ANNUAL REPORT 2006
PROJECT IP-06

Tropical Fruits, a Delicious Way to Improve Well-being

CONTENTS

1. Introduction

The mission of the Tropical Fruits Project is: *"To enhance competitiviness of smallholder producers of tropical fruits by providing information and tecnologies that lead to better production practices, facilitate access to markets and and will increase household income"*

The Tropical Fruits Project works on a external funding structure, and from small proportion of core funding from CIAT. Funding has been secured for the next 3 years to conduct work on 4 species highly important for the Andean Region: Solanum quitoense *(Fam: Solanacea, lulo or naranjilla)*, *Rubus glaucus (Moracea: Andean blackberry, mora*), *Persea americana* (*Fam Lauracea, avocado*) and *Musa spp. (Musacea: Plantain)*. Funding was granted through competitive grant scheme from Colombian Ministry of Agriculture (MADR), and Fondo Regional de Tecnología Agropecuaria for Latin America and the Caribean (Fontagro). The current research projects respond to needs identified by the fruit industry and will deliver solutions to production problems affecting the industries of Avocado, Lulo, Mora and Plantain. The framework for the Tropical Fruits Project is to develop principles of participatory variety selection, biotechnology techniques for improving planting material, strategies for sustainable crop and disease management, and to facilitate access to markets to generate economic impact in the rural communities for improving the livelihood of the rural poor.

Given the broad variability of "fruits"in the tropics, it is reasonable not to select a particular species, but to work on generic solutions and principles, and to develop guidelines for choosing those species to work on. In areas where climatic conditions are rather diverse as in the Andean region, focusing on a group of species with broad altitudinal distribution would deliver economic impacts on a larger population of smallholder producers. Consequentely, a set of criteria to select the species to pursue work on would include:

- Is a tropical fruit
- Have the commitment of partners
- Is grown by thousand of growers in a vast geographic area
- Is important in local economy and market could be expanded

This annual report includes ongoing research activities, which in some cases represent the beginning of research projects that will extend for the next 3 years.

List of ongoing special projects in 2006 – IP06

Project Title	Donor	Participating Institutions	2. Amount available in 2006 US$		Total project budget US$
			CIAT (lead scientist)	Participating Institution (lead scientist)	
Precision agriculture and construction of models for tropical fruit crops (2005 – 2007)	COLCIENCIAS Agencia Colombiana de Cooperación Internacional (ACCI) MADR Colombia	Corporación BIOTEC	(Alonso González/ Elizabeth Alvarez)	(Myriam Sánchez)	425,564 (CIAT: 25,000)
Lulo con valor agregado. Alternativas para el pequeño agricultor. (2006 – 2008). Added value lulo. Alternatives for small growers.	Ministry of Agriculture and Rural Development (MADR), Colombia	CORPOICA La Selva, Universidad de Antioquia	(Alonso Gonzalez, Zaida Lentini, Elizabeth Alvarez)	(Mario Lobo)	313,734 (CIAT: 153,000)
Integrated Water resource management by the implementation of improved Agro- Forestry concepts in arid and semi-arid areas in Latin America- WAFLA (Oct 2006- Mar 2009)	Sixth Framework- European Communitiy	TTZ (leading institutions) CIAT + 21 partners from Latin America and Europe	Alonso Gonzalez Carlos Ostertag Jorge Beltran	TTZ	Euros, 1'350.000.00 CIAT (80,000).

Project Title	Donor	Participating Institutions	2. Amount available in 2006 US$		Total project budget US$
			CIAT (lead scientist)	**Participating Institution** (lead scientist)	
Desarrollo y transferencia de medidas de manejo del Moko (*Ralstonia solanacearum*), en cultivos de plátano del municipio de Armenia (Capacity building and development of management strategies of Moko Disease (*Ralstonia solanacearum*) in Plantain in Armenia, Colombia.	Alcaldía de Armenia, Quindío, Colombia	Fedeplátano	(Elizabeth Alvarez)	Silverio González	5,217 (CIAT 5217)
Collection, characterization, and clonal multiplication of avocado with emphasis on identification of lines tolerant to Phytophthora spp. (2006-2008) (Collection, characterization, and clonal multiplication of avocado with emphasis on identification of lines tolerant to *Phytophthora* spp.)	Ministry of Agriculture and Rural Development (MADR), Colombia	CORPOICA, PROFRUTALES	(Alonso González Alvaro Mejia, Joe Tohme) (Elizabeth Alvarez)	(Juan Jaramillo, Danilo Rios)	411,580 (CIAT: 97,000)

2. Identifying Strategies for Managing Anthracnose (*Glomerella cingulata* (*Anamorph Colletotrichum gloeosporioides*) of Soursop *(Annona muricata L.),* Emphasizing Varietal Resistance

Alberto Rojas Triviño, Elizabeth Álvarez, and Danilo Sánchez

Rationale

Anthracnose is the most limiting disease for soursop production in Colombia and other countries. Incidence and losses can be 100% (Oliveros 2000), depending on agroecological conditions, crop management, and planting material. The disease is favored by inadequate cultural management practices. To successfully manage the disease, we must identify genetic resistance in soursop and understand the disease's epidemiology. Aiming to advance in our understanding of the pathogen's genetic variability we looked for molecular markers associated to pathogenicity. Equally important, we assessed the genetic variability of soursop clones selected for desirable agronomic traits and good performance in soursop production zones.

Materials and Methods

*Collection of plant material***:** Sampling of plant material infected with anthracnose was carried out in the Departments of Valle del Cauca, Cauca, Huila, Tolima, Meta, Santander del Sur, Norte de Santander, Quindío, Caldas, Córdoba, and Sucre in Colombia. Samples were taken from established crops and individual trees on which symptoms were observed in leaves, branches, stems, flowers, and/or fruits. Each sample was properly labelled and information of site of collection and predominant climatic conditions were recorded.

*Isolating, identifying, and storing the fungus***:** *Colletotrichum* isolates were obtained according to the direct method for isolating plant pathogenic microorganisms (Castaño-Zapata 1997), with modifications. Fungal growth, such as types of colony growth and the presence of acervuli, cirri, and spore types, was recorded using a compound light microscope. The fungus was purified on PDA agar, and monosporic cultures prepared on 2% water agar. The monosporic cultures were stored at 4º and --20ºC on Whatman No. 1 filter paper colonized by the fungus (Aricapa and Correa, 1994.).

Morphological characterization of Colletotrichum *isolates* **:** Fungus morphological characterization of the macroscopic variability was examined by looking at color and type of colony growth, cirrus color, length of conidia, presence of microsclerotia, and other parameters such as speed of growth, presence of the teleomorph stage, and length of asci and ascospore.

Molecular characterization of Colletotrichum *isolates* **:** DNA from each *Colletotrichum* isolate, was extracted as reported by Mahuku (2004) with modifications. We added 1.5 σL of proteinase K of 10 mg/mL, adding an equal volume of cold isopropanol to the supernatant obtained by adding chloroform and isoamyl at a ratio of 24:1, and washing twice with ethanol at 70%.

Amplifying the ITS region. To identify the species of each isolate, we used the following primers to amplify the internal transcribed spacer region (ITS) of rDNA: those specific to *Colletotrichum gloeosporioides* (CgInt) and C. *acutatum* (CaInt2) (Brown et al. 1996); and Col1 for the related C. *graminicola* and C. *dematium* that had not amplified with the first two primers (Afanador-Kafuri et al. 2003). These primers were coupled with the primer ITS4 (White et al. 1990). Table 1.1 presents the primers used and their corresponding sequences:

Table 2.1. Primers used in PCR analysis for amplifying specific fungal taxa.

Primer	**Sequence 5N3Nj**
CaInt2a	GGGGAAGCCTCTCGCGG
CgInta	GGCCTCCCGCCTCCGGGCGG
Col1b	GCCGTCCCCTGAAAAG
ITS4c	TCCTCCGCTTATTGATATGC

aBrown et al. (1996). b Afanador-Kafuri et al. (2003). c White et al. (1990).

To amplified products were separated on agarose gel with ethidium bromide at 1.0 mg/mL and adding buffer 10X TBE to a final concentration of 0.5X. A marker with a molecular weight of 100 bp was included, together with positive controls *(C. gloeosporioides and C. acutatum)* and a negative control composed of a PCR cocktail (Álvarez et al. 2005).

Amplifying RAM microsatellites at random. Genetic variability of isolates belonging to the same *Colletotrichum* species (previously determined by amplification of the ITS region), we used random amplification of microsatellites (RAMs), based on the polymerase chain reaction (PCR) (Hantula et al. 1996).

Pathogenic characterization of Colletotrichum *isolates***:** Pathogenecity of the *Colletotrichum* isolates, belonging to different RAM groups, is being evaluated by inoculating soursop plants of cv. Elita in the greenhouse.

Colletotrichum *inoculum.* Each isolate conserved on filter paper was planted on AA+E basal medium (Silveira et al. 2004) and incubated in an inverted box at 28oC for 15 days under 24 h of light. Alternatively, *Colletotrichum* spp. were propagated on Marthur's agar medium
(0.1% yeast extract, 0.1% Bacto™ Peptone, 1% sucrose, 0.25% MgSO4 1 7H2O, 0.27% KH2PO4, 1.2% agar supplemented with 25 mg ampicillin in 1 L sterilized distilled water). With this medium, the fungus produced considerable sporulation (Freeman et al., 1996).

After incubation, a suspension of spores was prepared, directly adding 10 mL of sterilized distilled water over the growing organisms in the Petri dish. This initial suspension was collected in 50-mL sterilized BD Falcon™ tubes, filtering with sterilized gauze to eliminate mycelia and fragments of medium. The suspension was then adjusted to a concentration of 1 × 107 spores/mL, using a hemacytometer (Reichert, Buffalo, NY, USA). Finally, Inex-A⊇ (COSMOAGRO S.A., Colombia) was added to disperse spores at a final concentration of 0.5%.

Preparing plants for inoculation. All the plants (including the checks) to be used in the experiment were cleaned of old leaves and any pests present on the stems and branches by rubbing down with cheese cloth. To create microscopic wounds, the upper surface of healthy young leaves was rubbed down with sterilized cheese cloth. impregnated with Carborundum®.

Inoculating the plants. On completing the treatment mentioned above, each isolate was inoculated on three plants, each constituting a replication, as follows (Figure 1.1):

- *Method of wounding.* Small rectangular cuts were made on stems with sterilized scalpels and 6-mm discs of isolate were placed in them. The isolate discs were taken from the center of the colony developed on PDA+E basal medium. Cuts were made on three parts of the stem, spaced at 10 cm, starting from the tree's canopy and finishing at its base, while ensuring that their locations were above the grafting point and in young tissues. Once completed, the points of inoculation were covered with Parafilm®.

- *Spraying method.* A suspension of 1×10^7 spores/mL of the fungus was used to spray the stem, apex, and youngest leaves (i.e., the first six leaves next to the apex). We used a vacuum pump and a DeVilbiss® sprayer.

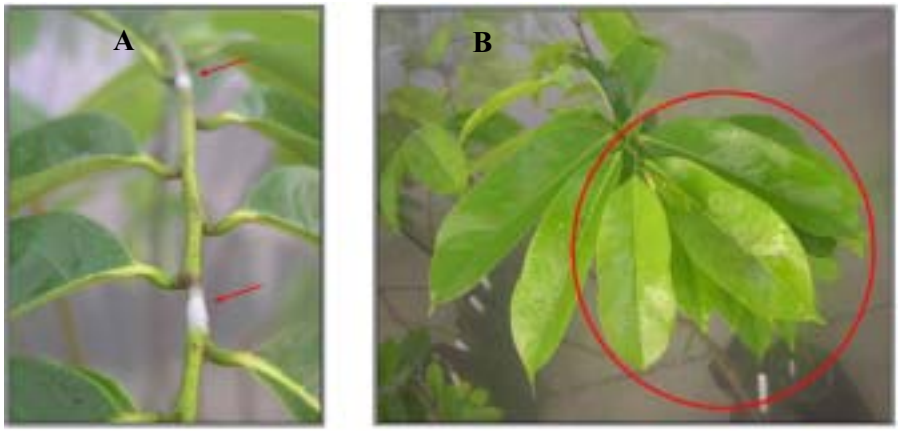

Figure 2.1 Inoculating soursop trees of cv. Elita. (A) Wounding method. (B) Spraying method

As control, three plants of the same cultivar were used, except that their wounds were not inoculated with fungus and they were sprayed with sterilized distilled water.

Incubating the inoculated plants. The inoculated plants were taken to a high humidity chamber, and each replication placed at random in three separate blocks, ensuring that they were not in contact. They were left for 72 h at a relative humidity of 90%–95% and an average temperature of 27o–29oC. The plants were then taken to a greenhouse with an average temperature of 27o–29oC and humidified for 1 min every hour for 17 days.

*Designing the severity scale and diagrams to evaluate anthracnose on leaves and branches of artificially inoculated soursop plant*s. The scale was designed according to percent values corresponding to 1, 5, 10, 25, and 50% (represented by grades 1, 3,

5, 7, and 9). To conduct the evaluation in the greenhouse, we prepared severity diagrams corresponding to each established value.
First, we determined the types of lesions most frequently observed on leaves and branches in the field, and then we examined symptom development in the same tissues inoculated in vitro.
Once we were able to reproduce the typical symptoms of anthracnose on leaves and branches of soursop observed in the field, we designed the diagrams to show the typical symptoms at different levels of severity. Each diagram was digitalized in an Epson Expression 1680 scanner, on a scale of grays, with a resolution of 300 dpi and stored in the TIFF format. The stored diagrams were interpreted with the WinRHIZO™ image analysis system (Regent Instruments, Inc., Quebec, Canada). With the values obtained on the infected area as interpreted by the system (black area = healthy tissue; white area = infected tissue), we selected diagrams that adjusted to the scale, ending up with three patterns of symptom development on leaves and one pattern for branches.

Evaluating the disease. To evaluate the disease, a scale was designed that took into account the presence of lesions on stems (considered by producers as the most serious symptom that impairs the most damage to the trees) and leaves. Evaluations began after 72 h of continuous wetting. The second and third evaluations were made on Days 10 and 20, starting from the first reading.

Evaluating germplasm in the greenhouse. Characterizing the pathogenicity of the *Colletotrichum* isolates and the RAM analysis, which permitted the formation of genetic groups, allowed us to select, at least one pathogenic isolate from each group and at least one isolate that was minimally pathogenic to cv. Elita. Thus, we could evaluate 20 accessions of soursop with participation of farmers (Table 2.2). The evaluations of disease's progress were conducted at equal intervals of time, using the same scale designed to characterize the pathogenicity of the *Colletotrichum* isolates.

Table 2.2. Soursop (*Annona muricata L.*) accessions evaluated for anthracnose

Accession name	Accession name	Accession name	Accession name
San Francisco	Rojas 1	Cítrica 1	Cítrica 6
Joya 1	Rojas 2	Cítrica 2	Cítrica 7
Joya 2	Cs1	Cítrica 3	Cítrica 8
Joya 3	Cs2	Cítrica 4	Cítrica 9
Costa Rica	Cs3	Cítrica 5	Cs4

Molecular evaluation of the germplasm:

To conduct a molecular characterization of the 20 accessions, we extracted DNA from each accession, and amplified it by PCR using the random amplification of microsatellites or RAM technique, and electrophoresis of single-stranded conformational polymorphisms (SSCPs).
The primers (synthesized by Technologies, Inc.) used to amplify the DNA extracted from soursop (Table 2.3) had been reported as polymorphic in assessments of plant and animal diversity (Piedrahita et al. 2005; Oslinger 2003; Álvarez et al. 2005.; Morillo et al. 2005: Espinosa et al. 2005 ;Arcos et al., 2005; Sanabria et al. 2006).

Table 2.3. RAM primers and nucleotide sequences.

RAM primer	Condensed sequence (5Nj to 3Nj	Sequence (5Nj to 3Nj
TG	HVH (TG)7T	5Nj HVH TGT GTG TGT GTG TGT 3Nj
CGA	DHB(CGA)5	5Nj DHB CGA CGA CGA CGA CGA 3Nj
CT	DYD(CT)7C	5Nj DYD CTC TCT CTC TCT CTC 3Nj
CA	DBDA (CA)7	5Nj DBD ACA CAC ACA CAC ACA 3Nj
GT	VHV (GT)7G	5Nj VHV GTG TGT GTG TGT GTG 3Nj
AG	HBH (AG)7A	5Nj HBH AGA GAG AGA GAG AGA 3Nj
CCA	DDB (CCA)5	5Nj DDB CCA CCA CCA CCA CCA 3Nj
ACA	BDB (ACA)5	5Nj BDB ACA ACA ACA ACA ACA 3Nj

Results and Discussion

Collecting plant materials : A total of 93 accessions of soursop were collected from Valle del Cauca, Cauca, Huila, Tolima, Meta, Santander del Sur, Norte de Santander, Quindío, Caldas, Córdoba, and Sucre. All of these being political departments of Colombia. We obtained 80 isolates of *Colletotrichum* from soursop trees infected with the disease; isolates were stored and used in the trials (Table 2.4).

Table 2.4. Sites (departments and municipalities) and number of *Colletotrichum* isolates collected from soursop with symptoms of anthracnose.

Department	Municipalities sampled	Isolates (no.)
Valle del Cauca	Palmira, Pradera, Tulúa, Cali, Toro, El Cerrito, Buga	41
Cauca	Caldono	1
Huila	Yaguará, Palermo	6
Tolima	Melgar	4
Meta	Villavicencio	4
Santander del Sur	Cimitarra	1
Norte de Santander	Bochalema	4
Quindío	Armenia, Montenegro	4
Caldas	Supía	4
Córdoba	Chinú	1
Sucre	Corozal, Sampués, Sincelejo	10
	Total	80

Morphological characterization of Colletotrichum *isolate*. Most of the initial isolates were *Colletotrichum spp*., except for isolate GM61-L01, which came from Norte de Santander and was isolated from leaves. Under microscopic observation, *Glomerella* spp. were determined as being present and lesions were atypical for anthracnose. Instead, lesions appeared as black spots (perithecia) on the main vein near the peduncle, the fungus having developed its asexual stage on culture medium. The sexual stage was also observed on culture medium for isolates GM57 and GM26 from Valle del Cauca and for which samples obtained were initially of *Colletotrichum* spp.

Molecular characterization of Colletotrichum *isolates:* amplifying the ITS region. With these amplifications, we sought to determine the presence of one unique genus by observing the typical electrophoretic patterns of the *Colletotrichum* genus (Figure 2.2).

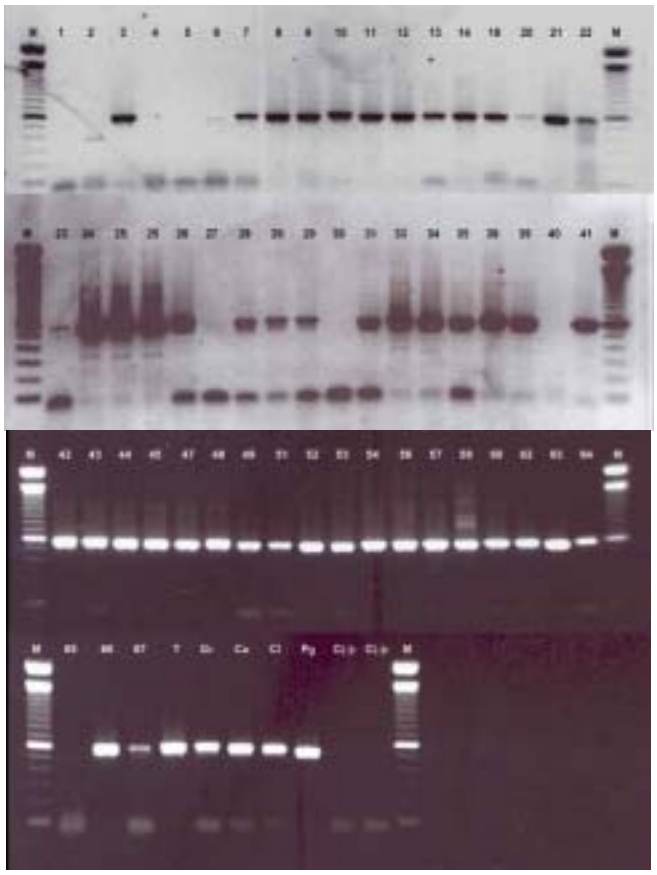

Figure 2.2. Electrophoretic profiles of DNA from 55 isolates of *Colletotrichum* spp., obtained from amplifying the ITS region, using primers ITS1 and ITS4. Lane M = marker with a molecular weight of 100 bp; positive control = *C. lindemuthianum*; negative control = PCR cocktail; check = *Phaeoisariopsis griseola*. The lanes identified as T, Gr, and Ca constitute *Colletotrichum* isolates from tea, granadilla, and cacao, respectively.

We partly determined the hybridization temperatures at which the primers in the PCR reaction would amplify the ITS region, coupling ITS4 primers with the specific primers CaInt2, CgInt, and Col1 to identify the species. With primers CgInt and Col1, we obtained the best results for hybridization at 60ºC, even though hybridization was more specific at this temperature for primer Col1 (Figure 2.3) and at 62ºC for CgInt (Figure 2.4).

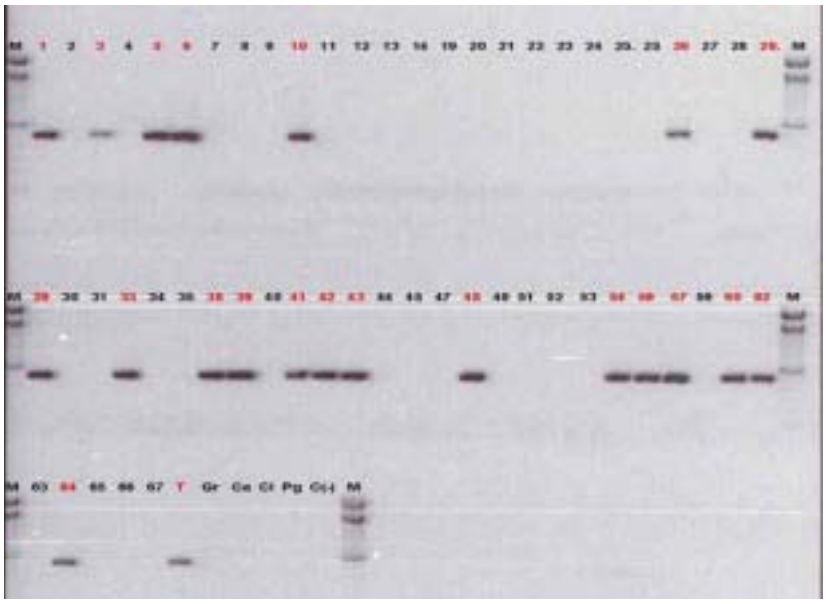

Figure 2.3. Electrophoretic profiles of DNA from 55 isolates of *Colletotrichum* spp., obtained by amplifying the ITS4 region + Col1 at a hybridization temperature of 60ºC. Lane M = marker with a molecular weight of 100 bp; positive control = C. lindemuthianum; negative control = PCR cocktail; check = Phaeoisariopsis griseola. The lanes identified as T, Gr, and Ca constitute isolates of *Colletotrichum* spp. from tea, granadilla, and cacao, respectively.

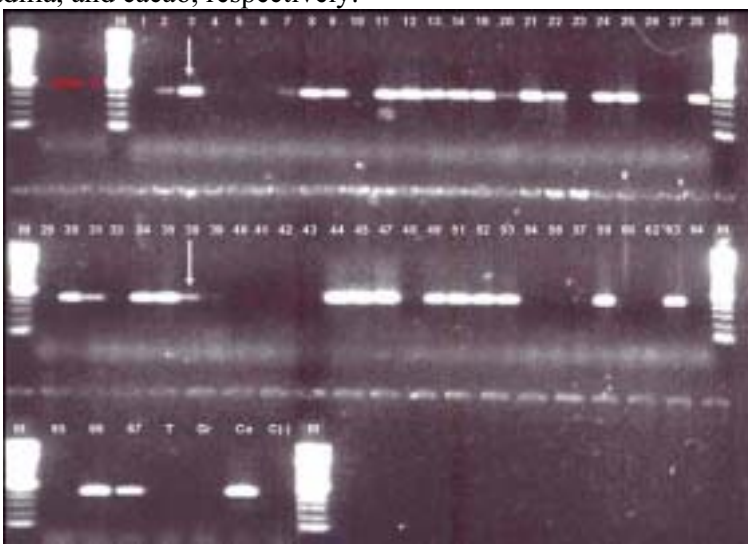

Figure 2.4. Electrophoretic profiles of DNA from 55 isolates of *Colletotrichum* spp., obtained by amplifying the ITS4 region + *Cg*Int at a hybridization temperature of 62ºC. Lane M = marker with molecular weight of 100 bp; positive control = *C. lindemuthianum*; negative control = PCR cocktail; check = *Phaeoisariopsis griseola.* The lanes identified as T, Gr, and Ca constitute isolates of *Colletotrichum* spp. from tea, granadilla, and cacao, respectively.

