalena, showing a 25% similarity and thus indicating that, between these two departments, strains possibly present the greatest genetic variation. To confirm this finding, however, a larger number of samples must be collected.

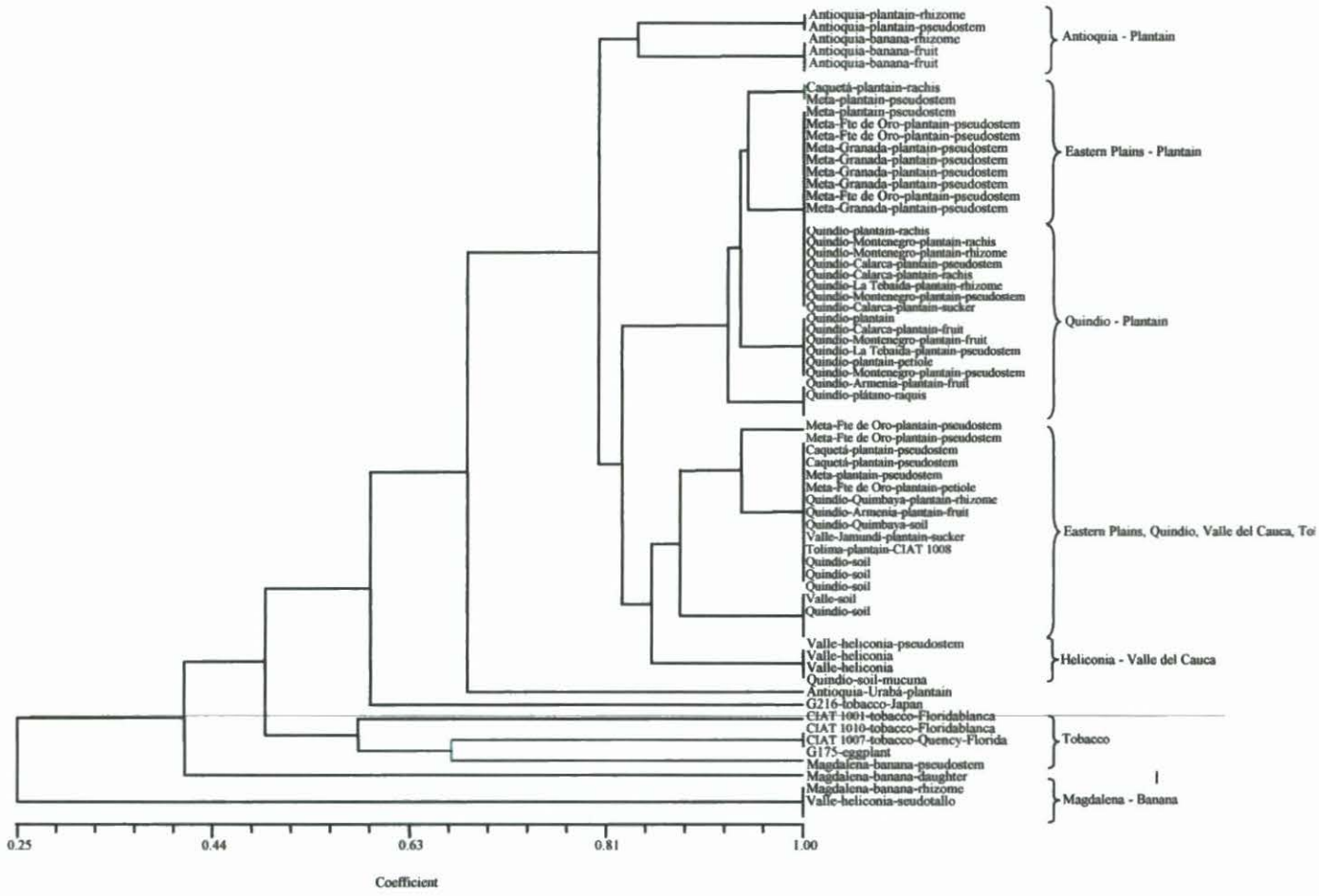

Figure 12.4. Dendrogram of similarity based on the Dice coefficient for 59 strains of Ralstonia solanacearum, with the RAM primer CGA.

12.4 Conclusions

The RAM technique enabled us to make an intraspecific and interspecific analysis of *R. solanacearum*, causal agent of bacterial wilt of Musaceae and of other plant species.

With the CGA primer, we obtained polymorphic band patterns that were reproducible for the selected strains. This primer also showed clear discrimination between strains according to crop type, based on the absence or presence of specific bands.

Ralstonia solanacearum strains from plantain tissue and soil from plantain crops in different parts of Colombia presented a similarity that ranged from 82% to 100%, according to the RAM analysis, showing close taxonomic affinity.

Because the band patterns were distinguishable among strains of different origin, clusters were formed according to crop type and geographical origin for which a coefficient of similarity of 0.87 was obtained. We deduced that a high level of variation does not exist among strains from plantain crops, heliconias of Valle del Cauca, and banana of Antioquia. However, the last mentioned showed less than 60% similarity with strains from banana in Magdalena, eggplant (race 3), and tobacco (race 1). Genetic variation increased among strains from different races.