The partial results obtained with primer CaInt2 are presumed to indicate the absence of *C. acutatum* as none of the isolates evaluated amplified with the primer specific to this species (data not shown).
The results so far obtained with amplifications, using three primers specific to *Colletotrichum* spp. are summarized in Table 2.5. The species so far established are *C. gloeosporioides and Colletotrichum sp*., as no isolate was observed to amplify for *C. acutatum.*

Table 2.5. Prior identification of *Colletotrichum* species.

Consecutive number	Sample code	Department of origin	Primer reaction		
			CaInt2/ITS4	CgInt/ITS4	Col1 /ITS4
1	GM01-L02	Huila	(-)	(+d)a	(+)
2	GM03	Huila	(-)	(+)	(-)
3	GM04-L01	Huila	(-)	(+)	(+d)
4	GM04-L02	Huila	(-)	(-)	(-)
5	GM05	Huila	(-)	(-)	(+)
6	GM06	Huila	(-)	(+d)	(+)
7	GM25-L01	Valle del Cauca	(-)	(+)	(-)
8	GM25-L02a	Valle del Cauca	(-)	(+)	(-)
9	GM25-L02b	Valle del Cauca	(-)	(+)	(-)
10	GM26	Valle del Cauca	(-)	(+d)	(+)
11	GM27	Valle del Cauca	(-)	(+)	(-)
12	GM28	Valle del Cauca	(-)	(+)	(-)
13	GM29	Valle del Cauca	(-)	(+)	(-)
14	GM30-L01	Valle del Cauca	(-)	(+)	(-)
19	GM35-L01	Valle del Cauca	(-)	(+)	(-)
20	GM35-L02	Valle del Cauca	(-)	(+)	(-)
21	GM36-L02	Valle del Cauca	(-)	(+)	(-)
22	GM37	Valle del Cauca	(-)	(+)	(-)
23	GM38a	Valle del Cauca	(-)	(+d)	(-)
24	GM38b	Valle del Cauca	(-)	(+)	(-)
25	GM39-L02	Valle del Cauca	(-)	(+)	(-)
26	GM40	Valle del Cauca	(-)	(+d)	(+)
27	GM41	Valle del Cauca	(-)	(+)	(-)
28	GM42	Valle del Cauca	(-)	(+)	(-)
29	GM44-L01	Quindío	(-)	(+d)	(+)
30	GM49-L01	Valle del Cauca	(-)	(+)	(-)
31	GM49-L02	Valle del Cauca	(-)	(+)	(-)
33	GM52- L01	Valle del Cauca	(-)	(-)	(+)
34	GM52- L02	Valle del Cauca	(-)	(+)	(-)
35	GM52- L03	Valle del Cauca	(-)	(+)	(-)
38	GM57	Valle del Cauca	(-)	(+)	(+)
39	GM58-L02	Valle del Cauca	(-)	(+d)	(+)
40	GM59a	Norte de Santander	(-)	(-)	(-)
41	GM59b	Norte de Santander	(-)	(+d)	(+)
42	GM60-L01	Valle del Cauca	(-)	(+d)	(+)
43	GM61-L01	Norte de Santander	(-)	(+d)	(+)
44	GM61-L02	Norte de Santander	(-)	(+)	(-)
45	GM62-L01	Valle del Cauca	(-)	(+)	(-)

47	GM62-L03	Valle del Cauca	(-)	(+)	(-)
48	GM63	Cauca	(-)	(-)	(+)
49	GM64-L02	Valle del Cauca	(-)	(+)	(-)
51	GM66-L01	Valle del Cauca	(-)	(+)	(-)
52	GM66-L02	Valle del Cauca	(-)	(+)	(-)
53	GM67-L01	Valle del Cauca	(-)	(+)	(-)
54	GM67-L02	Valle del Cauca	(-)	(+d)	(+)
56	GM68	Valle del Cauca	(-)	(+d)	(+)
57	GM69-L01	Valle del Cauca	(-)	(+d)	(+)
59	GM70	Valle del Cauca	(-)	(+)	(-)
60	GM71	Tolima	(-)	(+d)	(+)
62	GM73	Tolima	(-)	(+d)	(+)
63	GM74	Tolima	(-)	(+)	(-)
64	GM75	Santander del Sur	(-)	(+)	(+)
65	GM77	Quindío	(-)	(-)	(-)
66	GM78	Quindío	(-)	(+)	(-)
67	GM79	Quindío	(-)	(+)	(-)
T	Tea	Valle del Cauca	(-)	(-)	(+)
Gr	Granadilla	Huila	(-)	(-)	(-)
Ca	Cacao	Huila	(-)	(+)	(-)

a. (+d) indicates weak positive reaction

Pathogenic characterization of Colletotrichum *isolates*. Initially, an evaluation scale was designed for soursop plants inoculated artificially by the methods described above (Table 2.6), we prepared severity diagrams corresponding to each value, using the previous information on the types of symptoms that develop on branches, stems, and leaves (Figure 2.5)

Table 2.6. Severity scale to evaluate anthracnose on leaves and branches of soursop.

Grade	**Severity (%)**
1	1
3	5
5	10
7	25
9	50

Finally, we determined three patterns of symptom development on leaves and one pattern for stems and branches (Figure 2.5).

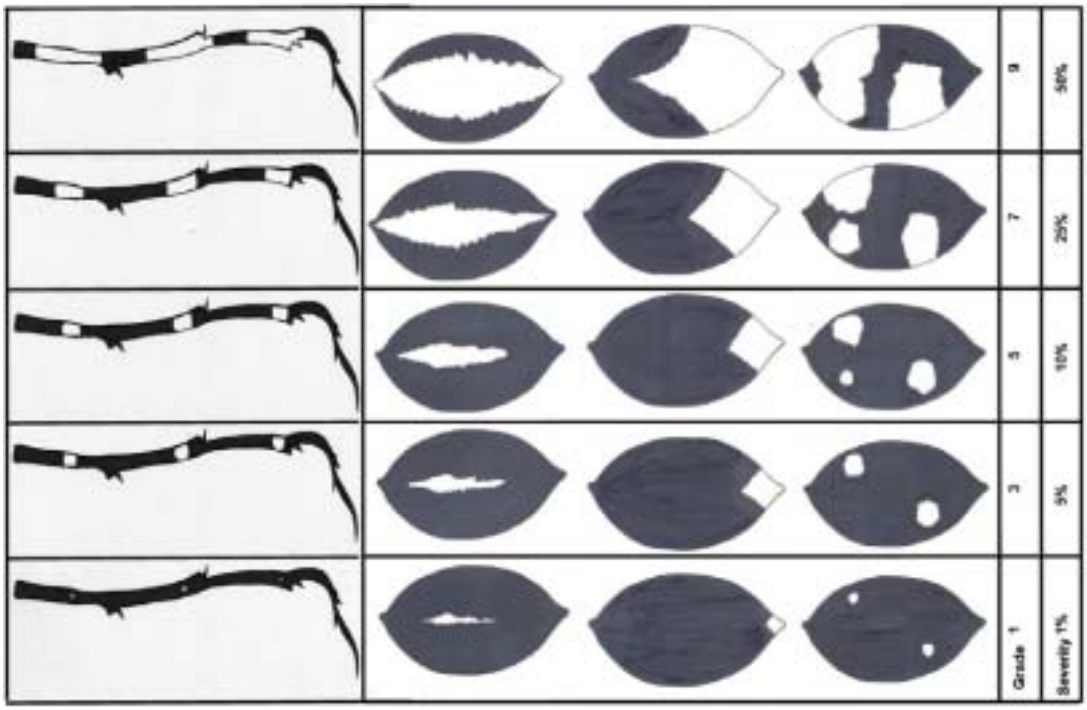

Figure 2.5. Severity scale (at right) and diagrams for evaluating anthracnose on leaves and branches of soursop. White areas indicate infected areas.

Evaluating the disease on plants of cv. Elita. We conducted the respective evaluations with the isolates inoculated on soursop cv. Elita. With the values obtained, we calculated the rates of development (r) for each treatment (Table 2.7) and conducted curves of disease development. We observed differences in disease progress occasioned by each isolate and by the rate at which each progressed over time.

Table 2.7 Rates of development of anthracnose in soursop according to *Colletotrichum* isolate.

Isolate	Origin	r (units per day)
GM01-L02	Huila	0.12
GM03	Huila	0.12
GM04-L01	Huila	0.01
GM04-L02	Huila	0.06
GM05	Huila	0.04
GM59a	Norte de Santander	0.04
GM59b	Norte de Santander	0.06
GM63	Cauca	0.04
GM68	Valle del Cauca	0.15
GM89-L01	Sucre	0.02
GM89-L02	Sucre	0.13
GM90-L01	Sucre	0.10
GM90-L02	Sucre	0.09

GM91-L01a	Sucre	0.05
GM91-L01b	Sucre	0.15
GM91-L02	Sucre	0.21
GM92a	Sucre	0.25
GM92b	Sucre	0.08
GM93	Sucre	0.08
GM94	Córdoba	0.06

Isolates GM91-L02 and GM92a presented the highest rates of development (0.21 and 0.25 units per day, respectively). That is, these two isolates showed more progress than the others evaluated, which fluctuated between 0.01 and 0.15 units per day (Figures 2.6, 2.7 and 2.8). These latter isolates were therefore less virulent.

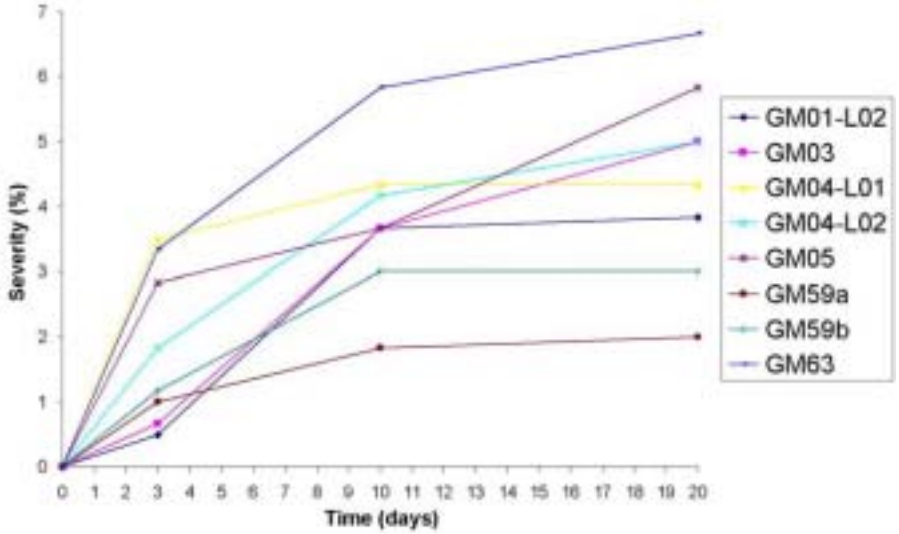

Figure 2.6. Progress curves of soursop anthracnose in cv. Elita inoculated with eight different isolates of *Colletotrichum* spp.

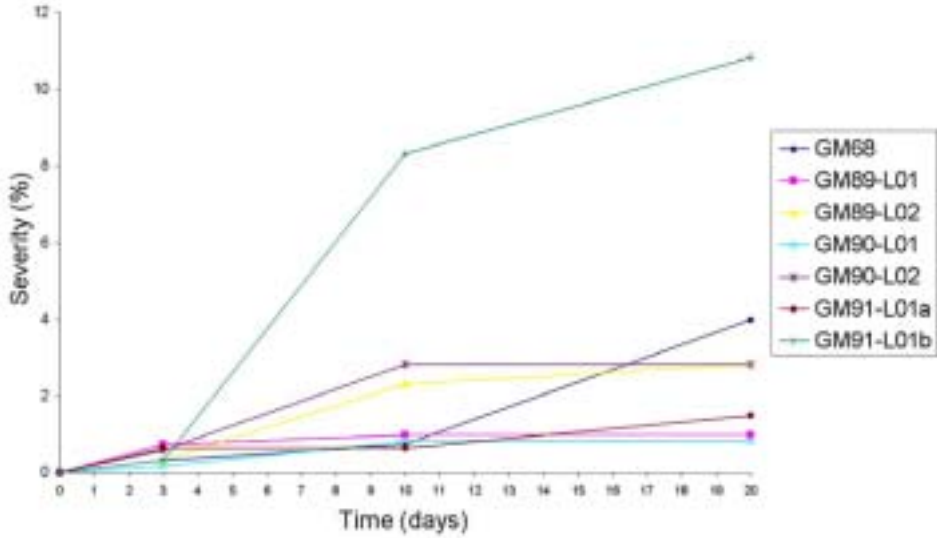

Figure 2.7. Progress curves of soursop anthracnose in cv. Elita inoculated with seven different isolates of Colletotrichum spp.

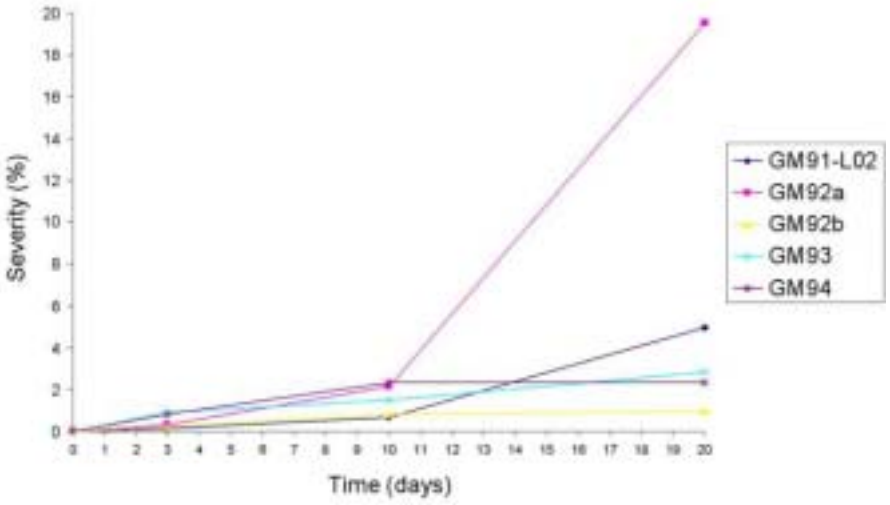

Figure 2.8. Progress curves of soursop anthracnose in cv. Elita inoculated with five different isolates of *Colletotrichum* spp.

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3. Molecular and Pathogenic Characterization of Isolates of *Colletotrichum* spp. Associated with Anthracnose of Andean Blackberry on Accessions from Valle del Cauca

Elizabeth Álvarez, Adriana Arenas, and Juan Fernando Mejía

Rationale

Anthracnose is an economically important disease that affects stems of 50% to 70% of Andean blackberry crops grown in Colombia (Tamayo, 2003). Incidence may even be as high as 100% in some crops (UNISARC and SENA 2006). Moreover, control of the disease is inefficient, despite the use of chemical products and cultural practices (Saldarriaga 2005). Tools are needed to generate technological alternatives that will contribute to the integrated management of the disease ((Saldarriaga 2005) This study assesses molecular characterization as a means of identifying pathogen species, and relating their variability and population composition to aspects of pathogenicity.

Objectives

To evaluate the variability between and among *Colletotrichum* spp., causal agents of anthracnose in Andean blackberry (*Rubus glaucus Benth*.) crops in Valle del Cauca, Colombia, through pathogenicity tests and evaluating the ITS region, using RAM molecular markers.

Materials and Methods

Sampling sites: Field sampling was conducted in 15 village districts and 29 farms of 10 municipalities of the Department of Valle del Cauca: Buga, Tuluá, Ginebra, Palmira, Cerrito, Bolívar, Guacarí, Trujillo, Jamundí, and Dagua (Figure 2..1). For most of the municipalities at least two 2 farms were visited.

Sampling. We collected 143 samples of tissues of Andean blackberry (Table 3.1), principally from young semi-woody stems, but also from fruit and petioles. Although most tissues showed symptoms of anthracnose, healthy ones were also sampled. We used pruning secateurs that were previously disinfected with hypochlorite at 2.5%. For each plant, 3 to 5 stakes (15 cm long) were placed inside a paper bag that was duly labeled. In the field, we also collected crop data on, or example, management, incidence, other phytosanitary problems, and geo-referencing (GPS coordinates). The samples were conserved in a cold room at 4°C until processed.

Monosporic culturing and storage. The samples were left to sporulate and morphologically identified as *Colletotrichum* spp. Then, for each sample, an aqueous suspension of spores was prepared with 500 mL of sterilized distilled water and placed in microcentrifugation tubes. Four drops were taken and added to a petri dish containing agar medium and water (18 g/L). About 15 h later, germinated spores were transferred, with the help of a dissection needle, to a petri dish containing PDA medium acidified with lactic acid at 25% to stimulate the development of individual colonies. The monosporic cultures were then stored on squares of filter paper previously colonized and dried.

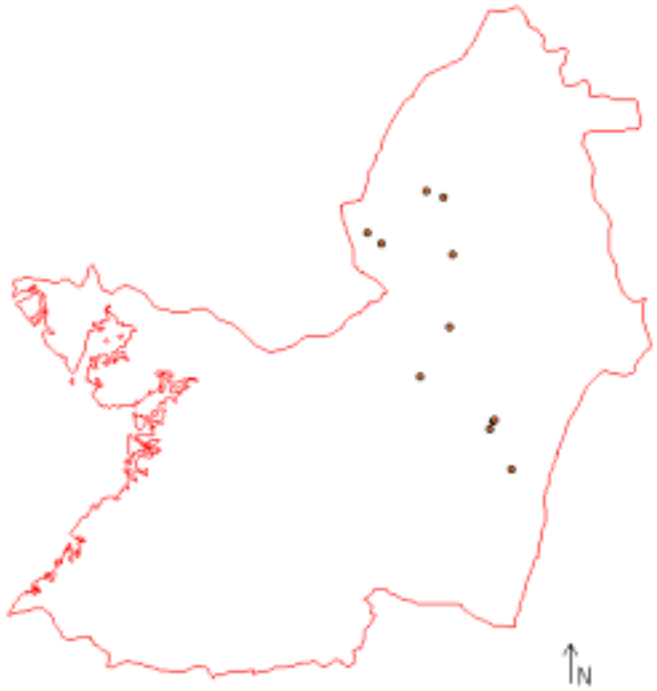

Figure 2.1 Location of sampling sites in Valle del Cauca, Colombia. Samples of Andean blackberry (*Rubus glaucus*), infected by *Colletotrichum* spp., were obtained according to coordinated geographic planes, using the program DivA-GIS (a geographic information system). This is a preliminary analysis of the location of points, as the exact locations of some sampled populations are yet to be refined.

Table 3.1 Samples of Andean blackberry collected mostly from stems, Department of Valle de Cauca, Colombia.

Municipality	Village district	Farms (no.)	Collected samples (no.)	Name and number of clones collected
Ginebra	Portugal, Costa Rica	3	18	Hartona Blanca (1), Hartona (2), Ranchona (2), Castilla (11), Zarzona Amarilla (1), Silvestre (1)
Palmira	Arenillo	2	18	Hartona (9), Castilla (9)
Dagua	Jordán	1	17	Regional (17)
Buga	Miraflores Unión	4	24	Castilla (3), Hartona (20), Regional (1)
Guacari	La Magdalena	2	21	Hartona (2), Ranchona (2), Castilla (17)
Tulua	La Mansión, Piedritas	4	10	Castilla (2), Hartona Negra (6), Hartona Mona (2)
Municipality	**Village district**	**Farms (no.)**	**Collected samples (no.)**	**Name and number of clones collected**
Trujillo	La Siria, Chuscales	6	10	Hartona Negra (6), San Antonio (1), Castilla (3)
Cerrito	Regaderos	2	7	Castilla (7)
Jamundí	Nueva Aventura	2	11	Castilla (11)

DNA extraction. DNA was extracted from monosporic isolates, following Mahuku's protocol (2004), with modifications by Álvarez (2005). The method involved inactivating proteins, using SDS/proteinase K, and precipitating polysaccharides in the presence of a high concentration of salts (Mahuku 2004). The quality of DNA was determined in agarose gel at 0.8% and quantified through fluorometry (Hoefer DyNA Quant™ 200 Fluorometer).

Amplifying the ITS region.To identify species from the *Colletotrichum* genus, we used the ITS4 universal primer in combination with primers specific to C. acutatum (CaInt2), C. gloeosporioides (CgInt), and *Colletotrichum* spp. (Col1) (Afanador et al. 2003).

For each PCR reaction, we used 10X Taq buffer at a concentration of 1X M/µL (100 mM Tris-HCl, pH 8; 2.5 mM MgCl2; and 500 mM KCl), 0.2 mM of each of the dNTPs, 0.5 µM of each primer, 1.5 mM MgCl2, 2 ng/µL of DNA, HPLC water (0.22 µm), and 0.1 U/µL of Taq polymerase (Bioloine). The amplification protocol for the DNA was carried out in a PTC–100 thermal cycler (MJ Research, Inc., Watertown, MA, USA). Initial denaturation was at 95oC for 5 min; one denaturation per cycle at 95oC for 30 s; annealing at 55ºC for 30 s for Col1; and extension at 72oC for 90 s, with a total of 40 cycles of amplification from step 2. The final extension was at 72oC for 4 min, finishing at 4oC for 30 min.

Because bands were not specific at the temperature suggested in Álvarez's PCR protocol (2005), temperatures for hybridization were tested at 55ºC, 60ºC, 62ºC, 64ºC, 65ºC, and 68ºC for primers CaInt and CgInt. Visualization of the bands was carried out on an agarose gel at 1.2%, with an electrophoretic current of 90 volts.

The 5.8S-ITS region of the rDNA was amplified, using the universal primers ITS1 and ITS4 (Álvarez 2005). The cocktail for the PCR was prepared with concentrations and quantities equal to those for the specific primers. Amplification was programmed with an initial denaturation at 94oC for 2 min, one denaturation per cycle at 94oC for 30 s, hybridization at 55oC for 30 s, and extension at 72oC for 120 s, making a total of 40 cycles of amplification. The final extension was at 72oC for 240 s, finishing at 4oC for 30 min.

SSCP and electrophoresis in polyacrylamide gel. To find single-stranded conformational polymorphisms (SSCP) that would permit rapid identification of *Colletotrichum* species, we mixed 2 µL of individual PCR product with 8 µL denaturing buffer (formamide at 95%, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol blue). The mixture was then centrifuged at a low pulse, heated in the thermal cycler at 96ºC for 10 min, and finally conserved on ice for 15 min. The mixture was placed in a Mini-PROTEAN 3-Cell chamber (Bio-Rad Laboratories, Hercules, CA, USA) with 1X TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0), and on a non-denaturing mini-gel with proportions of acrylamide to bis-acrylamide of 29:1 at 8%. Of the mixture, 10 µL were taken and the samples were run for 6 h at 15 mA (150–200 V). We included 3 µL of a marker with a molecular weight of 1 kb (Invitrogen, CA, USA) in the extreme right and left lanes of the gel to facilitate comparisons with SSCP patterns (Kong et al. 2004). Later, the bands were

visualized with ethidium bromide (1 mg/mL of final concentration) for 5 min and any excess washed off in a tray of water for 10 min.

Results and Discussion

From the field samplings and cultures, we obtained 83 monosporic isolates of *Colletotrichum* spp. stored on filter paper. Two species in particular were found to associate with stem tissue; these were C. *acutatum* and C. *gloeosporioides*. When these were further analyzed with the specific primers CaInt and CgInt, *C. acutatum* appeared to be the more frequent (62%) species than *C. gloeosporioides* (38%).

By standardizing the PCR protocol to amplify the ITS region, we determined that the hybridization temperatures adequate for the specificity of bands were 64ºC and 65ºC for primers CaInt and CgInt, respectively. This study attempted to genotype 50 isolates, of which 40 were evaluated with the specific primers CaInt, CgInt, and Col1 (Figure 3.2). We found that 21 isolates amplified for C. *acutatum*, 13 for C. *gloeosporioides*, none for Col1, and 6 did not amplify for any primer. Because these last isolates did not amplify, we analyzed them with primer Col1, varying the hybridization temperature.

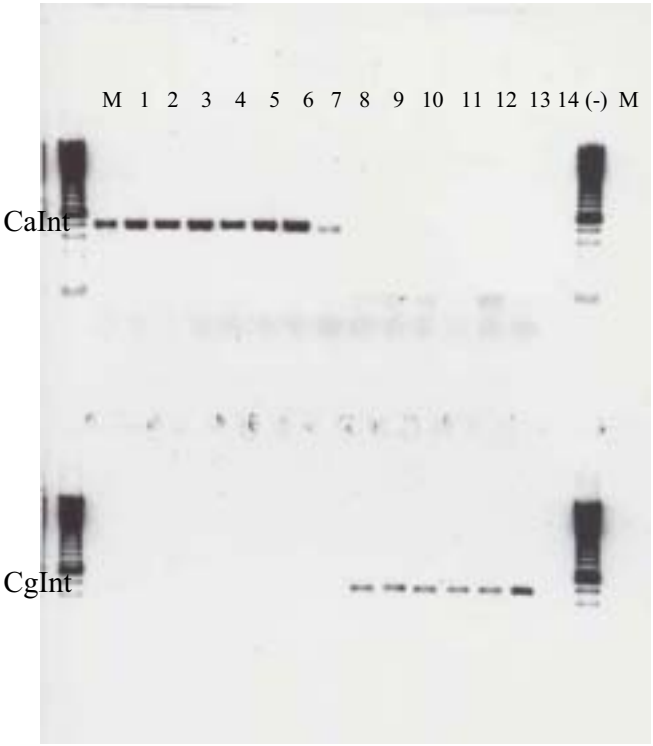

Figure 3.2 Isolates of *Colletotrichum* species were amplified with the specific primers CaInt and CgInt, and visualized on agarose gel at 1.2%. Lane M = marker with a molecular weight of 100 bp; lane 1 = 20t V; lane 2 = 23f V; lane 3 = 23t V; lane 4 = 26 V; lane 5 = 28 V; lane 6 = 66 V; lane 7 = 81 V; lane 8 = 89 V; lane 9 = 35 V; lane 10 = 46C1 V; lane 11 = 46C2 V; lane 12 = 50 V; lane 13 = 51 V; lane 14 = 59V; lane 15 =

Currently, an analysis of single-stranded conformational polymorphisms (SSCP; Figure 3.3) is being carried out and the samples evaluated have presented an apparent correlation with the amplification of the specific primers. Figure 3.3 shows that the pattern for C. *lindemuthianum* is different to the others, presenting a double band and below it, the fastest migration, thus indicating a different conformation. *Colletotrichum acutatum* presents a double band and a one band above that of C. *lindemuthianum* and one band below that of C. *gloeosporioides*. This last fungus presented only one band, and the band with the slowest migration. Accordingly, we determined that the six samples analyzed had the same pattern as C. *gloeosporioides*—a result that is coherent with the analysis on the specific primers.

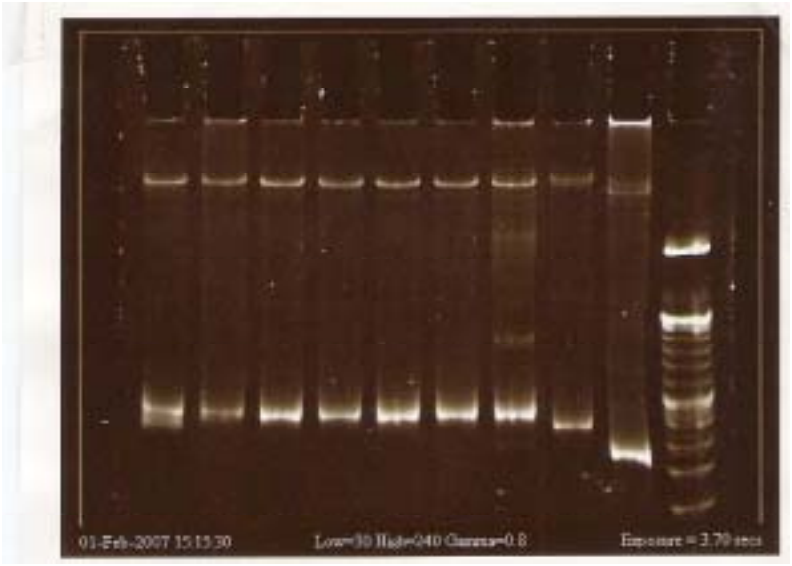

Figure 3.3 Electrophoresis of SSCP from *Colletotrichum* spp. on polyacrylamide gel. Lanes 1–6 = six isolates that amplified for *C. gloeosporioides* with the specific primers; lane 7 = *C. gloeosporioides*; lane 8 = *C. acutatum*; lane 9 = *C. lindemuthianum*; lane M = ladder with a molecular weight of 1 kb.

Conclusions

We consider that anthracnose of stems of Andean blackberry is caused mainly by C. *acutatum*. However, even though it is the major causal agent, it also appears to be part of a complex of *Colletotrichum* species attacking Andean blackberry. This hypothesis needs to be confirmed through further research.

Because the isolates have so far shown consistency with the genotyping, we believe SSCP analysis of the ITS region is probably an effective tool for identifying a *Colletotrichum* species. Again, further research is needed to confirm this finding.

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4. Anthracnose of Andean Blackberry (*Rubus glaucus* Benth.): Variability in Species and Races of the Causal Agent and Identification of Sources of Resistance to the Disease

Lucia Afanador Kafuri, Elizabeth Álvarez, and Alonso González

Central idea

The identification of species and races of *Colletotrichum* spp. is possible by combining morphological, molecular, and pathogenic characterizations of different isolates of the fungus. The use of these tools will make possible the identification of *Colletotrichum* species associated with anthracnose of Andean blackberry in regions where crop is produced in
Colombia.

Rationale

Anthracnose is found in all regions producing Andean blackberry in Colombia, with incidence ranging between 50% and 73%. Given the importance of this disease in the crop principal production regions, we need to initiate studies oriented towards a better understanding of its causal agents, and of the germplasm performance in the presence of pathogen populations. These aspects are indispensable for planning suitable strategies to manage and control the disease in the crop production regions. The correct and timely identification of the agents responsible for anthracnose of Andean blackberry and of the variability of their populations is indispensable for better understanding the epidemiology of this disease. Such understanding constitutes the basis on which to develop genetic improvement programs for this species.

Materials and Methods

Collecting germplasm of Andean blackberry and Colletotrichum *strains.*The collection of *Colletotrichum* strains and germplasm of Andean blackberry was carried out in 29 municipalities of the Departments of Antioquia, Caldas, Cundinamarca, Huila, Quindío, Risaralda, Santander, and Valle del Cauca (Table 4.1). In each municipality, four farms were visited and, from each farm, four samples were taken from stems, branches, and/or fruits with typical symptoms of anthracnose.

Table 4. 1 Germplasm of *Rubus* spp. collected from study areas in Colombia.

Source	Ecotype	Entries (no.)	Greenhouse	In vitro
Antioquia (CORPOICA)	Germplasm bank	38	2	10
Caldas	Castilla	2	2	0
Cundinamarca	Castilla (sexual seed)	4	3	0
Huila	Castilla	2	1	0

Source	Ecotype	Entries (no.)	Greenhouse	In vitro
Quindío	Sin Tuna, Castilla	3	1	2
Santander	Castilla, wild blackberry, wild raspberry	8	5	0
Valle del Cauca	Ecuatoriana, Castilla, Bejucuda, Ranchona, Sin Tuna	20	14	2
Nariño	Castilla	2	2	
	Total	79	30	14

Multiplying germplasm: The collected germplasm was propagated through germinated microstakes as described by Molina (1998). This method consisted of first treating with carbendazim (5 g/L) or benomyl (0.5 g/L) for 5 min, and then treating with Hormonagro® in powder. The treated stakes were planted at an angle in trays containing sterilized sand, and germinated in 25 to 30 days. We also evaluated in vitro propagation from cauline buds, using 4E culture medium (Roca et al. 1984).

Isolating, identifying, and storing the fungus: Monoconidial isolates of the fungus were developed on PDA medium. The fungal colonies were identified, in a preliminary way, through microscopic observation (400X) of the reproductive structures such as acervuli, spore masses, presence or absence of the sexual stage, and presence or absence of setae; and the study of growth characteristics of the colonies on the culture medium. The cultures were stored at 4ºC on squares of filter paper colonized by mycelia and spores.

Evaluating inoculation methods: We first evaluated a method of inoculating leaves, fruits, and stems removed from Andean blackberry. To inoculate the stems, we followed the methodology described by Stewart et al. (2003), which consisted of cutting portions of stems 20 cm long and 1 cm wide. The two extremes were sealed with paraffin and then the surfaces disinfected. An incision was then made in the central part of the stem, removing the external layer of tissue. A block of agar with mycelia from the fungus was placed on the incision and then sealed to prevent dehydration.

For inoculum we used a monoconidial isolate of the fungus that had 12 days' growth on PDA culture medium. As control, we used stems that were each inoculated with a block of water-agar with no fungus.

To inoculate leaves, we applied, on each side of the main leaf vein, 20-µL drops from an aqueous suspension of fungal spores, adjusted to a concentration of 1 × 106 spores/mL. Fruits were inoculated by adding one drop of the same suspension to the center of the fruit. Incubation was carried out under the same conditions as for the stems.

The inoculated tissues (stems, leaves, and fruits) were incubated at 22ºC, under 12 h of light and 12 h of darkness, in transparent plastic boxes with lids, and a plastic grid and film of sterilized distilled water on the bottom. Evaluation of each tissue reaction to the fungus began 2 days after inoculation and continued over 15 days.

Results and Discussion

Collecting blackberry germplasm and Colletotrichum *strains* : The fungus and Andean blackberry germplasm were collected in 10 departments, obtaining a total of 315 samples of tissues infected by anthracnose and 79 accessions of Rubus spp. Of these, 30 (38%) were established in the greenhouse, and 14 (18%) under in vitro conditions (Tables 4.1 and 4.2).

Table 4.2 Departments and municipalities in which sampling for anthracnose in Andean blackberry was conducted, together with a collection of *Rubus* germplasm.

Department	Municipalities	Village districts	Farms	Ecotypes
Antioquia	4	8	25	25
Caldas	1	1	1	1
Cauca	1	1	1	1
Cundinamarca	3	9	21	3
Huila	2	4	4	3
Nariño	2	2	3	–
Quindío	2	2	4	3
Risaralda	2	4	6	2
Santander	4	9	21	3
Valle del Cauca	10	12	20	11

The propagation by stake system was not very effective as a high rate of plants died when transplanted to plastic bags. An in vitro propagation system was identified, together with a culture medium for growing the explants and developing the plantlets.

We isolated 232 strains of *Colletotrichum* spp., as well as other types of fungi such as *Botrytis cinerea, Alternaria* sp., Phomopsis sp., *Mycosphaerella* sp., *Rosellinia* sp., *Kuehneola loeseneriana* (rust), and two types of viruses (a potyvirus and CMV (Table 4.3).

Table 4.3 Monosporic isolates of *Colletotrichum* spp. collected in the Departments of Antioquia, Caldas, Cauca, Cundinamarca, Huila,Nariño, Quindío, Risaralda, and Santander, Colombia.

Source	Date collected	Ecotypes	Symptoms	Total no. of isolates	Sexual phase
ANTIOQUIA Santa Elena, Guarne, La Ceja, Rionegro	2003 2004 2005	Pantanillo, San Antonio, Bogotana, San Rafael, Francesa, Guarne, Pantanillo, Germplasm bank at CORPOICA–La Selva	Black fruit, apical necrosis, mummified fruit	66	No
CALDAS Municipio Neira, Vereda La Mesa	2004	Not identified	Black fruit	1	No
CAUCA Corinto	2006	Castilla	Not identified	1	No
CUNDINAMARCA San Bernardo, Arbeláez, Gachetá	2006	Castilla, hybrid	Black fruit, pale stems, apical necrosis	46	Yes
HUILA La Plata, San José Isnos.	2006	Regional, Castilla, Santana	Black fruit	22	No
NARIÑO San Pedro, Cartago, La Unión	2006	Not identified	Black fruit, mummified fruit	4	No
QUINDIO Salento, Buenavista	2006	Castilla, San Antonio	Black fruit, pale stems, apical necrosis	7	No
RISARALDA Santa Rosa, Desquebradas	2006	Sin Tuna, Castilla	Black fruit, apical necrosis	11	Yes
SANTANDER Piedecuesta, Santa Bárbara, Charta, Floridablanca	2006	Castilla, Churca	Black fruit, pale stems, apical necrosis	74	Yes

Each isolate was given a preliminary morphological characterization, based on colony characteristics, morphological variants, and the presence or absence of the fungus's sexual phase (Figure 4.1).

Figure 4.1 Monosporic cultures of *Colletotrichum* spp. in which morphological variability occurs within strains (left), and the sexual phase is present (center and right).

Evaluating inoculation methods

In stems, the first symptoms began on Day 4 after inoculation as dark coffee-brown lesions around the site of inoculation. By Day 12, these lesions had expanded to completely cover the stem. Most present abundant sporulation of the fungus. In fruits, the first symptoms appeared on Day 5 after inoculation as depressed lesions that were dark coffee-brown in color. By day 10, the fruit was completely necrotic, and covered with mycelia of the fungus with abundant sporulation. In leaves, the first symptoms appeared on Day 9 after inoculation as necrosis of the central vein. It then expanded over most of the foliar blade (Figure 4.2).

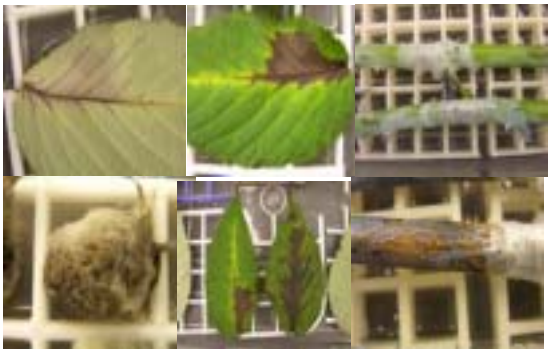

Figure 4.2 Symptoms of anthracnose in extracted stems, leaves, and fruits of Andean blackberry. At left are leaves and fruits inoculated with aqueous suspensions of spores from *Colletotrichum* strain 7(1) from Valle del Cauca; at right, inoculated stems and controls.

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5. Characterization and identification of phylotypes and sequevars of isolates of *Ralstonia solanacearum* obtained from plantain, banana, and *Heliconia* sp. in Colombia

Elizabeth Álvarez and Juan Fernando Mejía

Rationale

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 *Ralstonia solanacearum* race 2 from Colombia, causal agent of bacterial wilt, a major disease affecting crops of plantain, banana, and *Heliconia* sp.

Traditionally, *Ralstonia solanacearum* has been classified into five races according to differences in the range of hosts and into six biovars according to biochemical properties. Cook and Sequeira (1994) using restriction fragment length polymorphism (RFLP) analysis, showed that R. *solanacearum* could be classified into two divisions: 1, including biovars 3, 4, and 5, with isolates principally from Asia; and division 2, including biovars 1, 2, and N2, with isolates principally from the Americas. Other authors such as Taghavi (Taghavi et al., 1996), using sequence analysis of the 16S rDNA region, also confirmed the existence of these two divisions. The sequencing of the ITS region (16S-23S rRNA gene intergenic spacer region), the polygalacturonase gene, and the endoglucanase gene also corroborated the existence of these two divisions, but indicated the existence of another group of isolates originating from Indonesia (Fegan et al., 1998).

Poussier (Poussier *et al*., 2000) conducted a phylogenetic analysis of the *hrp* gene region, using PCR-RFLP and complementing it with amplified fragment length polymorphism (AFLP) and sequencing of the 16S rRNA gene. They observed a new cluster of isolates from Africa—biovar 1. Phylogenetic analysis of the endoglucanase and hrpB genes confirmed the presence of this group in strains originating from Africa. Under this classification system, members of the R. *solanacearum* species complex can be subdivided into four phylotypes, corresponding to the four genetic groups identified via sequence analysis (phylotypes I, II, III, and IV).
The phylotype to which a strain belongs can be rapidly identified through multiplex PCR, based on sequence information from the ITS region. This PCR employs four forward primers: one specific one for each phylotype and a single reverse primer that is specific for the species. It also includes a primer pair described by Opina (Opina et al., 1997). All were R. *solanacearum.* Blood disease bacterium (BDB; Pseudomonas syzygii) strains generate the 280-bp fragment that is specific to the R. *solanacearum* species complex (Allen et al., 2005).

Isolates of R. *solanacearum* infecting *Musa* spp. (also known as race 2 strains) are a major menace for crops such as plantain, banana, and heliconias all over the world, including Colombia. French and Sequeira (1970) defined five1 groups or ecotypes

[1]Group 1 = type A (Amazon Basin); group 2 = SFR (small, fluidal, and round); group 3 = B (banana); group 4 = D (distortion); and group 5 = H (Heliconia).

with strains of R. *solanacearum* race 2 that cause bacterial wilt of banana, plantain, and *Heliconia* sp. in Central and South America. The groups differ in virulence, where some are pathogenic to both plantain and banana (A, SFR, B, and D types) and others are pathogenic only to plantain (H). The groups also differ in transmission and aggressiveness.

Cook and Sequeira (1994) then discovered, through RFLP analysis, that all strains of R. *solanacearum* race 2 are found in three multi-locus genotypes (MLGs), designated as MLGs 24, 25, and 28. Moreover, from this description, Allen (Allen et al., 2005) conducted a classification based on the phylogenetic analysis of sequences of the ITS region (16S-23S) and the endoglucanase gene (egl). Under this scheme, strains of R. *solanacearum* race 2 were classified into phylotype II, sequevars 3, 4, and 6. On the basis of these results and specific classification, genomic DNA fragments were used to develop a multiplex PCR-based molecular test for R. *solanacearum* race 2 (Allen et al., 2005).

The main objective of our study was to determine the variability of *R. solanacearum* from *Musa* crops in different regions of Colombia. Our goal was to develop strategies to improve the acquisition of durable resistance to R. solanacearum.

Materials and Methods

Strains were chosen, based on studies of: 1. pathogenicity on the plantain hybrid 'Africa 1', 2. genetic diversity through RAM primers, and 3. the amplification of the pathogen's 16S rRNA gene by Álvarez (Álvarez et al., 2005). Of the 58 strains from the collection at CIAT, originated from infected crops of plantain (37), banana (5), and heliconias (3). The degrees of pathogenicity in the area under the disease progress curve (AUDPC) were between 10 and 70. The strains were collected from the Departments of Magdalena, Valle del Cauca, Quindío, Antioquia, Caquetá, and Meta (Colombia). The remaining 13 strains were controls and came from plantain (1), *Heliconia* sp. (1), tobacco (6), egg plant (1), tomato (1), potato (1), arrowroot (1), and capsicum (1) from Kenya, Japan, Asia, USA, and Colombia. Five nonpathogenic strains of *R. solanacearum* were isolated from soil for comparative purposes (Table 5.1) (Álvarez et al., 2005).

Table 5.1. Origin and pathogenicity of 58 strains of *Ralstonia solanacearum* race 2, causal agent of bacterial wilt, isolated from banana, plantain, and *Heliconia* sp.

							Phylotype			
No.	**Strain**	**Region**	**Host**	**Tissue**	**AUDPCa**	**Sequevar**	**I**	**II**	**III**	**IV**
1	1 S.A	Quindío	Plantain	Rachis	18.00	4		+		
2	3	Quindío	Plantain	Petioles	69.38	4		+		
3	5 (Sunisa 8)	Antioquia (Urabá)	Banana	Rhizome	38.75	4		+		
4	6	Antioquia (Urabá)	Banana	Fruit	31.83	4		+		
5	15	Quindío	Soil		37.63	4		+		
6	16b1	Quindío	Soil	Mucuna	0.00	–		+		
7	17	Valle (Jamundí)	Soil		69.50	4		+		
8	18	Valle	Plantain	Sucker	42.50	4		+		
9	32	Caquetá	Plantain	Pseudostem	33.88	4		+		

No.	Strain	Region	Host	Tissue	AUDPCa	Sequevar	Phylotype			
							I	II	III	IV
10	34	Caquetá	Plantain	Raceme rachis	27.63	4		+		
11	38	Quindío	Soil	Coffee pulp	59.50	4		+		
12	40	Quindío (Quimbaya)	Soil	Center of focus	15.75	4		+		
13	41	Quindío (Quimbaya)	Soil	Center of focus	56.25	4		+		
14	42	Meta	Plantain	Pseudostem	28.00	4		+		
15	43	Meta	Plantain	Pseudostem	20.75	4		+		
16	48	Quindío (Armenia)	Plantain	Fruit	37.13	4		+		
17	54a	Meta (Puente de Oro)	Plantain	Pseudostem	36.25	4		+		
18	58-1R	Meta (Puente de Oro)	Plantain	Petioles	56.63	4		+		
19	59	Meta (Puente de Oro)	Plantain	Pseudostem	0.00	4		+		
20	65	Meta (Granada)	Plantain	Pseudostem	47.63	4		+		
21	67	Meta (Puente de Oro)	Plantain	Pseudostem	41.63	4		+		
22	69-1	Meta (Granada)	Plantain	Pseudostem	27.00	4		+		
23	70	Meta (Granada)	Plantain	Pseudostem	5.75	4		+		
24	71aR	Antioquia (Urabá)	Plantain	Rhizome	21.25	4		+		
25	72b	Antioquia (Urabá)	Plantain	Pseudostem	10.75	4		+		
26	73a	Antioquia (Urabá)	Plantain	Pseudostem	10.75	4		+		
27	76	Quindío (Montenegro)	Plantain	Pseudostem	61.88	4		+		
28	78	Quindío (Montenegro)	Plantain	Rachis	73.38	4		+		
29	79	Quindío (Montenegro)	Plantain	Rhizome	66.88	4		+		
30	80	Quindío (Montenegro)	Plantain	Pseudostem	67.88	4		+		
31	81	Quindío (Montenegro)	Plantain	Fruit	10.88	4		+		
32	83	Quindío (Calarca)	Plantain	Fruit	55.00	4		+		
33	84	Quindío (Calarca)	Plantain	Pseudostem	61.00	4		+		
34	85	Quindío (Calarca)	Plantain	Sucker	68.38	4		+		
35	86	Quindío (Calarca)	Plantain	Rachis	59.75	4		+		
36	88	Quindío (La Tebaida)	Plantain	Rhizome	61.75	4		+		
37	89	Quindío (La Tebaida)	Plantain	Pseudostem	60.38	4		+		
38	97	Quindío (Quimbaya)	Plantain	Rhizome	28.63	4		+		
39	107	Quindío (Armenia)	Plantain	Fruit	68.25	4		+		
40	110	Magdalena	Banana	Pseudostem	63.25	6		+		
41	111	Magdalena	Banana	Rhizome	34.38	6		+		

No.	Strain	Region	Host	Tissue	AUDPCa	Sequevar	Phylotype			
							I	II	III	IV
42	112	Magdalena	Banana	Sucker	29.50	6		+		
43	113	Valle (Rozo)	Heliconia wameiana	Pseudostem	40.50	4		+		
44	114	Valle (Rozo)	Heliconia wameiana	Rhizome	40.38	4		+		
45	115	Valle (Rozo)	Heliconia catubea	Rhizome	33.63	4		+		
46	G175	Kenya	Egg plant	CIAT collection	–	–	+			
47	G216	Japan	Tobacco	CIAT collection	–	–	+			
48	CIAT 1008	Colombia	Plantain	CIAT collection	65.13	4		+		
49	CIAT 1001	Colombia	Tobacco	CIAT collection	–	–		+		
50	CIAT 1007	Florida (Quency)	Tobacco	CIAT collection	–	–		+		
51	CIAT 1013	North Carolina	Tobacco	CIAT collection	–	4		+		
52	G 177	Australia	Potato	CIAT collection	–	–		+		
53	CIAT 1017	Colombia	Arrowroot	CIAT collection	–	4		+		
54	CIAT 1077	North Carolina	Tomato	CIAT collection	–	–		+		
55	G 218	Philippines	Capsicum	CIAT collection	–	–	+			
56	G 217	Costa Rica	Heliconia	CIAT collection	–	4			+	
57	CIAT 1035	Colombia 37	Tobacco variety	CIAT collection	–	6		+		
58	CIAT 1054	Colombia	Tobacco	CIAT collection	–	–		+		

a. AUDPC = area under disease progress curve; data from Álvarez *et al*. (Álvarez *et a*l., 2005, Fitopat. Colom. 28(2):71-75).

The selected strains were amplified by multiplex PCR, their classification being evaluated according to phylotype with primers Nmult 21:1F, Nmult 21:2F, Nmult 22:InF, Nmult 23:AF, Nmult 22:RR, 759, and 760 (Figure 4.1B); and to sequevars in Musas with primers Mus 20-F, Mus 20-R, Mus 35-F, Mus 35-R, Mus 06-F, Mus 06-R, Si28-F, and Si28-R. Amplification conditions were as according to the methodology described by Fegan and Prior (2005) and cited in Allen (Allen *et al*., 2005).