The microsatellite marker CGA separated the plantain strains from strains from banana, heliconia, and the soil. This finding contrasts with the results on pathogenicity for which the strains show no differences among sites, hosts, and/or tissue types. In addition, geographical differentiation was shown for strains from Antioquia.

12.5 References

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12.6 Diagnosis

Table 12.1. Bacteria and fungi isolated from different crops and identified at the Cassava Pathology Laboratory, CIAT, Palmira, Colombia.

Location	Host plant	Disease	Detection method	Microorganism identified
Montenegro, Quindío	Plantain	Bacterial wilt	Isolation on selective media	*Ralstonia solanacearum*
Montenegro, Quindío	Banana	Panama disease	Isolation, pathogenicity	*Fusarium oxysporum*

13. Optimization of methodologies for massive production of clonal planting material of yellow passionfruit (*Passiflora edulis* f. flavicarpa Degener) free of diseases

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Collaborators: Corporación Colombiana de Investigación Agropecuaria - Corpoica

13.1 Background

The juice of the yellow passionfruit (*Passiflora edulis* f. flavicarpa Degener) is the third most important exotic juice in the world market. Colombia's productivity of passionfruit is considered the highest average yield with around 20 tons/ha; Colombia is the third largest producer of passionfruit concentrate after Brazil and Ecuador (Anonymous, 2005), and consequentely, the central government is promoting the establishment of new plantations as a strategy for the replacement of illegal crops.
A disease known as the "Secadera" is considered to be caused by a complex of soil borne fungi with predominance of *Fusarium* (Torres *et al.* 1999). Secadera is the main disease of the yellow passionfruit in Colombia, ana affects the most important producing regions of the country. This disease reduces de longevity of the plantations from 36 to 19 months (Rodriguez and Marmolejo, 1999) causing the loss of more than a half of production potential.
To minimize losses induced by "Secadera" one could identify commercial cultivars with tolerance to this disease. However, this is a long term task and requires a sustainable breeding program, as tolerant material might not have commercial attributes that make them appealing. A short term strategy to solve this lack of tolerance and to keep using the existing culvitars is to identify rootstocks tolerant to soil borne diseases. Rootstocks could be found in other *Passiflora* species resistant to them like *P. nitida* and *P. giberti* (Roncatto *et al.* 2004) or resistant selections of the same species (Newet, 2005).

Plantlets produced by seeds of resistant cultivars may be genetically unstable, or produce too weak stems to be used as rootstocks (Costa *et al.* 2004); therefore, the most appropriate tissues for the production of rootstocks are vegetatively propagated plants obtained from rooted cuttings.

Production of rooted cuttings will also be useful to fix other traits such as the productivity or fruit quality, if superior clones are vegetatively propagated and grafted onto resistant rootstocks.

Methods for the vegetative propagation of the yellow passionfruit have been developed using greenhouse based (Molina-Meletti *et al.*, 2002; Salomao *et al.*, 2002; Chamhum *et al.*, 2002) and tissue culture technologies (Gonzalez, 2003). In Colombia tissue culture derived plants have been commercialized in the past. However the adoption of this

technology for establishment of plantations has not been as expected, possibly due to the high costs of the clonally propagated plants or the little agronomic advantage of the propagated clones.

During 2005, as part of a project funded by the Ministry of Agriculture and Social Development of Colombia – MADR, and in collaboration with the Colombian Corporation for Agricultural Research, CORPOICA, we investigated the development of fast and inexpensive methodologies for clonal propagation of yellow passionfruit and other *Passiflora* species that could be applied for the massive production of disease tolerant grafted plants .

13.2 Methodology

Plants of the yellow passionfruit and of different *Passiflora* species included in this study were maintained in a greenhouse at room temperature. Stem segments with two or three nodes were cut from actively growing branches for producing cuttings. All leaves of the cutting with exception of that of the most upper node were cut away and discarded. The cuttings were treated with two concentrations of naftalenacetic acid (NAA; 10 and 100 mg/L) dissolved in water, for different periods of time (0, 10, 120, 180, 360 and 1440 minutes or 30 min in a second experiment). Then they were planted in sterile sand and placed under intermittent mist (from 6 to 18 hours, every hour for 1 minute) in a greenhouse (modified after Chamhum *et al.* , 2002)
The following variables were evaluated 45 days after planting: number of buds and roots developed, length of the longest root and plant recovery efficiency (PRE, the percentage of treated cuttings which produced plants). Means were analyzed with the Ryan-Einot-Gabriel-Welsch test of variance at a level of 5% of probability.

13.3 Results

Plant recovery from yellow passionfruit cuttings
Forty five days after experiment initiation most of the cuttings showed prolific adventitious root formation. With exception of the longest treatment with the highest NAA concentration (100 mg/l NAA for 1440 minutes, Table 13.1) which produced less than 60% recovered plants, all other treatments yielded over 80% plant recovery. Dipping the cuttings in a solution of 10 mg/l NAA for 120 minutes was the best treatment, yielding 100% plant recovery. However, for our surprise, also the control treatments in which no growth regulator was used, yielded plant recovery values over 98.6%, indicating that the growth regulator NAA is not necessary for an efficient root induction of cuttings with three nodes of the yellow passionfruit. Practically all of the rooted cuttings could be established in the greenhouse as a new plant, which served later on as a mother plant for production of further cuttings.