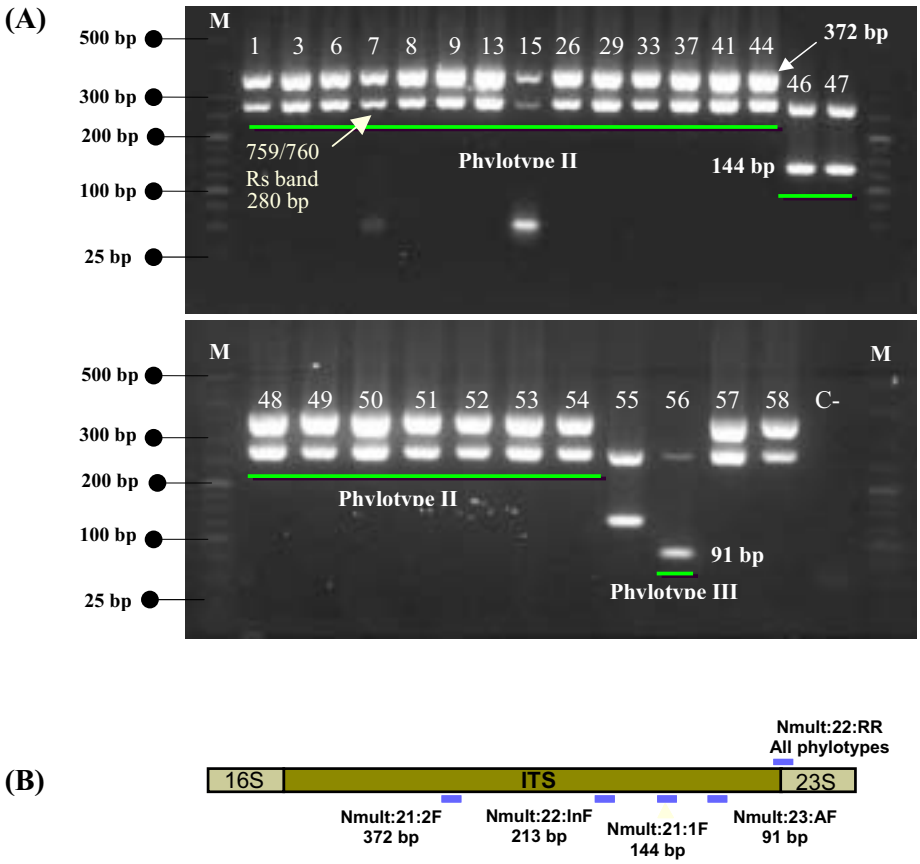


Figure 5.1A and **5.1B** Evaluation of phylotypes by multiplex PCR for 58 isolates obtained from banana, *Heliconia*, plantain, and controls from the collection held at CIAT. (B) Location of primers for multiplex PCR (phylotypes) in the ITS region. Lane M = marker with molecular weight according to HyperLadder V (100 lanes).

Results and Discussion

All the isolates obtained from Colombia, regardless of geographic region, host, tissue, or pathogenicity, belonged to phylotype II. In contrast, the control strains were characterized as belonging to phylotypes I, II, and III. Specifically, those from egg plant (Kenya), tobacco (Japan), and capsicum (Asia) belonged to phylotype I; those from plantain, arrowroot, tobacco (Colombia), tobacco (Quency, FL, and North Carolina), potato (Australia), and tomato (North Carolina), to phylotype II; and the sole *Heliconia* isolate (Costa Rica) to phylotype III (Figure 5.1A; Table 5.1).

According to Fegan and Prior (2005), the species complex of *R. solanacearum* can be subdivided into four phylotypes corresponding to four genetic groups identified according to sequence analysis. A phylotype is defined as a monophyletic cluster of strains that is revealed by phylogenetic analysis of sequence data, in this case, the ITS region, hrpB gene, and endoglucanase gene. The four phylotypes are:

- *Phylotype I* is equivalent to division I, as defined by Cook (Cook *et al.*, 1994). The strains in this phylotype all belong to biovars 3, 4, and 5 and were isolated

primarily from Asia.

- Phylotype II is equivalent to division 2, and the strains included belong to biovars 1, 2, and 2T and were isolated primarily from America. It also includes the *R. solanacearum* race 3 potato pathogen, which is distributed worldwide, and the race 2 banana pathogens.

- *Phylotype III* contains strains that belong to biovars 1 and 2T and were primarily isolated from Africa and nearby islands.

- *Phylotype IV* contains strains that had been isolated primarily from Indonesia and belong to biovars 1, 2, and 2T. These strains are also found in Australia and Japan. This phylotype includes the two close relatives of *R. solanacearum*: *P. syzygii* and the BDB.

In the sequevar analysis, we detected the multi-locus genotypes (MLGs) 25 and 28. Three isolates of banana from the Department of Magdalena, Colombia, were characterized as sequevar 6 (MLG 28), amplifying only one product of 220 bp with primers Si28-F/Si28R. The other isolates belonged to sequevar 4 (MLG 25), amplifying two products: one of 351 bp and the other of 167 bp for all the isolates with primers Mus20-F/Mus20-R and Mus06-F/Mus06-R, respectively. Isolates 3, 4, 24, 25, and 26 were absent from the fragment that amplified to 167 bp. Isolate 6 obtained from Mucuna-soil did not amplify for any sequevar, as likewise the nonpathogenic strains isolated from the soil. The 13 controls were characterized as follows:

- Sequevar 4 (with the presence of two bands) for isolates 48 from plantain (Colombia), 51 from tobacco (North Carolina) (data not shown[2]), 53 from arrowroot (Colombia), and 56 from *Heliconia* (Costa Rica) (data not shown[2]);

- Sequevar 6 for isolate 57 from tobacco (Colombia); and

- The remaining 8 isolates did not amplify for any sequevar (Figure 4.2).

[2] These amplifications were tenuous and could not be observed in Figure 2. They were re-amplified to confirm the results.

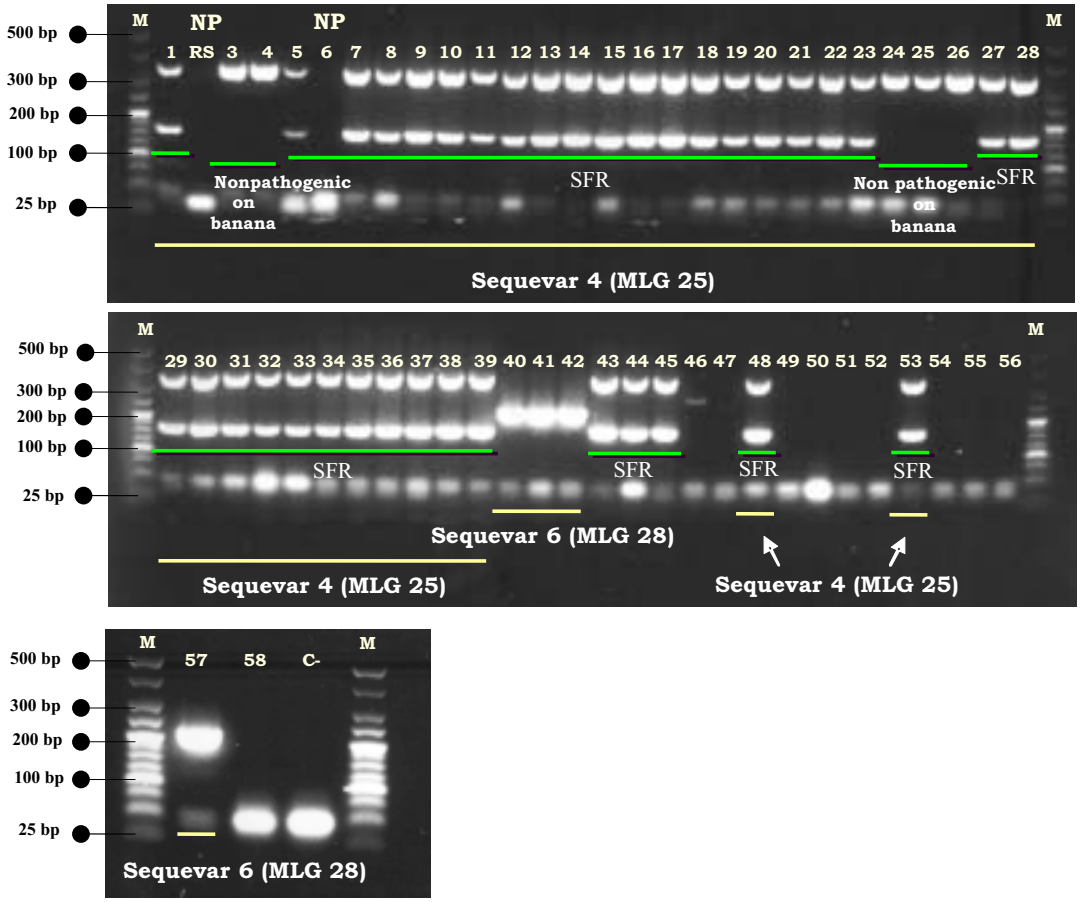
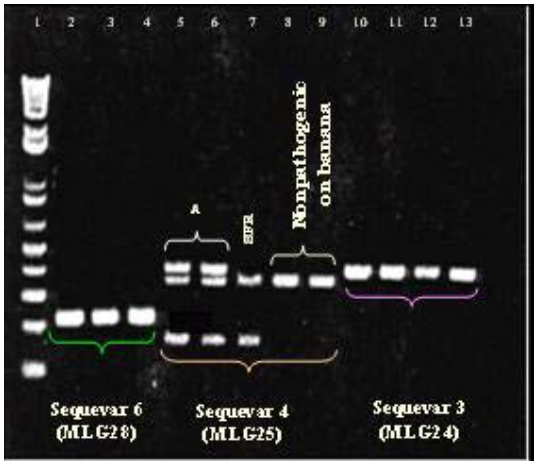


Figure 5.2 Evaluation of sequevars by multiplex PCR for 58 isolates obtained from banana, *Heliconia*, plantain, and controls from the collection held at CIAT. Lane M = marker with molecular weight according to HyperLadder V (100 lanes).

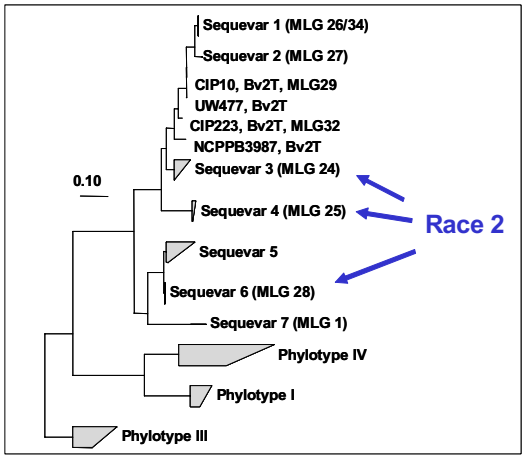
Based on Allen et al. classification (Allen *et al*., 2005), strains identified as "SFR" (small, fluidal, round colony form, insect transmitted) are found in MLGs 25 and 28. Strains identified as "D" (causing leaf distortion and slow wilting of banana) belong to MLGs 24 and 25. In contrast, strains designated as "B" (large elliptical colony form, rapid wilt of banana, not commonly insect transmitted) belong to MLG 24 only, as do the strains classified as "H" (slightly pathogenic on plantain but not pathogenic on banana). The authors' phylogenetic work showed that isolates belonging to MLGs 24 and 25 are closely related to each other, but are slightly more distant to MLG 28. Given that strains classified as SFR are present in MLGs 25 and 28, it is conceivable that these strains may have differing properties, including their capacity to survive in soil and host range (Figure 5.3A).

Comparing the band patterns for the different isolates from plantain, banana, and *Heliconia* spp. with those of the race 2 strains described above, the isolates that characterized as SFR-type strains, sequevar 4 (MLG 25), were isolates 1, 5, 7–23, 27–39, 43–45, 48, 51, and 53; those that are SFR-type, nonpathogenic on banana, sequevar 4 (MLG 25) were 3, 4, and 24–26; and SFR-type, sequevar 6 (MLG 28), were 40–42 and 57 (Figure 4.2; Table 4.1).

The primers designed for sequevar 4 (MLG 25) were each developed separately, using strains isolated from Peru, Colombia, Costa Rica, Martinique, and Florida (USA). Their host plants were banana, plantain, *Heliconia* sp., pothos (*Epipremnum aureum*), and anthurium. Strains isolated from bacterial wilt-infected anthurium from Martinique in the French West Indies and from pothos in Florida were found to cluster with *R. solanacearum* strains belonging to sequevar 4. The strains from anthurium were nonpathogenic on banana. However, the strains from pothos caused wilt of banana. The pathogenic potential of these strains for banana needs to be confirmed (Allen et al., 2005).



(A) Fegan and Prior (2005)



(B) Fegan and Prior (2005)

Figure 5.3A and 5.3B (A) Musa-specific region from the subtracted sequences specific to phylotype II, sequevars 3, 4, and 6, and used in a multiplex PCR. (B) Phylogenetic tree of phylotype II based on partial endoglucanase gene sequences. The corresponding sequevars can be seen where strains of *R. solanacearum* race 2 are classified. (Taken from Fegan and Prior [2005]).

Strains belonging to sequevars 3 (MLG 24) and 4 (MLG 25) are closely related and form a branch, together with sequevars 1 and 2, which contain potato disease-causing strains belonging to race 3/biovar 2 (Figure 4.3B). All strains previously identified as belonging to MLG 28 fell in sequevar 6. Strains in this sequevar were isolated from host plants of banana, plantain, and *Heliconia* sp. in Honduras, Venezuela, Hawaii, and Australia (where the disease has been eradicated).

Sequevar 6 is phylogenetically distinct from strains of sequevar 3 (MLG 24) and 4 (MLG 25) in which other bacterial wilt-causing strains are found. Hence, the R.

solanacearum race 2 strains are polyphylectic, which indicates a separate evolutionary origin for the two groups of strains. All strains of sequevar 6 were also found to belong to biotype 6 (Figure 4.3B) (Allen et al., 2005, Bacterial Wilt Disease, 1-510).

This finding is of vital importance, considering that strains isolated from banana in Magdalena, Colombia (Table 5.1), belong to sequevar 6 and are either moderately or highly pathogenic on plantain. The possibility of strains being introduced from Honduras or Venezuela exists and, hence, the danger of re-invasion of zones where the disease has been eradicated is constant.

During the course of this study, using primers specific to sequevars 3, 4, and 6 (MLGs 24, 25, and 28), we identified strains of *R. solanacearum* in different regions of Colombia. We observed a tomato crop naturally infected with sequevar 4 strains (MLG 25). This finding expanded the known host range of this organism and this study is the first report of tomato as a natural host of *R. solanacearum* race 2, biovar 1, in Colombia.

Conclusions

We used multiplex PCR to classify strains of *R. solanacearum* and confirmed that their current classification is composed of four genetic groups or phylotypes and, within these, subgroups or sequevars that corresponded to clusters or isolates with similar pathogenicity or isolates of common geographic origin. We could conclude that, to date (with about 40% of the collection at CIAT evaluated), 100% of strains isolated from Musas in Colombia belong to phylotype II, with 91% to sequevar 4 and 6.6% to sequevar 6.

The genetic and pathogenic characterization of *R. solanacearum* strains, although very important, must be complemented with information of the strains' biological, ecological, and epidemiological properties. By incorporating these different components, we can define a taxonomic scheme for predicting the pathogenicity of strains and thus contribute towards controlling this disease.

For a disease such as bacterial wilt of plantain, the genetic analysis of the pathogen's taxonomic structure as reported in this study will support the research so far carried out on the causal agent's biology and ecology. By being able to predict the genetic and pathogenic properties of the R. *solanacearum* race 2 strains in Colombia, we can begin to bring this disease under control.

6. Microbiological and Physicochemical Evaluation of Lixiviates from Decomposing Plantain Rachises and Pseudostems and their Effectiveness in Managing Bacterial Wilt

Elizabeth Álvarez, Luz Adriana Mesa, Victor H. Triviño, Germán Llano, and John Loke

Rationale

The plantain crop is affected by the vascular disease moko or bacterial wilt, caused by *Ralstonia solanacearum*. Currently, this disease is causing significant losses in Colombia, but it has not been successfully controlled because of a lack of effective management technologies and the nonexistence of resistant plantain varieties. Hence, research is needed to discover efficient alternatives that can be applied at low cost within an integrated management program, while generating a favorable impact on the environment. Preventive management of bacterial wilt of plantain is an excellent approach towards controlling the pathogen. This approach involves the use of natural substances extracted from organic residues such as lixiviate of compost of plantain rachises, pseudostems, and fruit.

Identifying microorganisms present in lixiviate from decomposing plantain rachises and pseudostems

Highlight

Lixiviates from decomposing plantain rachises and pseudostems contain bacteria that are useful for releasing nutrients and for acting as possible antagonists of pathogens.

Materials and Methods

Samples, from which the bacterial strains under study were obtained, came from the plantain variety Dominico Hartón, grown on seven farms located in the Department of Quindío, Colombia.

Isolating the bacteria.To isolate the microorganisms present in samples of lixiviates, we used a nutrient agar culture medium. The organisms were incubated for 24 h at 28ºC, after which different colonies were selected according to their morphology. The potassium hydroxide test was conducted on the various isolates to differentiate between Gram-negative and Gram-positive microorganisms.

Morphology of the bacteria assessed. To identify the type of microorganisms found in the lixiviate samples, we planted them in different culture media that were specific to different types of microorganisms (Table 6.1).

Table 6.1 Culture media used to characterize microorganisms in lixiviate from compost of plantain rachises and pseudostems and in a mixture of lixiviate from compost of plantain rachis, phosphoric rock, and french marigold.

Culture medium	Specific to:
Yeast extract, dextrose, and calcium carbonate (YDC)	*Xanthomonas, Erwinia*
Medium B of King et al. (KB)	*Pseudomonas fluorescens*
Casein agar and glucose (CAG)	*Bacillus*
Nystatin, polymyxin, penicillin, cycloheximide (NPPC)	*Streptomyces*
Salmonella–Shigella agar (SS)	*Salmonella, Shigella*
MacConkey	Enterobacteriaceae

Pure strains grown on nutrient agar with 24 h of incubation were planted on different culture media to observe their growth and later conduct biochemical tests to identify each microorganism.

Results and discussion

We obtained 22 bacterial isolates, of which 8 were Gram-negative and 14 Gram-positive, according to the KOH test. Table 5.2 presents the results of the microbiological analyses conducted on samples of lixiviate from the decomposition of various plantain parts. The largest number of bacteria were isolated from lixiviate of rachis.

Table 6.2 Presumed identification of bacteria present in four sources of lixiviates of plantain compost .

Rachis	Pseudostem	Mixturea [a]	Fruit
Bacillus	*Bacillus*	*Bacillus*	*Listeria*
Klebsiella oxytoca	*Streptococcus*		*Staphylococcus*
Actinobacillus	*Acinetobacter*		
Eikenella			
Pseudomonas			
Proteus vulgaris			

[a] Lixiviate of rachis, phosphoric rock, and french marigold.

We did not identify the bacteria *Escherichi*a *co*li or *Salmonella* spp., corroborating the results obtained by Larco (2004)), who reported the absence of Salmonella in lixiviate from banana and plantain compost. Through biochemical tests, we identified Pseudomonas bacteria, which are ecologically important microorganisms found in the soil and probably responsible for the degradation of many soluble compounds that derive from the monomeric rupture of plant materials in oxygenated habitats. These organisms are typically aerobic and contribute to the decomposition and discharge of nutrients, attacking the organic substrate, including humic acids and synthetic pesticides (Bess VH. 1998). Proteus bacteria were found because they live in soils, residual waters, and manure.

Conclusions

The lixiviates, being products generated by organic decomposition, presented different types of microorganisms according to their origin. Notable among them were beneficial bacteria, responsible for the initial and final stages of decomposition. To

ascertain its innocuousness, a more specific microbiological characterization of lixiviate of plantain compost must be conducted..

Through this study, we identified the bacteria present in samples of plantain lixiviates and *demonstrated that the variety of microorganisms changed according to the source of lixiviate.*

7. Physicochemical Characterization of Lixiviates from Decomposing Rachises, Pseudostems, and Fruit of Plantain

Elizabeth Álvarez, Luz Adriana Mesa, Víctor Hugo Triviño, Germán Llano, and John Loke

Highlights

Lixiviates from decomposing plantain rachises, pseudostems, and fruit are ideal ecological resources for use in managing the disease *moko* or bacterial wilt. These lixiviates contain various nutrients and minerals, in particular, high levels of potassium and manganese, which might help reduce the disease. Nonetheless, the lixiviate is a mixture of an large number of organic compounds.

Methodology

To determine the characteristics of lixiviates and thereby recommend suitable use in both their management and application, we conducted various analyses. We used molecular spectrophotometry to identify nitrogen, phosphorus, nitric nitrogen, ammoniac nitrogen, sulfur, boron, and carbon. To identify potassium, we used atomic absorption spectrophotometry (AAS), and the atomic absorption technique for calcium, magnesium, copper, zinc, manganese, and iron (García MN. 2005).

Color of the 10 sources of lixiviates from plantain compost collected from seven farms was visually assessed.

Results and discussion

Lixiviate from fruit was black, whereas lixiviates from other plant parts were either light or dark brown. Lixiviates from rachises also differed among themselves in color (Table 6.1).

Table 7.1 Colors of 10 lixiviates from decomposing plantain rachises, pseudostems, and fruit collected from seven farms, Colombia.

Origin	Source of lixiviate	Color
La Yalta Farm, Armenia	Rachis	Dark coffee brown
La Guaira Farm, Montenegro	Rachis	Light coffee brown
Las Américas Farm, Quimbaya	Rachis	Dark coffee brown
Las Américas Farm, Quimbaya	Fruit	Black
Guadualito Farm, Montenegro	Rachis	Dark coffee brown
Santa Elena Farm, Armenia	Rachis	Dark coffee brown
La Diana Farm, Armenia	Rachis	Dark coffee brown
La Diana Farm, Armenia	Pseudostem	Light coffee brown
La Diana Farm, Armenia	Mixture [a]	Light coffee brown
La Manigua Farm, Armenia	Rachis	Light coffee brown

[a] Mixture of lixiviate from decomposing rachis and phosphoric rock.

These results on color characteristics agree with those reported by Paúl and Clark (1996) when they described a commercially acceptable compost.

Table 4 shows the results of chemical analyses of lixiviate from decomposing rachises and pseudostems of plantain and a mixture (phosphoric rock, french marigold, and lixiviate of rachis) obtained from different farms in the Department of Quindío. Differences clearly existed among macroelements, according to the source and origin of lixiviates from compost analyzed in this study.

The largest percentage of phosphorus in lixiviate was found at Guadualito Farm with 404.93 mg/L. The smallest quantity (11.91 mg/L) of phosphorus was found at La Manigua Farm. Except for copper, the overall contents of elements in lixiviate of pseudostem was 10 times less than for lixiviate of rachis. The values for most elements were higher for fruit than for rachises and pseudostems. The average pH value ranged from 8 to 9, except for a pH 3.9 for lixiviate prepared from fruit at Las Américas Farm.

The chemical analyses of lixiviate indicated high concentrations of potassium in most samples. This element tends to be associated with inducing resistance to some diseases (Grajales and Villegas ,2002). For iron, values were higher than those reported previously. The lowest value (4.35 mg/L) for ammoniac nitrogen was found at La Manigua Farm and the highest (212.91 mg/L) was for lixiviate from fruit.

The correlation between disease progress of bacterial wilt (expressed as the area under the wilt progress curve over 7 weeks or AUWPC) in trials conducted in the greenhouse and the chemical composition of five lixiviates showed values of -0.77 for potassium content and -0.75 for manganese content. Although these correlations might indicated that these two elements might help in reducing the progress of the disease, it is not possible to deny the role of other organic compounds present in the lixiviate.

The chemical analyses of different sources of lixiviate showed that most presented high values for phosphorus, possibly because of the high quantity of residues (rachises, leaves, pseudostems, and corms) generated in each harvest of banana and plantain. These results agree with those reported by Muñoz (2003).

Lixiviates are an ideal resource for use in managing bacterial wilt, as the quantity of nutrients and minerals released is high, especially of potassium and manganese. Another important aspect is the quantity of water present in these residues, as it facilitates rapid decomposition and transformation into organic matter (Mojica,1994). Moreover, lixiviates can be applied in the field or greenhouse to manage diseases or as fertilizer.

Table 7.2. Chemical composition of samples of lixiviate from decomposing rachises, fruit, and pseudostems of plantain from seven farms in the Department of Quindío and one farm in Department of Meta (Ariari), Colombia.

S [a]	Source (farm and tissue)	pH (null)	C (mg/L)	N (mg/L)	P (mg/L)	K (mg/L)	Ca (mg/L)	Mg (mg/L)	S (mg/L)	B (mg/L)	Na (mg/L)	Fe (mg/L)	Mn (mg/L)	Cu (mg/L)	Zn (mg/L)	N (NH4) (mg/L)	N (NO3) (mg/L)
1	Las Américas, rachis	8.67	2780.00	420.09	157.95	18,185.42	82.92	38.66	111.52	0.83	12.55	2.67	1.25	0.16	0.29	6.98	0.00
2	Las Américas, fruit	3.91	11,680.00	1781.61	375.68	11,343.41	5137.17	824.74	197.37	6.61	22.34	2949.81	23.32	0.11	10.33	212.91	4.52
3	La Diana, rachis	8.38	1640.00	278.55	317.16	16,332.48	74.75	51.68	149.93	0.86	7.51	2.94	1.39	0.00	0.31	19.20	0.01
4	Santa Elena, rachis	9.28	1565.00	249.30	212.00	20,937.91	41.37	34.77	183.82	0.67	28.54	1.75	1.01	0.16	0.53	6.27	0.00
5	Ariari (Meta), rachis	9.36	2690.00	466.58	311.05	26,680.96	51.77	51.62	398.46	2.38	6.77	3.82	4.12	0.00	23.36	7.44	0.00
6	Guadualito, rachis	9.34	2996.07	893.38	404.93	28,838.90	69.11	31.30	206.50	0.56	6.05	3.05	1.91	0.04	0.60	144.64	0.00
7	La Guaira, rachis	8.58	2160.06	205.10	187.88	15,588.58	64.58	43.37	55.72	0.12	6.13	0.50	0.51	0.00	0.06	47.21	0.00
8	La Manigua, rachis	8.54	408.95	45.76	11.91	324.83	5.54	2.04	0.00	0.00	4.91	1.62	0.41	0.00	0.27	4.35	2.88
9	Mixture [b]	8.89	2.67	283.63	367.40	18,391.39	379.76	59.82	86.75	2.19	5.40	40.14	0.00	0.00	0.82	32.65	0.00
10	La Diana, pseudostem	8.43	1.29	70.03	66.89	4,458.62	44.91	22.65	21.46	0.98	1.46	0.88	0.00	2.23	0.01	24.97	0.00
11	La Yalta, rachis	8.97	2.84	217.82	161.59	12,749.92	46.89	24.45	87.60	1.16	5.62	0.69	1.11	0.11	0.28	73.24	0.00

[a] S = sample

[b] Mixture of phosphoric rock, french marigold, and lixiviate of rachis.

Conclusions

Lixiviates produced from decomposing plantain rachises and fruit contain high concentrations of potassium.The chemical analysis of sources of lixiviate showed that most lixiviates were high in phosphorus. The exact nature of what compound or element is responsible for disease control using lixiviate is not known. Such information is only possible to gather by conducting studies where individual compounds are isolated, which is beyond the goal of these studies.

8. Detecting *Ralstonia solanacearum* in Lixiviates from Decomposing Rachises and Pseudostems of Plantain

Elizabeth Álvarez, Luz Adriana Mesa, Víctor Triviño, John Loke, and Germán Llano

Rationale

Moko, *maduraviche*, or *ereke* is a bacterial wilt of plantain and banana caused by *Ralstonia solanacearum*. It is the most important bacterial disease of these crops in Colombia, affecting 125,000 families who depend directly on them for their livelihoods. The use of lixiviate from decomposing plantain rachis has been effective as a practice for managing the disease. The presence of *R. solanacearum* in samples of lixiviate from decomposing plantain residues is a phytosanitary risk if it is applied directly to the crop without first verifying the absence of the pathogen. In this study, we determined whether *R. solanacearum* is present in lixiviates from infected plants.

Methodology

The pseudostems of 6-week-old plantain seedlings, variety Dominico Hartón, were inoculated with 0.5 mL of lixiviate obtained from plantain harvest residues from the Department of Quindío, specifically from the farms of La Guaira, La Manigua, Santa Elena, Guadualito (rachises only), Las Américas (fruit and rachises), La Diana (rachises; pseudostems; and mixture of lixiviate of rachis, phosphoric rock, and french marigold or *Tagetes patula*), and La Yalta (rachises). We used a randomized complete block design, with five replications and an experimental unit of two plants. We evaluated the effect of different sources of lixiviate, using the *R. solanacearum* strain CIAT No. 78 as a positive check and sterilized deionized water as the negative check. The inoculated plants were kept for 3 days under constant wetting and later microsprayed every 24 h for 26 days.

Evaluations of severity were conducted daily, taking into account the development of symptoms of wilt between Days 5 and 30 and recording the appearance of symptoms such as flaccidity in leaves and wilt. To measure disease development, a scale of 0 to 6 was generated, where:

0 = absence of symptoms
1 = leaves presenting flaccidity
2 = leaves showing a slight but noticeable wilt, not only in their shape but also in the loss of their intense green color
3 = leaves showing a highly noticeable flaccidity and, in some cases, yellowing
4 = leaves showing yellowing with necrosis in some sites and highly advanced flaccidity, losing their shape
5 = advanced necrosis and leaves have totally lost their turgidity
6 = plants are entirely dead

In addition to the experiment described above, the SMSA medium was added to two petri dishes and, in each dish, a 0.1-mL sample of each lixiviate was suspended. Incubation was carried out at 28∀C. The dishes were examined every day for the presence of the pathogen. Possible colonies were purified and incubated for 2 weeks. The samples were evaluated three times over the experimental period.

Results and discussion

Healthy plants that had been inoculated with 10 different sources of lixiviate showed no symptoms of the disease. The positive check inoculated with *R. solanacearum* showed typical symptoms of the disease. Some plantain seedlings injected with lixiviate of fruit presented leaves showing some small burns. When these tissues showing burns were cultured on SMSA medium, no R. *solanacearum* isolates were obtained. This symptom was probably caused by a phototoxic substance in the lixiviate or low pH.

Lixiviates planted in SMSA medium did not show colony growths typical of *R. solanacearum*, thus confirming the results obtained in the greenhouse trial.

The absence of *R. solanacearum* in the lixiviate samples collected from the seven farms therefore ascertains the innocuousness of lixiviates for plantain crops for either managing bacterial wilt and sigatoka or using as biofertilizer (García and Apezteguia, 2001; Larco, 2004).

Conclusions

The different sources of lixiviate evaluated were considered not to contain *R. solanacearum* because the plantain plants inoculated with the lixiviates did not develop symptoms of the disease after 30 days of evaluations under greenhouse conditions. The absence of *R. solanacearum* from the lixiviate samples evaluated demonstrated that applications of lixiviate of plantain compost do not cause either residual or pathogenic effects on plantain plants, thus indicating its suitability for use in this type of crop.

9. Identifying Live and Dead Cells of *Ralstonia solanacearum* Exposed to Lixiviates from Plantain Residues, Phosphoric Rock, and French Marigold

Elizabeth Álvarez, Luz Adriana Mesa, Víctor Triviño, and John Loke

Rationale

Laboratory trials on culture medium and in the greenhouse showed that lixiviate of plantain rachis and pseudostem inhibits the *R. solanacearum* bacterium. One limitation of these tests is that nobody knows the effect lixiviates have on the viability of the pathogen's inhibited cells. The bacterial cells could live without multiplying and so continue to be a phytosanitary risk.

Methodology

Bacterial viability is determined by using the fluorescence kit LIVE/DEAD® L-13152 (Molecular Probes, Leiden, Netherlands), which contains two nucleic acid markers. The fluorochrome Syto9 is a small molecule that can penetrate bacteria that possess an intact plasmatic membrane, giving off a green fluorescence when observed under a epifluorescent microscope. The fluorochrome propidium iodide (PI) penetrates damaged membranes, which are therefore not viable, giving off a red fluorescence (Defives et al. 1999). The bacterial strain used was CIAT No. 78, isolated from plantain rachis from Montenegro (Quindío, Colombia). Treatments were:

1. Cumbre® (gentamicin sulfate at 10.7% and oxytetracycline hydrochloride at 32.3%)
2. Lixiviate from decomposing rachis of plantain variety Dominico Hartón from La Guaira Farm
3. Lixiviate of rachis from Las Américas Farm
4. Lixiviate from decomposing pseudostem from La Diana Farm with phosphoric rock (29% P_2O_5)
5. French marigold (*Tagetes patula*)
6. Mixture of lixiviate of rachis compost from La Diana Farm with phosphoric rock and french marigold

As checks, we used:

1. Components A (Syto9) and B (propidium iodide) from the kit LIVE/DEAD® without *R. solanacearum*
2. Components A and B with the bacterium
3. Sterilized deionized water
4. Sterilized deionized water with the bacterium
5. Ethanol at 70% (Molecular Probes, 2004)
6. Ethanol at 70% with the bacterium

The treatments were filtered twice with a vacuum pump using Whatman No. 1 filter paper and once through a 0.2-µm Millipore filter (Whatman). They were then stored in sterilized, BD Falcon™, centrifuge tubes of 50 mL. They were kept sealed with

Parafilm® and conserved at -80oC. From each solution, 2 mL were placed per microcentrifuge Eppendorf tube, sterilized twice at 121oC and under 20-lb pressure for 20 to 30 min.

Preparing inoculum

A bacterial suspensión was prepared in sterilized deionized water. Under aseptic conditions, the concentration of the suspensión was determined in a Turner spectrophotometer, model 390, with a wavelength of 600 nm, using Fisher cells (ref. Spectronic 20). The concentration was adjusted by diluting with sterilized deionized water until a 0.3 absorbance was obtained, corresponding to about 1 × 106 colony-forming units per milliliter (He et al. 1983).

Observing R. *solanacearum* cells tinted with LIVE/DEAD® L-13152 with the epifluorescent microscope

We took 100 µL of *R. solanacearum* and added them to the Eppendorf tubes that contained the treatments and vortexed. In another 0.5-mL tube, we mixed the Syto9 coloring (component A) with 0.75 mL of propidium iodide dye (component B). The tube with the mixture of colorings was wrapped in aluminum foil to keep out light and conserved at -20ºC. We took 0.25 µL from each Eppendorf tube containing a treatment with the bacteria and placed them on one microscope slide per treatment. Immediately, 0.25 µL of the mixture of colorings were also added. The mixture was gently moved about with the point of a micropipette and a cover slip placed over it. The slides were kept in darkness for 15 min, after which time, the sample was examined under an epifluorescent microscope with a wavelength of 490 nm to determine the percentage of live (green) and dead bacterial cells (red) in the sample.

Results and discussion

The total count of the bacterial population was obtained by counting the percentages of green (viable) and red (dead) cells that were observed in the field of the epifluorescent microscope. Observation over time allowed us to determine the number of days needed for each product analyzed to act on the *R. solanacearum* cells. On Day 2, we observed that the mixture of phosphoric rock, french marigold, and lixiviate of plantain rachis from the La Diana Farm destroyed 80% of the pathogen's cells (Table 8.1).

Table 9.1 Microscopic observations conducted with the kit LIVE/DEAD® baclight L-13152 to determine the effect of different types of lixiviate on Ralstonia *solanacearum* cells.

Treatment	Inocul. with *R. solanac.*	Red cells (dead) of *R. solanacearum* (%) at days after experiment begins						
		1	2	5	7	14	21	24
Checks								
1 Components A (Syto9) + B (PI) from the kit	No	0	0	0	0	0	0	0
2 Components A (Syto9) + B (PI) from the kit	Si	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
3 Sterilized water	No	0	0	0	0	0	0	0
4 Ethanol at 70%	No	0	0	0	0	0	0	0
5 Sterilized water	Yes	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
6 Ethanol at 70%	Yes	100	100	100	100	100	99.9	100

Products									
1	Gentamicin and tetracycline[b]	Yes	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
2	Lixiviate[c] of plantain rachis (La Guaira Farm, Montenegro)	Yes	0[a]	0[a]	25	25	50	90	99.9
3	Pure lixiviate[c] of plantain rachis (Las Américas Farm, Quimbaya)	Yes	0[a]	0[a]	10	0[a]	0[a]	99	99.9
4	Lixiviate[c] of plantain pseudostem (La Diana Farm, Armenia)	Yes	0[a]	0[a]	0[a]	20	20	20	50
5	Phosphoric rock[d]	Yes	0[a]	0[a]	40	20	10	20	40
6	French marigold[e]	Yes	0[a]	0[a]	2	60	90	90	99.9
7	Lixiviate[c] of plantain rachis, with phosphoric rock[d] and french marigold[e] (La Diana Farm, Armenia)	Yes	0[a]	80	90	90	90	100	100

[a] Green.
[b] Cumbre® (gentamicin sulfate at 10.7% and oxytetracycline hydrochloride at 32.3%)
[c] At a pure concentration, with no water added
[d] Concentration at 30 g/50 mL.
e Concentration at 10 g/50 mL

Conclusions

Bacterium, of R. *solanacearum* were killed when exposed to the mixture of lixiviate from decomposing plantain rachis, phosphoric rock, and french marigold as demonstrated through epifluorescent microscopy. Lixiviates without disinfection by phosphoric rock and french marigold killed most of the cells; however, a small percentage remained viable. This is the first microscopy study that demonstrated the effect of different types of lixiviates on the viability of the *R. solanacearum* bacterium.

10. Determining the Control of Bacterial Wilt in Plantain Seedlings by Different Types of Lixiviate

Elizabeth Álvarez, Luz Adriana Mesa, Victor Triviño, John Loke, and Germán Llano

Rationale

Different greenhouse trials were conducted to discover the effect on plantain seedlings of applying lixiviates of rachis and pseudostem to the soil. The aim was to evaluate the potential of lixiviates as an ecological practice for controlling the disease.

Trial 1: Inoculating plantain plants with Ralstonia solanacearum *at different concentrations*

Methodology

We aimed to determine (1) the time between inoculation with R. *solanacearum* and expression of symptoms of bacterial wilt, and (2) the minimum concentration of inoculum needed to produce symptoms. We used the R. *solanacearum* strain No. 78 from the collection held at CIAT, first growing it for 24 h in nutritive agar. We then prepared three suspensions of the strain in sterilized deionized water at different concentrations. Under aseptic conditions, the concentration of each suspension was determined by reading the absorbance in a Turner spectrophotometer, model 390, with a wavelength of 600 nm, using Fisher cells (ref. Spectronic 20). The concentrations were as follows:

Absorbance 0.5 = 1×10^8 colony-forming units (cfu)/mL
Absorbance 0.3 = 1×10^6 cfu/mL
Absorbance 0.1 = 1×10^3 cfu/mL (He et al. 1983)

The inoculation method used was injection with sterilized 1-mL syringes and needle size 27G × 1/2". For each suspension, 0.5 mL was inoculated into the pseudostem of each of eight plantain plants at a height of 2 cm from the soil surface. Another eight plants were used as the negative control and were injected with sterilized deionized water. The 32 inoculated plants were then placed in three randomized complete blocks and left to incubate in a humid chamber for 3 days before being microsprayed for 30 days. The plants were evaluated daily between Days 5 and 30 after inoculation.

Results and discussion

After inoculating plantain seedlings with R. *solanacearum,* the disease's progress was observed under controlled conditions to determine the speed at which the pathogen infected the plants. Of the three concentrations tested, those seedlings receiving 1 × 108 cfu/mL had symptoms by Day 7, and by Day 18 they were dead. The disease was also observed to advance by the vascular system, as described by Gómez (Gómez, 2005), beginning at the site of inoculation and advancing along the stems to the leaves. The trial showed that the more concentrated the inoculum, the more quickly the disease was expressed and the more severe the symptoms (Table 9.1).

Table 10.1 Development of bacterial wilt in plantain seedlings inoculated with different concentrations of *Ralstonia solanacearum* and evaluated for 3 weeks under greenhouse conditions.

Treatment (concentration of R. *solanacearum* at cfu/mL)		Disease progress at day of evaluation[a]		
		7	14	21
1	Water	0	0	0
2	1 × 103	0.8	1.5	4.0
3	1 × 106	2.2	3.0	5.0
4	1 × 108	2.5	3.5	6.0

[a] Scale of 0 to 6, where 0 is absence of disease and 6 is plant death

Conclusions

Through this trial we showed that the three concentrations of inoculum of R. *solanacearum* caused plant death within 3 weeks. By using different concentrations of inoculum of the pathogen, we could better assess the lixiviates' capacity to reduce the damage caused by bacterial wilt.

***Trial 2: Inoculating sterilized soil with* Ralstonia solanacearum**

Methodology

The goal was to identify the minimum bacterial concentration detectable in inoculated sterilized soil. We used dilution methodology to determine concentrations and indicator plants to establish disease progress. We first grew the *R. solanacearum* strain CIAT No. 78 for 24 h in nutritive agar and then prepared suspensions of it in sterilized deionized water. Under aseptic conditions, each suspension's concentration was determined by reading its absorbance from the spectrophotometer. The concentration of each bacterial suspension was adjusted with dilutions in sterilized deionized water starting with absorbance 0.3. We added 1 mL in a test tube, completing to 100 mL with sterilized deionized water. From this solution, 1 mL was taken and completed to 100 mL with sterilized deionized water in a test tube. For the last tube, the same procedure was carried out to obtain the fourth concentration. The concentrations of the bacterial suspension were as follows:

Treatment no. 1 = 1×10^8 colony-forming units per milliliter (cfu/mL)
Treatment no. 2 = 1×10^6 (cfu/mL)
Treatment no. 3 = 1×10^4 (cfu/mL)
Treatment no. 4 = 1×10^2 (cfu/mL)
Negative check = Inoculation with sterilized deionized water

For each concentration, 30 mL of bacterial solution was placed in flowerpots containing soil previously sterilized at 121°C under 20 lb of pressure for 30 min. Later, 9-week-old plantain plants were planted into each pot. After inoculating the soil, the plants were incubated in a humid chamber (90% RH) for 3 days. The plants were then transferred to a greenhouse and microsprayed at intervals of 1 min throughout the day. On Day 7 after inoculation, evaluations were begun, continuing for 1 month.

We inoculated 50 plants, distributed across 5 treatments, with 5 replications and a negative check. The experimental unit was 2 plants in a randomized complete block distribution. Evaluations were carried out every day for 30 days, checking all leaves on each plant to determine wilt. If a leaf presented symptoms of the disease, a tissue sample was taken from the infected plant and cultured onto the semi-selective SMSA medium and again inoculated onto other healthy plantain plants.

Results and discussion

The healthy plants planted into soil inoculated with *R. solanacearum* presented typical symptoms of the disease, starting from Day 10 (Table 9.2).

Table 10.2. Reaction of healthy plants to applications of different concentrations of *Ralstonia solanacearum* to the soil.

Treatment (concentration of R. *solanacearum* at cfu/mL)		Disease progress[a] at day after inoculation			
		7	14	21	28
1	Water	0	0	0	0
2	1 × 102	0	0.2	0.5	1.2
3	1 × 104	0	0.3	0.7	1.6
4	1 × 106	0	0.8	1.2	2.0
5	1 × 108	0	0.8	2.2	4.3

[a] Scale of 0 to 6, where 0 is absence of disease and 6 is plant death

Table 10 shows that, at higher bacterial concentrations, symptoms of the disease were expressed in the plantain plants in less time and with greater severity. The table also shows that when the bacterium is inoculated into the soil, symptoms take longer to manifest in the plants (10 days) than when it is directly inoculated into plants (7 days). The disease appeared even at concentrations of 1 × 102 cfu/mL, which is the equivalent to 3000 colonies per flowerpot.

***Trial 3: Applying lixiviate before and after inoculating seedlings with* Ralstonia solanacearum**

Methodology

To evaluate the effectiveness of lixiviate of plantain in managing bacterial wilt, 30 mL of pure lixiviate was applied to the soil before or after inoculating with the pathogen. Lixiviate was obtained from plantain compost from the following farms: Las Américas, La Guaira, La Manigua, Santa Elena (all rachises), and La Diana (pseudostem and a mixture of lixiviate of rachis, phosphoric rock, and french marigold). All the farms were located in the Department of Quindío, Colombia.

In the greenhouse, 6-week-old plantain plants were planted in polypropylene sacks with a 1-kg capacity and containing a mixture of sterilized sand and soil at a rate of 3:2. One set of 168 plants were inoculated on Day 15 after planting, using sterilized 1-mL syringes. Their pseudostems were inoculated at a height of 2 cm from the soil

surface with a concentration of absorbance 0.1, determined as previously described for preparing inoculum for plantain plants. Each plant received 0.2 mL of the suspension. After inoculation, 30 mL of lixiviate from different sources (rachises, pseudostems, and fruit) was applied at 100% to the soil of each of the 168 plants. Another set of 168 plants received lixiviate before they were inoculated with *R. solanacearum.*

We established 46 treatments, the experimental unit being 3 plants. The experimental design was split-plot in different blocks separated by treatment, with 4 replications. The main plot was the time of applying lixiviate (before or after inoculation) and the subplot was the source of lixiviate. Lixiviate was applied at Days 3, 7, and 15 before inoculation and at Days 3, 5, and 7 after inoculation.

To establish the positive check, 72 plants were inoculated with the *R. solanacearum* strain CIAT No. 78. The negative check comprised another 72 plants that were inoculated with sterilized deionized water and also received the antibiotic Cumbre® (gentamicin sulfate at 10.7% and oxytetracycline hydrochloride at 32.3%) at 8 g/L, injecting 0.5 mL into the stem and 1 mL to the soil for each plant.

The inoculated plants were kept for 3 days under constant humidification and then given 7 lots of microspraying at 1 min per day of 24 h for 30 days. Evaluations were made daily between Days 5 and 30, examining leaf by leaf in each plant for the appearance of symptoms of wilt such as flaccidity and yellowing. The area under the disease progress curve (AUDPC) was calculated for the variable of severity, according to a graded scale of severity of disease and, through the statistical program Statistix 8.0, an analysis of variance was conducted for the AUDPC.

To determine differences between treatments in terms of their effectiveness in controlling bacterial wilt on Days 7, 14, 21, 28, 35, 42, and 49, an analysis of variance was conducted, together with tests on the separation of means (Tukey's; Ŭ= 5%).

Results and discussion

On average, 10 days after inoculation, leaves showed the first symptoms, presenting flaccidity on touch (grade 1). After about 18 days, the leaves showed some wilting and began losing their intense green color (grade 2). After 4 weeks, the leaves were noticeably flaccid and, in some cases, yellow (grade 3). For grade 4, 40 days after inoculation, the leaves were yellow, necrotic, and very flaccid, having lost their shape.

These findings contrast with those of Gómez (Gómez, 2005), who reported that, by Day 5, symptoms of the disease such as flaccidity and/or wilt in the leaves can appear. Possibly, the size and origin of the seedlings influence the disease's progress.

Only those plants inoculated with bacteria and treated with water presented grade 5 on the disease scale, showing the most severe symptoms of advanced necrosis and loss of shape in the leaves. The other treatments did not present this grade of disease until the seventh evaluation (i.e., 49 days after inoculation). The symptoms observed and the re-isolation demonstrated that the symptoms are caused by R. solanacearum. Uninoculated plants and treated only with water did not show wilt during the

experiment. At 3 weeks after inoculation, no significant differences were observed between treatments, using Tukey's test at 5%.

By the fourth week, significant differences were observed between products and the positive check. During the fourth and fifth weeks, the lixiviate from Las Américas Farm in Quimbaya (Quindío) significantly reduced the disease's progress. At the end of the trial, all the products had a similar effect on the progress of the wilt with an average grade of 4.2, as expressed according to the severity scale, where the leaves presented yellowing with necrosis and advanced flaccidity, losing their shape (Table 9.3). The inoculated plants treated only with water suffered grade 6.0 and died (Figure 9.1).

Table 10.3 Analysis (Tukey, 5%) to compare the effect of time between the application of products and day of inoculation with *Ralstonia solanacearum* on disease progress in plantain seedlings established in the greenhouse. Values in the table refer to scores on a disease scale of 0 to 6, where 0 is absence of disease and 6 is plant death.