Table 13.1. Average number of buds and roots produced, length of the longest root and plant recovery efficiency of cuttings of the yellow passionfruit treated with different periods of time and concentrations of NAA (average followed by the same letter indicates a statistical similarity within each column to 5% of probability).

Exposure time (min)	Number of buds			Number of roots			Length of the longest root (cm)			Plant recovery efficiency* (%)		
	0 mg/L	10mg/L	100mg/L	0 mg/L	10mg/L	100mg/L	0 mg/L	10mg/L	100mg/L	0 mg/L	10mg/L	100mg/L
0	1,8 bcd			8,6 bc			13,1 c			98.6		
10		3,7 a	3,1 ab		14,9 bc	24,7 ab		18,1 bc	17,4 bc		83,3	87,5
120		3,0 ab	2,3 abc		14,0 ab	40,8 a		21,2 b	20,8 b		100	92,5
180		1,9 bcd	1,4 cd		12,1 bc	18,7 ab		17,6 bc	16,3 bc		89,5	90,7
360		1,9 bcd	1,4 bcd		16,3 ab	23,5 ab		16,4 bc	16,2 bc		84,6	96,4
1440		2,9 ab	0,6 d		25,5 ab	16,4 c		19,9 b	28,8 a		94,9	52,6

* % treated cuttings which produced plants

Plant recovery from cuttings of different Passiflora species

In other set of experiments, cuttings of two or three nodes from different Passiflora species, , were treated with one or two concentrations of NAA for 30 min and then placed under mist conditions. In a first experiment with three node cuttings plant recovery efficiency ranged between 43 and 56%. In a second experiment with two node cuttings plant recovery efficiencies increased to values between 89 to 100% (Table 13.2). This increased plant recovery efficiencies of the second experiment do not seem to be related to the number of nodes of the explants, but to the physiological conditions of the mother plants from which the cuttings were isolated. The mother plants from the second experiment were younger than plants used in the first experiment. Thus, it seems likely that plant recovery efficiencies of cuttings of Passiflora species may be improved by maintaining the mother plants in optimal conditions.

Table 13.2. Plant recovery efficiency from cuttings of different *Passiflora* species treated for 30 min with one or two NAA concentrations

Species	Number of Nodes	Plant recovery efficiency (%)	
		10mg/L	100mg/L
P. alata	3	56.7	43.3
P. maliformis	3	50	50
P. mollisima	3	51.5	48.5
P. alata	2	100	
P. edulis	2	89.8	
P. ligularis	2	100	
P. quadrangularis	2	90.9	

Additionally to the above mentioned *Passiflora* species, several other tested with the developed propagation methodology (*P. cincinnata, P. subpeltata, P. lehmani, P. foetida* and *P. popenovii*) could be cloned with efficiencies over 50% (data not shown).

Determination of the number of nodes needed for efficient clonal propagation
To be able to use cuttings with less than two or three nodes for propagation will allow one to produce more propagules from a single mother plant.
Analysis of the buds of two node cuttings participating in the formation of the new cloned plant, indicates that the first (the upper) bud participates in the formation of most of the recovered plants, while the second stayed dormant. This suggests that it may be possible to reduce the number of nodes of the cuttings to single node ones, and increase by this way the propagation efficiency. Experiments are underway to confirm this.

The methodology developed is much simpler than that reported by Molina Meletti et al. (2002) who used hydroponic cultures with complex recycling forms of nutrient solutions. Also, with the possibility of using single node cuttings as initial explant, our propagation methodology may be applied with higher efficiencies than that developed by Chamhum *et al.* 2002, who used only selected portions of the stem of the mother plants as source of cuttings of three or more nodes.

Taking the necessary measures for maintaining mother plants in good health in the greenhouse, this propagation methodology may represent a simple alternative for tissue culture propagation of selected cultivars of the yellow passionfruit or other *Passiflora* species.

Figure 13.1. Rooted cuttings of *the yellow passionfruit (P. edulis* f. flavicarpa Degener*)* and of the sweet passionfruit (*P. alata* Dryand.) after 45 days of treatment with NAA solution and maintainedeunder intermittent mist conditions.

13.4 Conclusions

An efficient and inexpensive methodology has been adapted and optimized for the clonal propagation of the yellow passionfruit and other *Passiflora* species. This methodology can be used for the propagation of selected cultivars or for the production of rootstocks tolerant to pests or diseases.

13.5 Future plans

To test the the propagation efficiency of single node cuttings. To produce grafted yellowpassionfruit plants using rootstocks of *Passiflora* species reported as resistant to *Fusarium* wilts, and test its tolerance to Secadera in the field.

This project will be continued with minimal core funding until a proper donor is found,.

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