Time between product application and inoculation of seedlings	**Evaluation on day after inoculation**						
	7	**14**	**21**	**28**	**35**	**42**	**49**
Days before inoculation							
15	0.49 bc	1.02 c	1.59 c	2.20 b	3.01 b	3.27 b	3.45 b
7	1.44 a	2.38 a	3.01 ab	3.35 ab	3.84 ab	4.31 ab	4.60 ab
3	0.97 ab	2.08 ab	3.11 ab	3.62 a	4.05 ab	4.42 ab	4.66 a
Days after inoculation							
3	0.26 c	1.43 abc	2.54 abc	3.04 ab	3.53 ab	4.06 ab	4.40 ab
5	0.29 c	1.41 bc	2.31 bc	3.11 ab	3.44 ab	3.88 ab	4.33 ab
7	0.28 c	2.18 ab	3.49 a	4.11 a	4.39 a	4.81 a	5.17 a

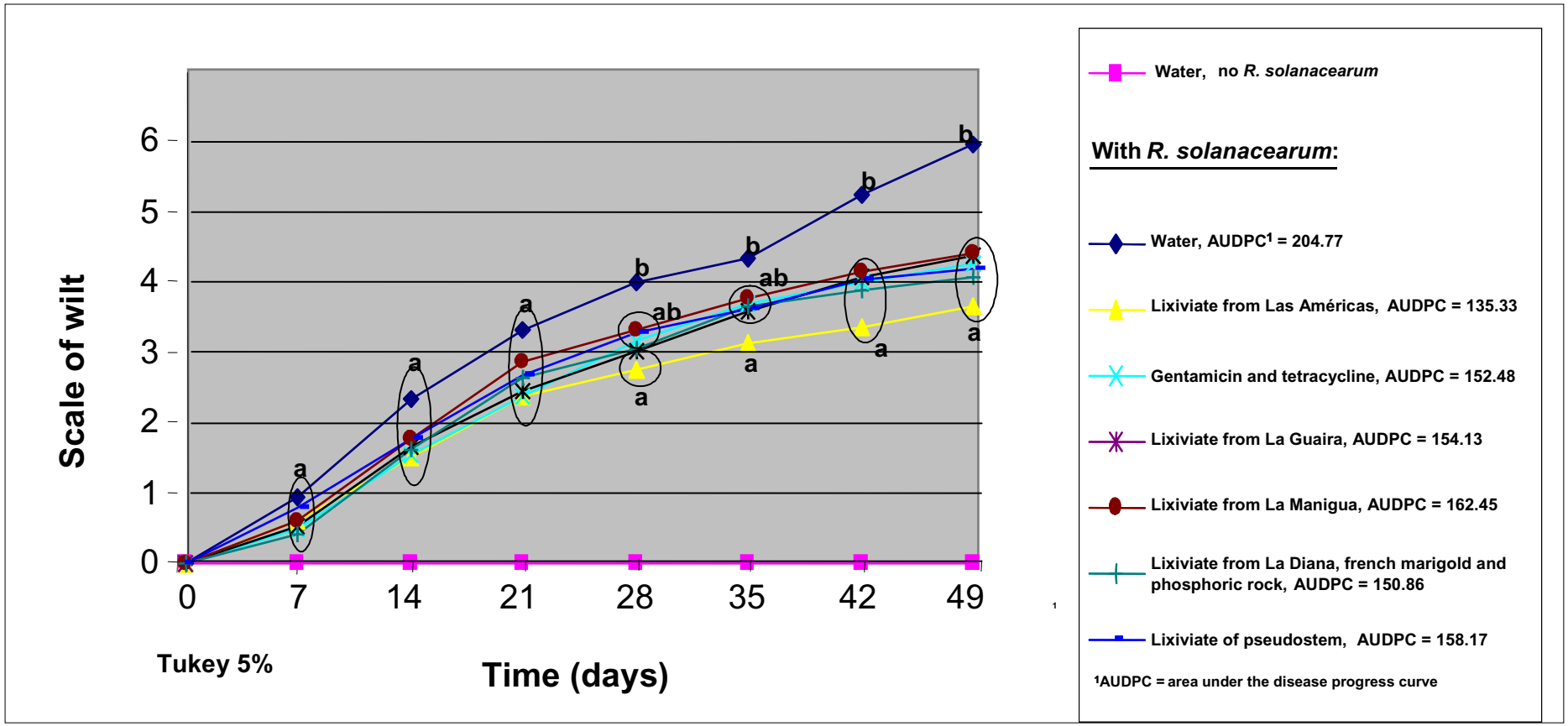


Figure 10.1. Effect of lixiviates from decomposing residues of plantain on the progress of bacterial wilt in plantain seedlings growing in the greenhouse over 7 weeks.

With respect to the four replications, two had a greater concentration of inoculated pathogen than the other two. Highly significant differences (Tukey, 5%) occurred between replications 1 and 2 and replications 3 and 4, with symptoms appearing much more quickly in replications 1 and 2 than for replications 3 and 4.

Conclusions

The mixture of the two antibiotics gentamicin and tetracycline was effective in inhibiting the bacterium *R. solanacearum* from infecting plantain seedlings. This finding is similar to that reported, where Cumbre® was shown to be effective in controlling this pathogen in tomato. Taking into account that the lixiviates used in this study are as equally effective as the antibiotics, we do not recommend the use of Cumbre®. On observing the effect of the lixiviates on disease progress, we recommend that plantain farms apply this ecological alternative every 2 weeks to reduce disease incidence and expression of wilt symptoms and as a preventive measure to protect the crop from infection. Of the three lixiviates obtained from plantain rachises, that collected from Las Américas Farm was the most effective in reducing the wilt's progress over time.

The lixiviate obtained from decomposing pseudostem was equally effective as the antibiotics and other lixiviates obtained from rachises or fruit. In this trial, only one application of each product was used and, even so, the reduction in the rate of increase in wilt was notable. For the first 6 weeks of the experiment, no product inhibited the disease completely. The products showed no major controlling effect over the wilt when applications were made after the pathogen was inoculated. We recommend that trials be established in the field to determine the effectiveness of extracts from pseudostems for managing the disease, considering their high potential to control it and their availability on farms.

The application of organic products or noncontaminating minerals such as lixiviate of plantain to control *R. solanacearum*, does not disturb the ecological equilibrium, and is thus an ecological alternative to the use of conventional chemical products.

11. Effect of Lixiviates on Controlling Bacterial Wilt in Soil under Field Conditions at the Santa Elena Farm, Municipality of Armenia, Quindío

Elizabeth Álvarez, Luz Adriana Mesa, Victor Triviño, John Loke, and Germán Llano

Rationale

Bacterial wilt of plantain is disseminated not only by work tools but also through survival in the soil and dissemination through water. Plantain producers do not have access to practices for disinfecting or inhibiting the multiplication of the pathogen in the soil. This study aims to evaluate the effect of three sources of lixiviates of plantain rachis, phosphoric rock, and french marigold for managing, in the soil, foci of bacterial wilt of plantain.

Methodology

We identified 14 foci on farm located in the Municipality of Armenia, Quindio, Colombia The farm Santa Elena was selected because plants presented typical symptoms of bacterial wilt. The farm is located in the village district of La Pradera, corregimiento El Caimo, Municipality of Armenia in Quindío, Colombia. The foci are managed by surrounding the area with polypropylene fiber and guadua or bamboo to restrict access to the area and prevent workers from disseminating the disease. Then, for each focus, infected plants and surrounding healthy plants are pulled up and chopped on the site. All tools that had been used in the foci are disinfected with sodium hypochlorite at 2.25%.

Selecting treatments for managing wilt in the field: To evaluate the effectiveness of lixiviate of rachis compost in the ecological management of wilt, we selected at random the treatments described in Table 10.1.

Table 10.1 Distribution of soil treatments, number of applications, and plantain plants infected by bacterial wilt on the Santa Elena Farm, Municipality of Armenia, Quindío. The field trial was established in November 2005a and evaluated over 13 months.

Soil treatment	**Unit**					
Lixiviate of plantain rachis from Las Américas Farm applied to plot no.:	1	7	9	12	15	18
Number of plants in that plot infected by bacterial wilt at start of trial, Feb. 2006	5	2	6	3	3	10
Months in 2006 when lixiviate applied	March, April, May	March, April, May	March	March, April, May	March	March
Lixiviate of plantain rachis from La Guaira Farm applied to plot no.:	3	5	6	8	11	14
Number of plants in that plot infected by bacterial wilt at start of trial	3	1	1	3	1	10
Months in 2006 when lixiviate applied	March,	March	March,	March,	March	March

	April, May		April, May	April, May
Lixiviate of plantain rachis from Santa Elena Farm applied to plot no.:	13	17		
Number of plants in that plot infected by bacterial wilt at start of trial	2	2		
Months in 2006 when lixiviate applied	March	March, April, May		

[a] Plots 1, 7, 9, 12, 15, and 18 received applications of phosphoric rock, extract of french marigold, and lixiviate of plantain rachis

The lixiviates were applied only to the site where the diseased plants had been eradicated in each focus. In May, french marigold was chopped up and mixed with the lixiviate 2 days before applying, partly to allow a release of metabolites that inhibit bacteria and partly to facilitate the infiltration of these metabolites, together with the lixiviate, in the area where the roots of the eradicated infected plants are found.

*Treatments established in foci of bacterial wilt***:** Inside the focus and over the material of diseased plants that had been eradicated and chopped as described previously, , we applied the following products:

- Leaves, stems, and flowers of french marigold (fresh weight) at 1 kg/m^2
- Phosphoric rock at 25 kg (Bolivariana de Minerales Ltda., Bogotá, Colombia; active ingredients = total phosphorus 29%)
- Efficient Microbes (EM), which comprised the principal groups of beneficial microorganisms (phototrophic bacteria, lactic acid bacteria, yeasts, and fungi) at 0.5 L for 20 L of lixiviate
- Lixiviate of plantain rachis at 20 L per site

Each focus was numbered and labeled with the treatment applied. It was kept free of weeds by applying glyphosate and manually eradicating any plantain shoots. The entrance into each focus was defined by a stake, where a tray containing sodium hypochlorite at 2.25% was set down to disinfect shoes on entering and leaving the focus. The precaution was also taken to shake the earth off the shoes before wetting them with the disinfectant.

Detecting Ralstonia solanacearum *in soil and tissue samples taken from foci infected by bacterial wilt*: Soil and plant tissue samples were obtained to determine the presence of bacteria in the plots, designated as foci, where wilt-infected plants were eradicated. Samples were taken every 30 days and processed at the Pathology Laboratory, CIAT as described below.

Processing soil samples: Soil samples were collected from wilt-infected foci at a depth of 20 to 35 cm, placed in 1-lb polypropylene bags that were duly marked, and conserved in a styroform ice-box for transport from the farm to CIAT. In the laboratory, 3.3 g of soil was weighed from each sample and mixed with 30 mL of sterilized deionized water by vortexing. Serial dilutions were conducted in TE buffer at pH 7.6, taking 1 mL of the mother solution for a 10-1 dilution and, from this, 100 µL for a 10-2 dilution. The diluted

solutions were then planted in petri dishes containing semi-selective SMSA medium and incubated at 28ºC for 7 days. As a comparative reference for typical colony growth, we used strain CIAT No. 78 of R. *solanacearum* race 2 from the collection held at the Cassava Pathology Laboratory, CIAT.

After incubation, we selected those colonies whose growth in the SMSA medium presented morphological characteristics that were similar to those of the reference strain. The selected colonies were re-chopped and planted in drop form on nutritive agar (NA) to obtain individual colonies. After 24 h of culturing in NA, a suspension was prepared in sterilized deionized water. Under aseptic conditions, the concentration of the suspension was determined by reading the absorbance from a spectrophotometer (Turner, model 390) with a wavelength of 600 nm, using Fisher cells (ref. Spectronic 20). The concentration was adjusted by diluting with sterilized deionized water until an absorbance of 0.3 was obtained, corresponding to about 1 × 10-6 colony-forming units per milliliter (He et al. 1983).

The colonies were inoculated onto 45-day-old plantain plants of the variety Dominico Hartón, in thermic chambers located in the Municipality of La Tebaida, Quindío. The plants, with naked roots, were transported in carton boxes to CIAT greenhouses, where they were planted in plastic 1-kg bags containing a sterilized mixture of sand and soil at a rate of 3:2. The plants were not watered for 24 h before inoculation (EPPO 1990).

The inoculation method used was injection with sterilized 1-mL syringes and needle size 27G × 1/2". Half a mL of the bacterial suspension was inoculated into the pseudostem of each plant at a height of 2 cm from the soil surface. After inoculation, the plants were incubated for 3 days in a humid chamber to guarantee optimal development of the pathogen. They were then placed in the greenhouse under controlled conditions with temperaturas between 24º and 29ºC (minimum night and maximum day, respectively), relative humidity between 91% and 80% (maximum night and minimum day, respectively), and light for 13 h.

Processing plant tissue samples: Samples of different types of infected plant tissues (corms, pseudostems, and leaves) were selected from plantain plants that presented typical symptoms of the disease such as reddish streaks on the pseudostem, and wilt, flaccidity, and yellowing of leaves. These had been ascertained from evaluations conducted during visits to the farm to determine the presence of the pathogen.

Fragments of infected tissue were washed with deionized water for 30 min, disinfected in sodium hypochlorite at 1% for 30 s, then submerged in ethanol at 50% for 1 min, and finally rinsed twice with sterilized deionized water for 10 s to remove residues of the disinfectants. This procedure was conducted in a laminar flow chamber under aseptic conditions, using materials sterilized at 121ºC under 20 lb of pressure for 20 to 30 min. To isolate the bacteria present in the tissue, the disinfected fragments were macerated in a mortar sterilized at 121ºC under 20 lb of pressure with a TE buffer solution (10 mM Tris-HCl and 1 mM EDTA, with a pH 7.6).

The resulting suspension was planted, using a sterilized microspade, in petri dishes containing semi-selective SMSA medium (10 g/L peptone, 5 mL/L glycerol, 1 g/L casaminoacids, and 18 g /L agar). Antibiotics were added under aseptic conditions when the temperature of the SMSA medium was 50ºC. This stock was added as follows: 100 mg/L (600,000 U) polymyxin ƃ-sulfate; 25 mg/L bacitracin (source: 36 mg/L Baneocin®); 0.5 mg/L (82.5 U) penicillin; 5 mg/L chloramphenicol; 50 mg/L 2,3,5 chlorotriphenyltetrazole; and 5 mg/L crystal violet (Englebrecht 1994; Elphinstone et al. [1996], cited by Martins 2000; Denny and Hayward 2001). The solution of antibiotics was sterilized by filtering, using Millipore filters with pore size of 0.22 µm, and adjusted for use in syringes.

The dishes that were planted with the suspension were incubated for 5 to 7 days at 28ºC. Colony growth was compared with that of strain CIAT No. 78 of R. *solanacearum* race 2, itself isolated from samples of plantain rachis from Montenegro, Quindío.

The procedure for samples from colonies isolated in SMSA medium was the same as for the soil samples.

Decomposition of materials from infected plants: To accelerate the decomposition of materials chopped up from infected plants, we prepared 12.5 g of *Trichoderma harzianum* or *T. viride* (1 × 1010 conidia/g) in 20 L. Four applications were made every 15 days. The two strains were alternated every 15 days. To compare the plots where the product was applied, we had to take into account the appearances of new cases of plantain plants infected by wilt where the material was chopped. In all the treated foci and 10 others, plants infected with wilt and surrounding healthy plants were chopped up, including corms. Each infected plant received an application of 4 L of a mixture of 200 L of water, 10 kg of molasses, 20 L organic matter decomposer facilitated by Sanoplant (Palmira, Valle del Cauca), 200 g of a mixture of T. *harzianum*, *T. viride*, and *T. koningii* (concentration at 1 × 1012 conidia/g), and 200 g of *Paecilomyces lilacinus* (concentration at 1 × 1012 spores/g). The application was repeated 30 days later.

Results and discussion

From the soil samples taken from foci infected with wilt, we found that, of 172 samples analyzed, only 3 were positive. This finding ratified the difficulty in isolating and identifying viable cells of *R. solanacearum* in the soil, where the percentage of detection of this pathogen in this study was low (1.7%), confirming findings by Gómez (Gómez. 2005), who detected only 8% in soil samples.

The mechanisms of *R. solanacearum* for surviving in the soil are complicated and little studied. One reason for the pathogen was not readily detected in the soil samples is perhaps that populations decay progressively over time to an undetectable level (Coutinho 2005). This finding contrasts with that reported by Martins (2000), who suggested that the bacterium can persist in many soils under different crops, with diverse conditions of management. The bacterium's survival is linked directly with the presence of water. The soils of most of the farms in Quindío had good drainage, which made it difficult for the pathogen to stay in the soil.

In this study, the bacterium was not detected between November 2005 and March 2006 in any of the foci. The pathogen was isolated only in April and October 2006 (Table 10.2). In August and September 2006, no samples were taken because seedling indicator plants had been planted to improve the detection of *R. solanacearum* in the different experimental plots. Indicator plants were planted at 3 per plantain plant infected with bacterial wilt.

Table 11.2. Detection of *Ralstonia solanacearum* in soil and plant tissue samples, using SMSA culture medium to determine the effect of a mixture of lixiviate from decomposing plantain rachis, phosphoric rock, and french marigold applied to the soil of foci infected by bacterial wilt at the Santa Elena Farm, Quindío, Colombia. Eight samplings were conducted over 13 months.

		Soil samples		Plant tissue samples	
Month and year in which samples were taken		**(no.)**	**Positive for *R. solanacearum* (no.)**	**(no.)**	**Positive for *R. solanacearum* (no.)**
1	Nov, 2005	21	0	15	1
2	March, 2006	21	0	7	1
3	April, 2006	23	2	3	0
4	May, 2006	23	0	0[a]	0
5	June, 2006	23	0	0[a]	0
6	July, 2006	23	0	0[a]	0
7	Oct, 2006	18	1	0[a]	0
8	Nov, 2006	20	0	0[a]	0
Total of samples		172	3	25	2
Efficiency of detection of *R. solanacearum* (%)			1.7		8.0

[a] The sampling of plant tissue could not continue because of the chopping up of infected plants. The gradual decomposition of plant tissue made sampling impossible.

Throughout the experiment, we also processed 24 samples of plant tissues from new cases of plants infected with bacterial wilt around the treated foci. Results were positive, thus confirming the presence of the bacterium around the foci being treated.

Visits to the farm allowed us to monitor the area around the treated plots and so identify the pathogen's dissemination by the appearance of new cases. We saw an increase in August, noting that new cases of infected plants consistently appeared very close to the old foci, probably because of deficient eradication of healthy plants around the infected plants at the beginning of the experiment. To create soil conditions more conducive to pathogen development, we planted indicator plants in plastic bags and placed them inside the experimental plots. The bags were placed below soil level, thereby permitting the accumulation of moisture in each bag for lack of drainage. In each of the 18 plots, 3 indicator plants were planted. However, none of these plants became diseased with this method of detection.

Of 145 plantain indicator plants planted in July 2006 in 15 experimental plots, none were diseased 4 months later. Nor, in October 2006, was *R. solanacearum* detected in 16 plants processed in the laboratory, using SMSA culture medium.

Conclusions

A very low (1.7%) efficiency of detection of *R. solanacearum* was observed for soil samples from the experimental plots, using SMSA medium. For future studies, we recommend using molecular markers and real-time PCR to detect the pathogen in the soil and plant tissues. During the frequent visits made to the farm, we went through fields recording the appearance of plants newly infected with bacterial wilt. In the site occupied by each infected plant in each focus, we planted three plantain plants after the last application of lixiviate to discover the efficacy of the treatments established. On Day 60, a sampling of soil and plant tissues was conducted to detect the presence of *R. solanacearum* in the soil and living materials of plantain plants.

Over 9 months, we watched 54 new plants become diseased with bacterial wilt. We concluded that the disease spread in this farm largely because inadequate management practices were used such as the use of tools (e.g., machete and hacking knife) without disinfecting them, lack of early detection and eradication of foci, and not marking infected areas. We did not find evidence for dissemination of the disease through the soil. After 2 months, we saw that the 48 plants planted in plastic bags did not become diseased, even with the rainy conditions of the zone. We conclude that the pathogen was not found in plot soil. Because *R. solanacearum* was absent from the soil, we could not determine the effect control practices had over bacterial wilt. We recommend that plans be established to prevent losses in crops and, hence, drastic applications of chemical products. Likewise, we suggest the use of integrated management packages to control this disease.

12. Socializing Research Results on Managing Bacterial Wilt of Plantain

Elizabeth Álvarez, Luz Adriana Mesa, Victor Triviño, and John Loke

Rationale

Bacterial wilt has spread widely because of poor practices in prevention and management such as using infected suckers, contaminated tools and clothes, and lack of early detection and eradication.

This activity aims to diffuse information on the integrated management of bacterial wilt to farm owners and workers, technicians, and agronomists in the Municipality of Armenia, Department of Quindío, Colombia.

Methodology

To emphasize the importance of this disease, explain its management, and describe research advances, we conducted two meetings directed by CIAT functionaries and students from the Universidad de Quindío.

Results and discussion

The first meeting was held on 26 June 2006 at the Santa Elena Farm, Armenia (Quindío), to which 42 persons attended, including farm owners and workers, technicians, and agronomists. At this meeting, the importance of bacterial wilt was discussed, together with possible causes of its dissemination. Management of this problem was explained, and research advances described.

The second socialization was conducted on 31 August 2006 at the La Yalta Farm, which had successfully adopted and adjusted management practices for bacterial wilt. At this meeting, 20 people actively participated, clearing their doubts and discussing recomendations on managing the disease.

Referentes

Gómez E. 2005. Aislamiento, identificación y caracterización del agente causal del moko del plátano, *Ralstonia solanacearum* raza 2, proveniente de plantaciones afectadas en Colombia. Faculty of Sciences, Pontificia Universidad Javeriana, Bogotá, DC, Colombia.

He LY; Sequeira L; Kelman A. 1983. Characteristics of strains of *Pseudomonas solanacearum* from China. Plant Dis 67(12):1357–1361.

Larco E. 2004. Desarrollo y evaluación de lixiviados de compost y lombricompost para el manejo de sigatoka negra (*Mycosphaerella fijiensis* morelet), en plátano. MSc thesis. Program of Education for Development and Conservation, Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Turrialba, Costa Rica.

13. Designing and Standardizing a TaqMan® Probe for the Specific Detection of *Ralstonia solanacearum* Race 2 in Plantain through Real-Time PCR

Elizabeth Álvarez and Juan Fernando Mejía

Rationale

Ralstonia solanacearum race 2, causal agent of bacterial wilt of plantain, also known as *moko*, is considered to be the disease that most limits plantain production. The wilt is characterized for its aggressiveness, speed, easy dispersion, and high genetic variability. It causes significant economic losses. Currently, the most effective practice for managing the disease is to eradicate foci of diseased and apparently healthy plants. This practice generates high costs of renovation and treatment for the farmers.

Despite the publicizing of preventive measures and management, the disease continues to increase to the point where 95% of plantain fields have at least one plant with wilt (R. Galindo, personal communication 2004, Instituto Colombiano Agropecuario, Bogotá).

To improve efficiency in detecting viable cells of this pathogen, especially in the soil, several methods currently exist such as the BIO-PCR technique, developed by Schaad et al (1995). This technique consists of isolating colonies in semi-selective medium South Africa (SMSA) (Denny and Hayward, 2001) and amplification with the specific primer OLI1, together with the nonspecific primer Y2. Another method is to inoculate plantain plants and use the tobacco hypersensitivity reaction test to identify race 2 strains that are pathogenic to plantain. A third method is to characterize biovars through biochemical probes.

However, these methodologies are not very sensitive, specific, or quantifiable. They also demand considerable time to identify the bacterium. The development of a detection tool that is sensitive, quick, and specific, and which would quantify minimal concentrations of the bacterium's cells would facilitate early detection of the pathogen. It would also help reduce the high costs of managing and eradicating foci.

Various methodologies exist that can replace these procedures such as real-time PCR (sometimes called kinetic PCR). Unlike conventional PCR, amplification and detection occur simultaneously in the same closed vial, without needing further action. This results in greater speed, reduces risk of cross contamination, and increases the flow of samples and trials for evaluation.

Moreover, this technique uses fluorescence for detection. Hence, during amplification, we can measure the quantity of DNA synthesized at a given moment, as the emission produced in the reaction is proportional to the quantity of DNA formed. Thus, we can quantify the initial concentration of nucleic acid present in the samples in a much simpler and precise way that is, above all, within a much greater range (5–6 log) than with conventional

procedures (2–4 log). As well, the probes are highly specific. Equipment for real-time PCR can be used for qualitative and quantitative trials, determination of mutations, multiple PCR, and other activities, whereas a lot of equipment is needed with conventional procedures.

Materials and methods

Designing the probe: To identify the region for pathogenicity in strains of *R. solanacearum* race 2 in plantain, we had to design the TaqMan® probe to be specific for these strains. We took as reference the work described previously, which evaluated, through multiplex PCR, the ITS region (phylotype II, sequevar 4) of strains pathogenic to plantain and isolated from different zones of Colombia. The association of the region was made by correlating strain pathogenicity with absence or presence of amplified fragments for sequevar 4.

Once the region was identified, we used the program Primer Express® Software v2.03 to design the TaqMan® probe and the forward and reverse primers. To design the TaqMan®, we:

1. Kept the G-C contents in the 30%–80% range
2. Avoided runs of an identical nucleotide; this was especially true for guanine, where runs of four or more Gs had to be avoided
3. Did not put Gs on the 5N end
4. Selected the strand that gave the probe more Cs than Gs
5. For single-probe assays, melting temperature (T_m) was kept between 68° and 70°C when using Primer Express® Software

To design the forward and reverse primers, we:

1. Chose the primers after the probe
2. Designed the primers as close as possible to the probe without overlapping it
3. Kept the G-C contents in the 30%–80% range
4. Avoided runs of an identical nucleotide; this was especially true for guanine, where runs of four or more Gs had to be avoided
5. When using Primer Express® Software, the T_m was kept between 58° and 60°C
6. Ensured that the five nucleotides at the 3N end had no more than two G and/or C bases

Sequencing: The region or fragment with the highest correlation with the strains pathogenic to plantain was sequenced in two ways:

[1] Primer Express® Software is a primer design program from Applied Biosystems that also allows for an easy design of compatible TaqMan® probes, for use in real-time PCR or end-point PCR analyses. It can also be used to design primers alone when the user expects to analyze samples with SYBR® Green only.

1. PCR purification by adding a volume of a 20% solution of polyethylene glycol (PEG) with 2.5 M NaCl for later sequencing with BigDye® Terminator Kit (Applied Biosystems) in an ABI Prism® 377 sequencer.

2. PCR products purified, using the QIAquick PCR Purification Kit (QIAGEN, Inc.), ligated in the pGEM®-T Easy Vector System, introduced into the *Escherichia coli* strain DH5-ζ by electroporation at 2.4 kV/cm^2.

PCR products purified, using the QIAquick PCR Purification Kit (QIAGEN, Inc.), ligated in the pGEM®-T Easy Vector System, introduced into the Escherichia coli strain DH5-ζ by electroporation at 2.4 kV/cm2.

Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media. Plasmids were extracted with a Plasmid Miniprep System Kit (Gibco-BRL). Positive inserts were observed by plasmid restriction with EcoRI and electrophoresis in 1.5% agarose gel. Different-sized fragments were selected for sequencing by automated dideoxy sequencing (ABI Prism® 377-96 DNA Sequencer), using a DNA-sequencing kit from Applied Biosystems. Sequencing alignment analysis was conducted with the programs Sequencher 4.1 and BioEdit (Hall, T.A. 1999. Nucl. Acids. Symp. Ser. 41:95-98).

Evaluating the probe: To evaluate the TaqMan® probe, we used Opticon 2 (MJ Research, Inc.) to standardize annealing temperatures and concentrations for the probe and primers. Two replications were carried out for each condition evaluated, allowing us to choose the cycle threshold (Ct) values with the least variability.

Amplifications were conducted in a final 25-µL volume of 1X Taq buffer, 1.5 mM MgCl2, 0.2 mM dNTP, 0.2 µM of each primer and of the TaqMan® probe, and 1.5 units of Taq DNA polymerase (Bioline USA, Inc.).

The specificity and reproducibility of the plantain probe was compared with a TaqMan® probe that had detected all biovars of *R. solanacearum*, as reported by Weller et al, 2000. Appl. Environ. Microbiol. 66:2853-2858, (RS-P4; RS-I-F/RS-II-R). We also used primer pairs OLI1/Y2 and RS-I-F/RS-II-R with the SYBR® Green fluorescent dye.

An evaluation of sensitivity was conducted through successive dilutions from 2 × 100 to 2 × 108 of total DNA, and detection compared between conventional and real-time PCR.

To standardize the probe and sensitivity trials, we worked with the control strain CIAT 1008, which is pathogenic to plantain.

[4]. TaqMan® probe, labeled with a FAM dye, partly homologous with the OLI1 primer from Seal et al, 1993. J. Gen. Microbiol. 139: 1587-1594.

Results

Designing the probe and sequencing: Based on results from the multiplex PCR analysis for sequevar 4 (Activity 1.4.1.1), we ascertained the specificity of primers Mus 20F/Mus 20R (351 bp) for strains pathogenic to plantain, obtaining a correlation of 100% for strains with high, intermediate, and low pathogenicity. The region that amplified with primers Mus 06-F/Mus 06-R (167 bp) is absent from isolates obtained from Urabá (Department of Antioquia), comprising two isolates from banana and three from plantain. The isolate obtained from Mucuna-soil did not amplify for any sequevar and neither were the strains isolated from soil pathogenic.

On the basis of the previous results, we sequenced the 351-bp region amplified with primers Mus 20F/R, which correlated with pathogenicity on plantain for strains of R. *solanacearum* race 2. With the two methodologies, we obtained 19 sequences, which were then aligned and compared with the Sequencher 4.1 and BioEdit programs (Figure 13.1).

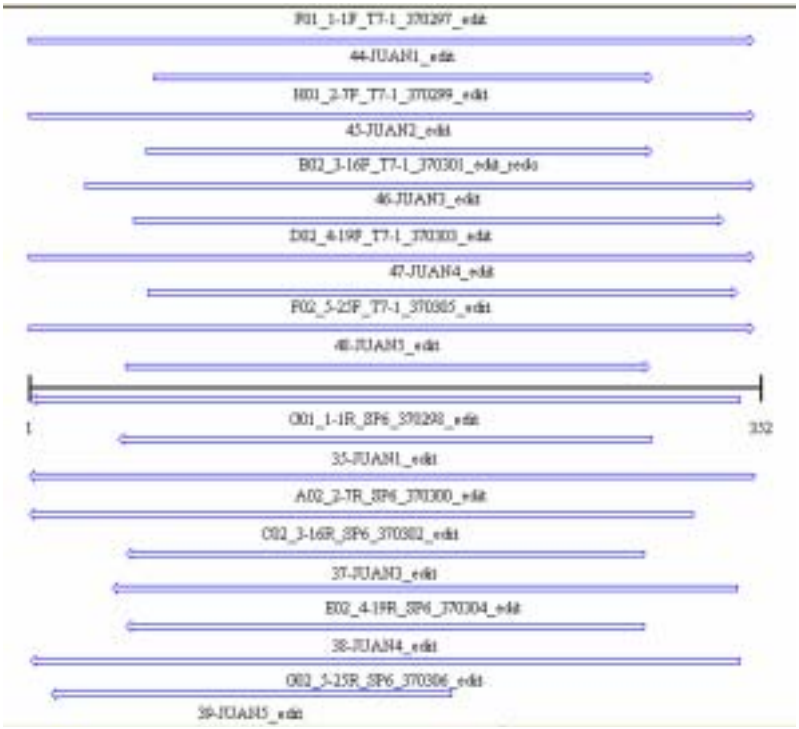


Figure 13.1 Assembly into one group of 19 sequences from the 351-bp region that had been amplified with primers Mus 20F/Mus 20R, themselves designed from subtracted sequences specific to phylotype II, sequevar 4.

Once we had obtained the consensus sequence, we designed the reverse primer (Mus 20RP) and the TaqMan® probe (Mus 20P) according to recommendations from the Primer Express® Software v2.0 program. For the reverse primer, we conserved a %GC of 45, a Tm of 59, and a window size of 22 nucleotides; and for the probe, the %GC was 57, Tm was 70, and the window size 21 nucleotides. The 5' terminal nucleotide was marked with a suitable fluorophore (reporter dye) such as FAM, specifically 6-carboxyfluorescein, and the 3' terminal nucleotide with a quencher dye such as the Black Hole Quencher® 1. For

the forward primer, the original sequence of primer Mus 20F was conserved (%GC = 57; Tm = 60) (Figure 13.2).

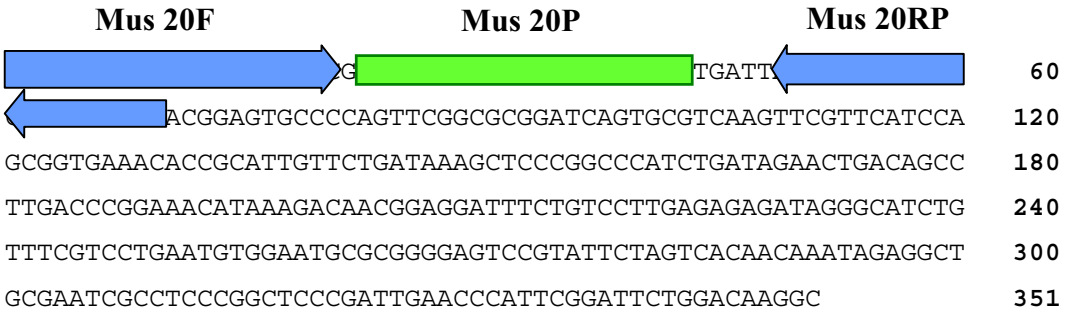


Figure 13.2 Design and location of primers Mus 20F/Mus 20RP and the TaqMan® probe (Mus 20P) specific to strains pathogenic to plantain and obtained from the 351-bp sequence of sequevar 4.

The amplicon obtained was 70 bp, permitting greater speed and specificity in amplification. The primers were located near the probe and the probe's temperature was kept higher than that of the primers to prevent nonspecificity and provide optimal alignment of the primers.

Evaluating the probe: We evaluated the annealing temperatures of 58°, 60°, 62°, and 65°C, and conducted combinations in concentrations of primers at 50, 300, and 900 nM (Figure 3). The temperature and concentration that showed least variation in the two replications were 62°C and 300 nm, respectively, of each primer, with Ct values of 20.616 and 20.607 for a variation of 0.009. For the probe, the concentration was 250 nM (Table 12.1, Figure 12.4).

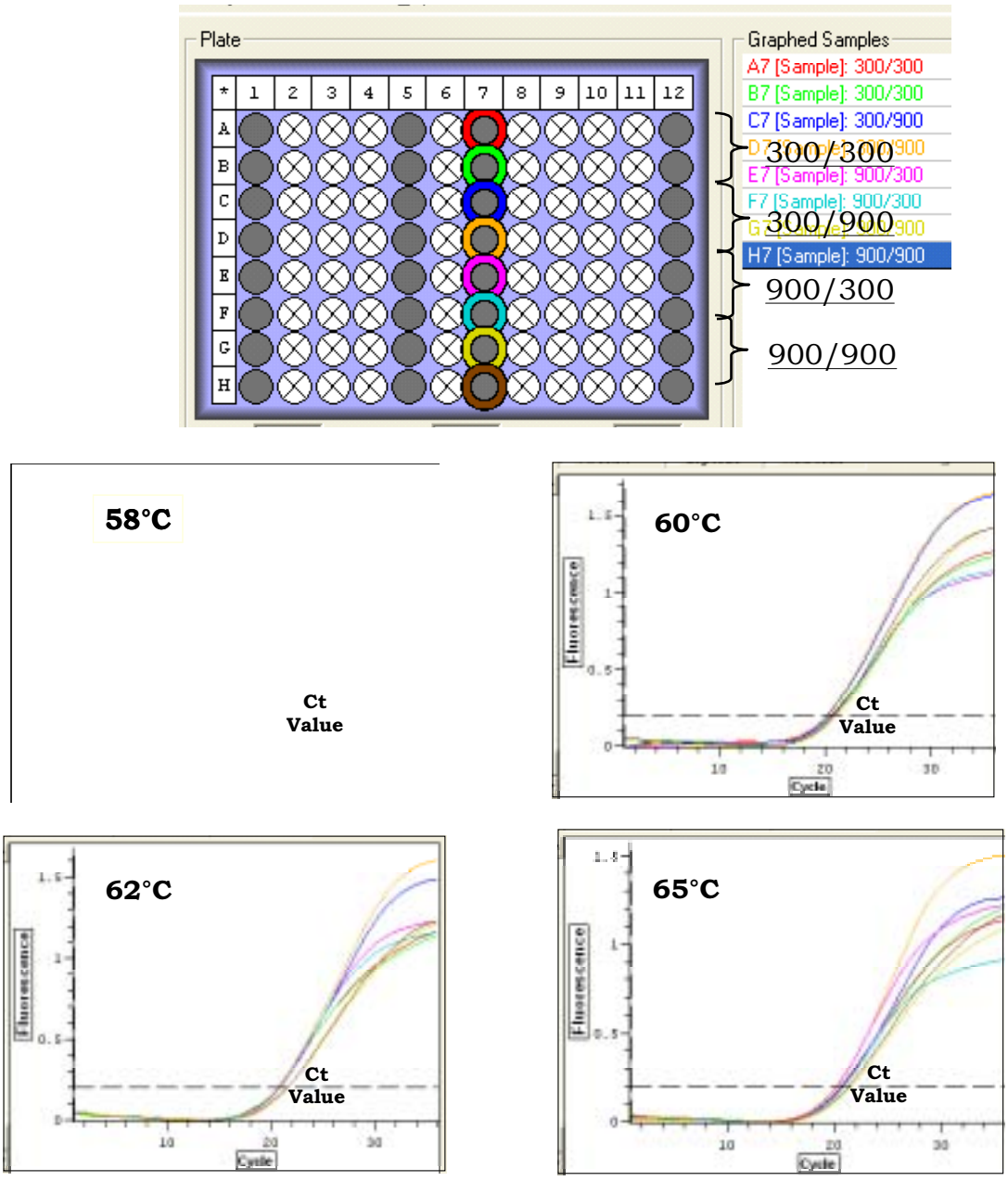
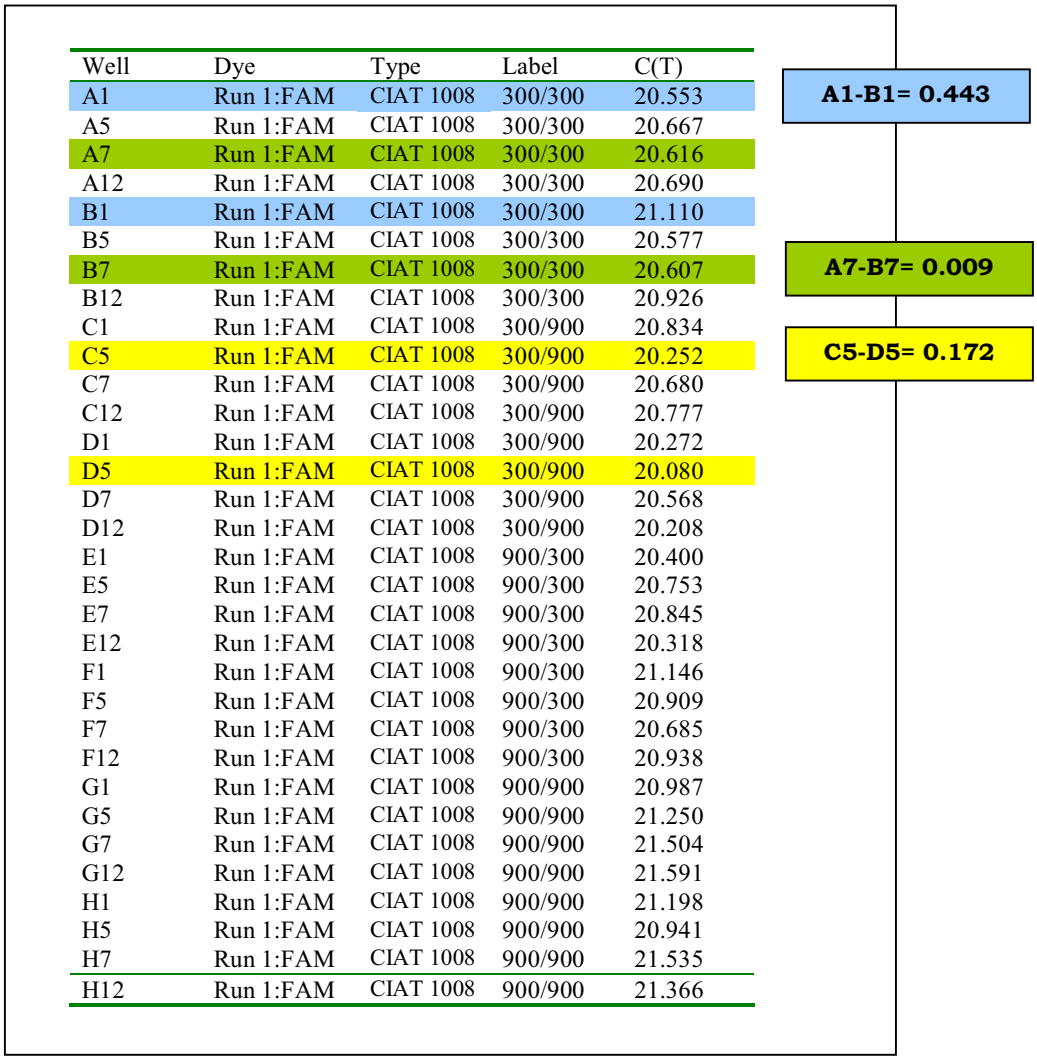


Figure 12.3 Evaluation of annealing temperatures (58 °C, 60°C, 62°C and 65°C) and combinations of concentrations of primers at 50, 300, and 900 nM.

Table 13.1 Combination of different concentrations for primers Mus 20F/Mus 20RP.

Con-centration	Mus 20F/Mus 20RP (nM)		
	50	300	900
50	50/50	300/50	900/50
300	50/300	300/300	900/300
900	50/900	300/900	900/900



Well	Dye	Type	Label	C(T)
A1	Run 1:FAM	CIAT 1008	300/300	20.553
A5	Run 1:FAM	CIAT 1008	300/300	20.667
A7	Run 1:FAM	CIAT 1008	300/300	20.616
A12	Run 1:FAM	CIAT 1008	300/300	20.690
B1	Run 1:FAM	CIAT 1008	300/300	21.110
B5	Run 1:FAM	CIAT 1008	300/300	20.577
B7	Run 1:FAM	CIAT 1008	300/300	20.607
B12	Run 1:FAM	CIAT 1008	300/300	20.926
C1	Run 1:FAM	CIAT 1008	300/900	20.834
C5	Run 1:FAM	CIAT 1008	300/900	20.252
C7	Run 1:FAM	CIAT 1008	300/900	20.680
C12	Run 1:FAM	CIAT 1008	300/900	20.777
D1	Run 1:FAM	CIAT 1008	300/900	20.272
D5	Run 1:FAM	CIAT 1008	300/900	20.080
D7	Run 1:FAM	CIAT 1008	300/900	20.568
D12	Run 1:FAM	CIAT 1008	300/900	20.208
E1	Run 1:FAM	CIAT 1008	900/300	20.400
E5	Run 1:FAM	CIAT 1008	900/300	20.753
E7	Run 1:FAM	CIAT 1008	900/300	20.845
E12	Run 1:FAM	CIAT 1008	900/300	20.318
F1	Run 1:FAM	CIAT 1008	900/300	21.146
F5	Run 1:FAM	CIAT 1008	900/300	20.909
F7	Run 1:FAM	CIAT 1008	900/300	20.685
F12	Run 1:FAM	CIAT 1008	900/300	20.938
G1	Run 1:FAM	CIAT 1008	900/900	20.987
G5	Run 1:FAM	CIAT 1008	900/900	21.250
G7	Run 1:FAM	CIAT 1008	900/900	21.504
G12	Run 1:FAM	CIAT 1008	900/900	21.591
H1	Run 1:FAM	CIAT 1008	900/900	21.198
H5	Run 1:FAM	CIAT 1008	900/900	20.941
H7	Run 1:FAM	CIAT 1008	900/900	21.535
H12	Run 1:FAM	CIAT 1008	900/900	21.366

Figure 13.4 Evaluation of variation of Ct values in two replications for each primer and temperature (see Figure 13.3).

The specificity and reproducibility of the probe Mus 20P and primers Mus 20F/Mus 20RP from plantain, compared with the probe RS-P and primers RS-I-F/RS-II-R, showed a positive amplification for 44 strains pathogenic to plantain, with Ct values between 21.613 and 23.487. No amplification occurred for the five non-pathogenic strains and the six

isolates from other species used as control. This contrasts with what we found with probe RS-P, which amplified positively for pathogenic and non-pathogenic strains, and for isolates from other species. The Ct values were between 17.012 and 30.651 (Figure 12.5).

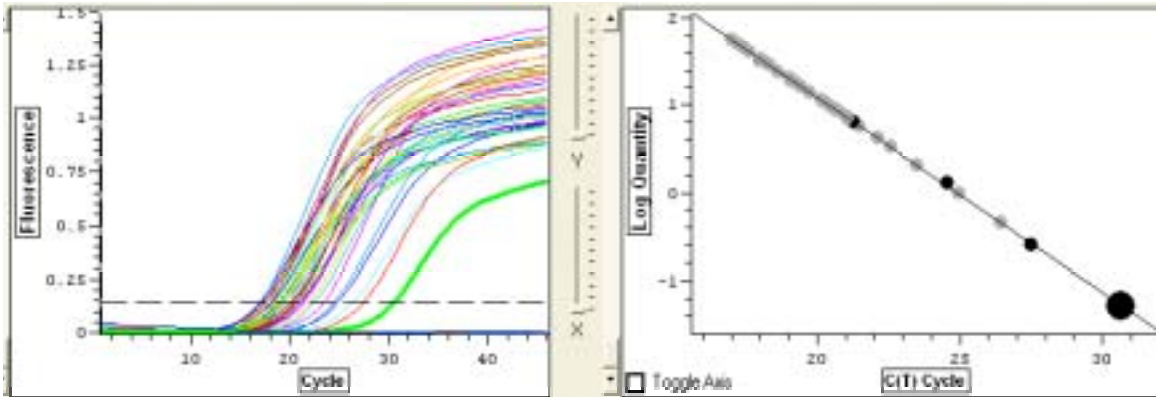


Figure 13. 5 Positive amplifications with probe RS-P for 58 strains, pathogenic and non-pathogenic to plantain and from other species, with Ct values between 17.012 and 30.651. Positive amplification for 44 strains pathogenic to plantain, with Ct values between 21.613 and 23.487, and negative amplifications for five non-pathogenic strains and six isolates from other species.

The evaluation of OLI1/Y2 and RS-I-F/RS-II-R with the SYBR® Green dye, did not discriminate between pathogenic and non-pathogenic strains and isolates from other species. However, reproducibility was good (Figure 13.6, Table 13.2).

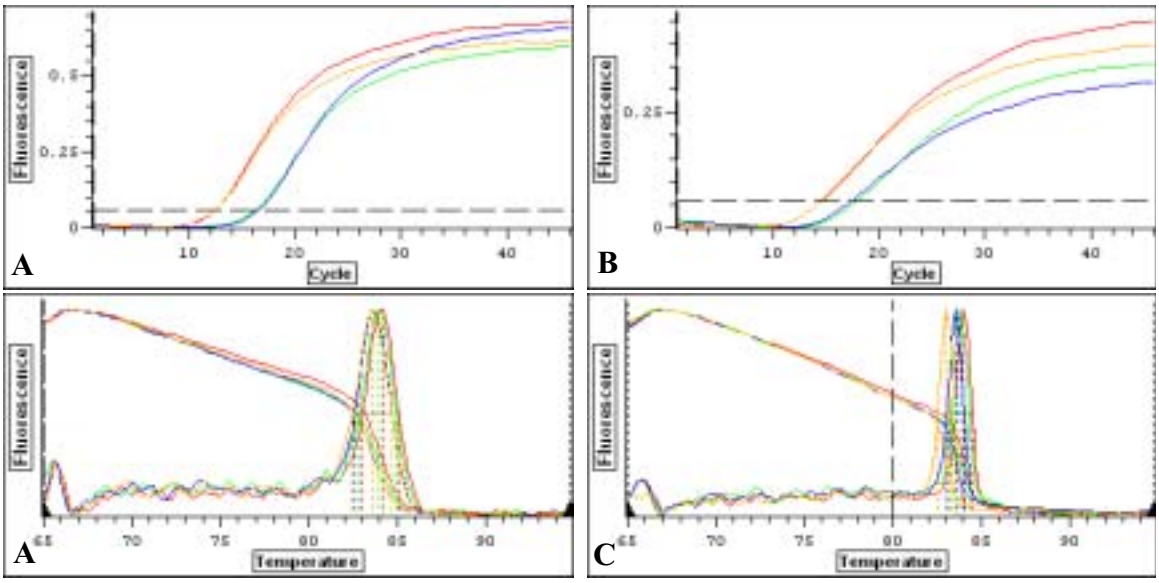
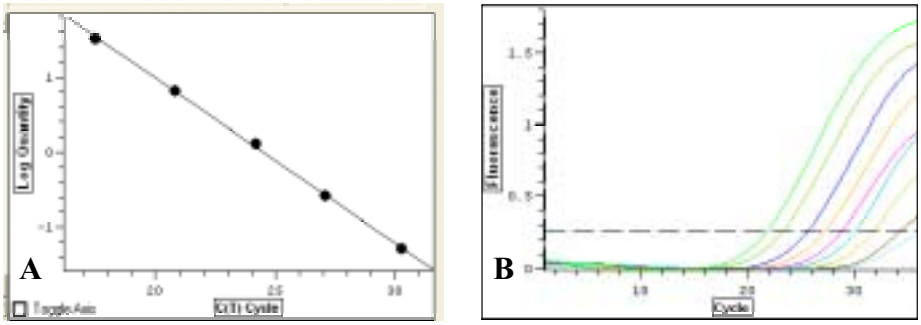


Figure 13. 6 Amplifications of primers RS-I-F/RS-II-R (A) and OLI1/Y2 (B) with the SYBR® Green dye.

Table 13.2 Isolates evaluated with primers RS-I-F/RS-II-R and OLI1/Y2 with the SYBR® Green dye.

Primer pairs	Isolates (origin), no. or name
Pair RS-I-F/RS-II-R (10 µM)	
E (Red)	Plantain pseudostem (Caquetá), 32
F (Green)	Plantain-soil (Quindío), 38
G (Blue)	Potato, G177
H (Orange)	Tobacco, CIAT 1054
Pair OLI1/Y2 (10 µM)	
E (Red)	Plantain pseudostem (Caquetá), 32
F (Green)	Plantain-soil (Quindío), 38
G (Blue)	Potato, G177
H (Orange)	Tobacco, CIAT 1054

The evaluation of sensitivity by successive dilutions, comparing conventional PCR with real-time PCR, was carried out by evaluating a standard curve for quantification, Ct values, and presence or absence of bands in agarose gel. Dilutions were made from 32 to 8.1 × 105 ng (2 × 100 to 2 × 108). With conventional PCR, we could detect as low as 1024 × 102 ng (2 × 104) (Figure 13.6), whereas with probe Mus 20P, we detected at 4.8 × 104 ng (2 × 106), with Ct values between 18.020 and 35.111 (Figure 13.7).



[Ct 18.020 – 35.111] [32 ng – 4,8x10-4ng]

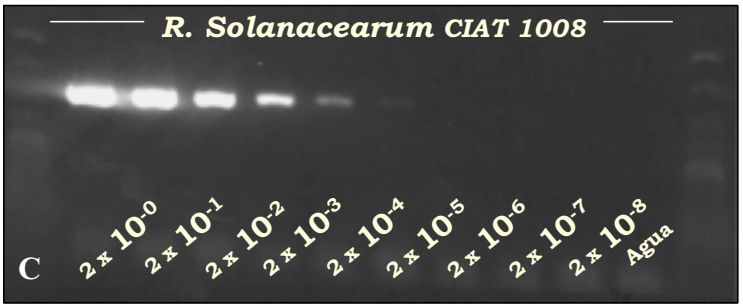


Figure 13.7 Successive dilutions for the evaluation of sensitivity of conventional PCR versus real-time PCR, from 32 to 8.1 × 105 ng (2 × 100 to 2 × 108). (A) Standard curve for quantification. (B) Positive and negative amplifications, with Ct values between 18.020 and 35.111. (C) Presence or absence of bands in agarose gel at 1.5%.

Conclusions

We designed primer Mus 20RP and the TaqMan® probe Mus 20P, successfully associating it with pathogenicity to plantain, and standardized annealing conditions to 62°C and concentrations to 300/300 nM for the primers and 250 nM for the TaqMan®. We obtained a sensitivity for the probe that could detect as little as 4.8 × 104 ng (2 × 107) of DNA.

We need to quantify for BIO-quantitative real-time PCR (qRT-PCR) to measure colony-forming units per milliliter (cfu/mL) from bacterial cells on semi-selective SMSA culture medium. Moreover, we need to standardize direct detection in tissue and soil samples from foci of the disease.

14. Added value Lulo: Alternatives for Smallholder producers.

Armando Muñoz, Maria Eugenia Buitrago, Zaida Lentini, Elizabeth Alvarez, Alonso González
(Collaborating institutions: Corpoica, Mario Lobo; Productora de Jugos S.A)

Sources of Funding: Colombian Ministry of Agriculture (MADR)

Abstract

The Solanacea family is highly diverse containing about 2500 species, many of which are edible and economically important crops like tomatoes, sweet papers, eggplants, potatoes, tobacco etc. Within this family, the Lasiocarpa section includes between 11 and 13 species of shrubs and trees. Species of the Lasiocarpa section distribute in the northeast of Southamerica, and some produce edible fruits, like Solanum quitoense and Solanum sessiliflorum which are economically important in Latin America. The center of diversity for Solanum quitoense includes Colombia, Ecuador and Peru. Some wild related species are found in Venezuela, Brazil and Central America.Lulo or Naranjilla es currently cultivated in Venezuela, Panama, Guatemala and Costa Rica, but do not have the same economic importance as in Colombia and Ecuador. The species is grown by smallhoders in plots that average 0.6 ha (Colombia) and 1.5 ha (Ecuador), with an total area in these two countries of about 10 to 12,000 ha. This represents about 20,000 families that use this crop for household income.

The plant is grown in the hilldside of the Andes between 1200 and 2400 masl, produce fruits 8 months after planted for about 2 years, fruits are harvested weekly offering a constant income for the smallholder family. After the crop cease production, growers move to new areas avoiding phytosanitary issues (nematodoes, antracnose, phytophtora, insect pests, etc) that normally arise if crop is planted again in the same plot. Moving the crop to new areas result in new land clearing and disruption of the production clusters.

The crop is normally propagated by sexual seeds, which result in a high level of hetereogeinity that results in lack of interest from the fruit processing sector. In Colombia about 95% of the area planted in Lulo corresponds to the so called Lulo de Castilla, which have attractive organoleptic properties but highly variable, and is marketed through wet markets and large surface supermarkets. Only one variate exists in Colombia and two in Ecuador, which are interspecific hybrids. These varieties are propagated using cuttings or tissue culture produced plants.

Juice and pulp processing industries offer future contracts to growers, but mainly of the interspecific hybrid because it has more homogenous quality, some level of resistance to nematodes and demand less use of pesticides and insecticides. Demand for this fruit is high and increasing, but quality needs to improve to make growers more competitive and able to supply pesticide free products. CIAT research on Lulo is aiming to develop inexpensive mass multiplication of planting materials, identify elite clones of lulo Castilla that suits the needs of growers and industry, and to identify sources of genetic resistance to major diseases (fungi and nematodes).

The research funded for the Colombia Ministry of Agriculture (MADR) address several of the issues described here. Funding for three years was granted to ciat and collaborating institutions.

Project Leader: CIAT, Tropical Fruits Project.
Partners: Corpoica (Colombia), Productora de Jugos SA (local fruit industry, private sector).

Objective: To develop technologies that allow smallholders to cultivate native species with added value for national and international markets, and that contribute to the conservation of the native biodiversity. Specifically, this project aims at enhancing the competitiviness of small lulo growers through the use of improved varieties, native varieties and the use of tissue culture techniques applied at the farm level.

Main activities:

1. Develop and adapt a methodology for *in vitro* propagation of planting material to be implemented in rural laboratories, and to design a strategy for germplasm conservation.
2. To train and empower smallholders in the lulo production areas in clonal propagation of elite planting material using *in vitro* technologies in rural laboratories.Rural laboratories will be developed using minimum cost materials and reagents.
3. To assess productivity, disease resistance and fruit quality characteristics of elite planting material (40 clones). Evaluation of plant material will be carried out in greenhouse, experimental plots in grower's field in ther representative lulo growing areas in Colombia.
4. Assess the performance of interespecific hibrids derived from hibrid La Selva, selected populations of the National Collection of Lulo, and accessions identified by growers through participatory methods. New materials identified by growers will be incorporated to the National Collection to preserve local genetic variability.
5. Promote genetic variability in local populations used by growers by delivering high quality populations to be planted in their fields. Over time, enhanced populations will outcross with local populations used by smallholders and new introgressed populations could be used for selecting elite planting material.

Results to date:

Development of the consortium.New partners joint the consortium after funding was secured. The Universidad del Cauca, located in Popayán (Cauca) and the Universidad de Santa Rosa de Cabal, located in Risaralda, Colombia. The Universities would be implementing activities related to in vitro propagation of planting material and assessment of performance of elite clones and new hybrids.
The location of these two universities is considered as an asset to the consortium. The Universities are the focal point of many lulo growers in their areas, and some of their family members are actually students in these Universities. The universities would provide planting material produced in vitro in their labs to local nurseries, which will propagate elite clones using conventional methods (cuttings) after facilities are designed to avoid phytosanitary issues that could emerge by propagating cuttings without the required care.

Directly in fields of a growers association (to be defined) in the Risaralda region, a low cost in vitro propagation laboratory will be developed. Materials produced in these laboratories should be lower price than those produced in commercial laboratories. The feasibility of those laboratories and the medium and long term impact would need to assessed.

Disease resistance assement of elite clones.

Collection of isolates of Colletotrichum and Phytophthora. The fungi P. *infestans* and C. *gloeosporioides* limit lulo production. Characterizing different lulo materials for their reactions to these diseases is important when taking into account the significant role that genetic resistance plays in any program for the integrated management of diseases. Collection of isolates of these fungi is an ongoing activity. So far, 26 isolates have been isolated and cultivated using purified. These isolates, and others, would be used to characterize their pathogenicity againts lulo elite clones (Table 1).

Table 14.1. List of *Colletotrichum spp*. and *Phytophthora infestans* isolates obtained from samples of infected lulo.

No.	Code	Municipality	Department
***Colletotrichum* gloeosporioides**			
1	LMc 04	Trujillo	Valle del Cauca
2	LMc 06	Río Negro	Antioquia
3	LMc 07	Río Negro	Antioquia
4	LMc 12	Tambo	Nariño
5	LMc 18	San Pedro de Cartago	Nariño
6	LMc 19	Tambo	Nariño
7	LMc 40 a	Santa Rosa	Risaralda
8	LMc 40 b	Santa Rosa	Risaralda
9	LMc 41 b	Belén de Umbría	Risaralda
10	LMc 41 c	Belén de Umbría	Risaralda
11	LMc 42 a	Santa Rosa	Risaralda
12	LMc 43 a	Santa Rosa	Risaralda
13	LMc 43 b	Santa Rosa	Risaralda
14	LMc 44 a	Santa Rosa	Risaralda
15	LMc 44 b	Santa Rosa	Risaralda
16	LMc 44 c	Santa Rosa	Risaralda
17	LMc 45	Neira	Caldas
18	LMc 46	Gachetá	Cundinamarca
19	LMc 48	Timbio	Cauca
20	LMc 50	Timbio	Cauca
21	LMc 63	Timbio	Cauca
22	LMc 65	Timbio	Cauca
23	LMc 69	Timbio	Cauca
24	LMc 71	Timbio	Cauca
25	LMc 73	Timbio	Cauca
26	LMc 74	Pitalito	Huila
Phytophthora infestans			
27	LM75p	Manizales	Caldas
28	LM76p	Manizales	Caldas

Cultivation and purification of pathogen isolates. .We collected samples of infected tissues from plants presenting lesions of the fungi P. *infestans* and C. *gloeosporioides* in the Departments of Valle del Cauca, Antioquia, Caldas, Nariño, Risaralda, Cauca, and Cundinamarca. Each sample was geo-referenced, labeled, and assigned a code for further identification. Both leaves and fruits were disinfected with hypochlorite at 1% for 60 s and alcohol at 70% for 60 s, washed with sterilized distilled water, dried at room temperature, and conserved at 20°C on filter paper for later use.

Colletotrichum gloeosporioides, *causal agent of anthracnose:* In the field, under favorable conditions, depressed circular lesions appear on fruits, becoming salmon in color as the fungus' spores become concentrated (Figure 14.1). Where lesions appear on leaves, they are found on the main vein as depressed, black, oval spots

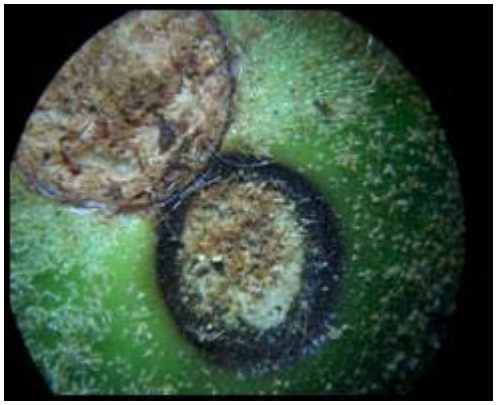


Figure 14.1. Lesions on lulo fruit caused by *Colletotrichum* spp.

Tissues, disinfected as described above, were placed on PDA with lactic acid (at 1 mL per 100 mL of medium) and left for 3 days. The growing colonies were examined for their morphology and under the microscope to determine which of these growths corresponded to *Colletotrichum spp*. Each suspected colony was taken to a medium that favored sporulation (Marthur agar) and, from this new culture, spores were obtained to carry out monosporic culturing to multiply and conserve the fungus.

Adjusting the methodology for inoculating lulo fruits and leaves with *Colletotrichum* gloeosporioides

Before we could begin testing lulo materials, we needed to standardize and adjust the methodology for inoculating lulo fruits and leaves. We conducted the following procedure.

We used the puncture method to inoculate lulo leaves and fruits, using a sterilized dissection needle. The leaves had previously been disinfected with hypochlorite at 1% for 5 min, alcohol at 70% for 5 min, and washed with sterilized distilled water. The main vein was inoculated by depositing, in the puncture site, aliquots at 1 µL, 5 µL, and 10 µL of a suspension of spores at 1 × 106 . The control was sterilized distilled water. After inoculation, the leaves and fruits were placed on grids inside plastic boxes, with each box containing 20 mL of sterilized distilled water. The boxes were closed hermetically to

simulate a humid chamber and favor the development of infection. The development of symptoms was evaluated 5 days later.

Five days after inoculation typical disease symptoms were observed on lulo leaves (Figure14.2).

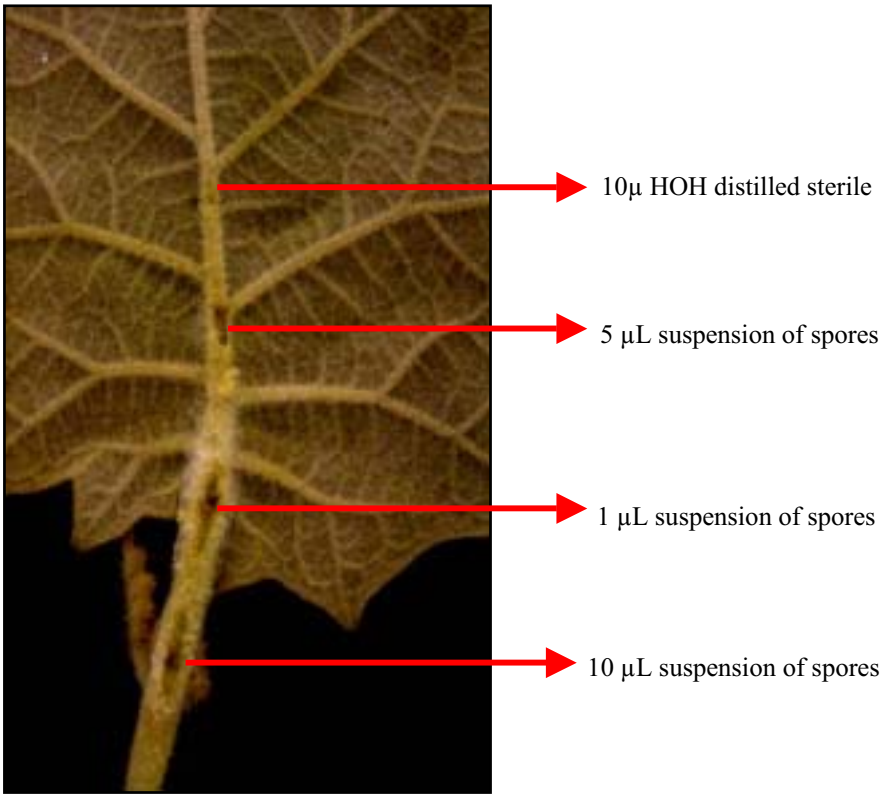


Figure 14.2. (Disease symptoms on lulo leaves 5 days after inoculation

In fruits disease symptoms were obseved 5 days after inoculating 10µL of 1 x 106 spores/mL (Figure 14.3).

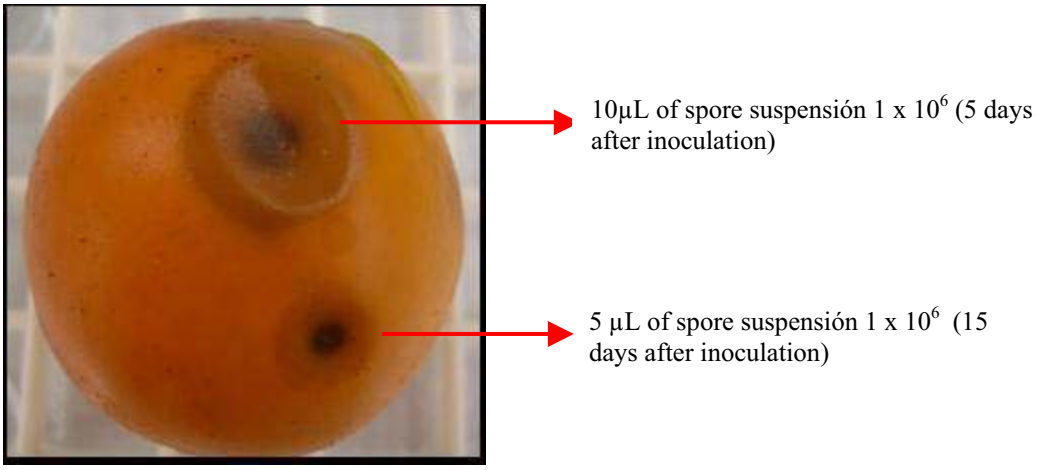


Figure 14.3. Disease symptoms on lulo fruits observed 5 days after inoculation

Phytophthora infestans*, causal agent of blight or drop: I*n fruits the disease appears first as irregular small lesions. These lesions rapidly enlarged and become necrotic. The infected fruits showed dark depressed areas that extend deep into internal tissue of the fruits.

Our results so far showed that we obtained 26 isolates of C. *gloeosporioides* and 2 of P. *infestans* (Table 14.1). Both P. *infestans* isolates were collected from the Montelindo Farm belonging to the University of Caldas and SENA in Manizales (Figure 14.4).

Figure 14.4. Disease symptoms caused by *Phytophthora spp*

Nematode resistance assessments. Solanum quitoense performance is serioulsy impaired by *Meloidogyne incognita*. This root nematode reduce plant productivity and lifespan, forcing growers to locate new plots to plant the next crop. Resistance to this nematode has been found in closely related *Solanum* species, and the interspecific hybrid, La Selva (*S, quitoense x S hirtum*) has higher tolerance to this nematode than accessions from Lulo Castilla (*S. quitoense*)
Pure nematode population was increased by inoculating tomatoes (*Solanum lycopersicum*) cv Rutgers. From propagated inoculum, lulo plants of S. *quitoense* (susceptible) and S. hirtum (tolerant) were inoculated to test the pathogenicity of the inoculum. Each lulo plant, grown in pots, was inoculated with 7000 eggs of M incognita. Evaluation of root nodules is pending.

Agronomic performance of elite clones: Tissue culture propagated plantlets were planted at Corpoica la Selva, experimental Station, located at 2120 masl. These plants are being used to characterize attributes of elite clones collected in different regions, against two test plants (*Solanum quitoense var. Septentrionale and Solanum quitoense var. quitoense*). Two plants of each accession were planted on Sept 5, 2006. Descriptors of those accessions were developed by Corpoica. Same materials would be planted in at least two other locations in Risaralda and Cauca to further test the performance of these materials in 2007.

15. Collection, characterization, and clonal multiplication of avocado (*Persea americana*) with emphasis on identification of lines tolerant to *Phytophthora spp*.

Alvaro Mejía Jimenez, Johanna Villamizar, Maria Luisa Orozco, Joe Tohme, Elizabeth Alvarez and Alonso González
(Collaborating institutions: Corpoica, J. Jaramillo, and Profrutales, Danilo Rios)

Sources of Funding: Colombian Ministry of Agriculture (MADR), and Asohofrucol (Colombia).

Abstract:

Avocado is becoming a very important subtropical fruit in the international market. From today's export market (excluding bananas), the most important especies is mango, followed by pineapple, papaya and avocados (4.8%, Faostats, 2004). Latin America and the Caribbean share 62% of the world avocado production. In 2004, Mexico was the main producer, followed by Indonesia and USA. Recently, plantings in Chile are entering production and this country could then become the world largest producer of avocado. However, avocado is a very important food in many countries, and fruits for local markets are mainly supplied by smallholder producers. The local markets in many developing countries is dominated by local landraces and not by the commercial varieties like Hass, Duke 7, etc. Small growers, and those pioneering by planting large areas are faced with a serious threat, the most limiting soil borne disease, *Phytophtora cinnamomi*, a pseudo-fungus that kill trees very rapidly after tree get infected. Currently, there are not known rootstocks for the tropics that can tolerate this disease and protect small or large growers from loosing their orchards. Commercial rootstocks are sold in international nurseries (like Duke 7) at prices that are not affordable for smallholders, and with unknown response to strains of *Phytophtora spp*. found in the tropics.

To address this problem, a project was funded jointly by the Colombian Ministry of Agriculture and the National Fund for Horticultural products (FNHF, managed by Asohofrucol). The project is to be implemented between 2006 to 2009. Here will describe the main aspects of the project and advances to this date.

Project Leader: CIAT, Tropical Fruits Project.
Partners: Corpoica (Colombia), Profrutales (local nursery, private sector).

Objective: To enhance the competitiviness of commercial avocado orchards through the use of commercial varieties grafted onto rootstocks tolerant to Phytophtora spp.

Main activities:

1) To collect germplasm with putative tolerance to *Phytophtora spp*. Germplasm will include *Persea americana* and other species of genus Persea that could be used as rootstocks. Collected material will be conserved in the avocado collection held by Corpoica.
2) Establish a broad collection of isolates of *Phytophtora* to be used to test tolerance of avocado germplasm.
3) Seek tolerance to *Phytophtora cinnamomi* in germplasm collected in Colombia and in imported commercial varieties.
4) Optimize methodologies for clonal propagation of avocado rootstocks.
5) To train commercial nurseries in clonal propagation of rootstocks.
6) Establish demonstration plots using commercial varieties grafted on cloned rootstocks tolerant to *Phytophtora cinnamomi.*

Progress to date.

Collection of vegetative material. Protocols for collection of avocado sticks that have high probability of success of being grafted onto rootstocks was established following protocols of the commercial nursery. This protocol is being used in the collection of avocado germplasm in Colombia from the following geographic regions and Departments within those regions :

a. Southwest: Valle del Cauca, Cauca, Nariño y Putumayo
b. Northwest: Risaralda, Caldas, Quindío, Antioquia
c. Cariben Coast:Cordoba, Bolívar, Magdalena
d. Northeast: Santanderes, Cesar, Meta
e. Center: Tolima y Huila

To this date 15 accessions have been collected from several municipalities, commercial farms where the disease have been observed. Collecte sticks and and fruits have ocurred from avocado tress and from closely related species (*Persea caerulia* (39 stics-30 fruits), *Ocotea caracasana* (10 sticks-3 fruits) and, *Beilschmiedia pendula* (5 sticks-6 fruits). Variable number of sticks and fruits collected is related to vigour of trees where material was collected.
Commercial farms visited for germplasm collection are selected based on the history of the farm. The local nursery participating in the project have records of trees sold, locations where farms were established and identified and unknown rootstocks delivered to the grower. We are targetting farms where disease ocurred in the past and we target surviving trees, when exist. Since many commercial avocado farms were planted on rootstocks originated from seeds collected in the local wet market (a practice that this project will aim to erradicate), high genetic variability is expected. However, to be able to recover the rootstocks from living trees, it is necessary to induce “chupones”or regrowth from the stock.
Induction of regrowth of avocado rootstocks: Currently, a methodology is being developed that minimizes damage to commercial trees (avoiding the need for detopping). Initial trials

trials show promising results; cutting the bark above the point of grafting and adding a paste containing several dose of Bencyl Amino Purine (BAP; 50 - 250 mg/l). The higher concentration of BAP resulted in 15% induction of shootsbelow the wounded and from the rootstock. Collected shoots were then micro-grafted on recently germinated seedlings, and the genotype was rescued. Further experiments will include higher dose of the BAP, beginning with 250 mg/l and applying the past to either a) roots, b) trunk by drilling little holes and injecting the paste (to avioid cutting the bark), or c) cutting the bark and applying the paste directly to the wound, as in the first trial.

Clonal propagation of the rootstock. Once the shoots or sticks of promising genotypes that could become rootstocks is collected, it has to be grafted onto existing seedlings. However, for the promising genotype become a rootstock, it has to develop roots. This procedure (Frohlich and Platt, 1970) is already in used in commercial nurseries (Method of root stock propagation William Henley Brokaw (http://www.google.com/patents?vid=USPAT4012866&id=UtErAAAAEBAJ&dq=brokaw+avocado+propagation), and we are adapting it as *in vitro* protocol. Developing of protocols for sterilizing avocado seeds is neccesary, because to be used as initial rootstocks are collected from local wet markets and come with all sort of problems. Our data indicate that treating seeds at 45C for 15 min results in good germination rates (80%) and eliminate the presence of fungi. Seeds take between 20 to 40 days to germinate. At the same time, protocols to introduce genotypes into tissue culture is ongoing. Some difficulties are observed with seeds from some genotypes (Hass, Booth 8) because show high levels of contamination when grown in vitro, compared to seeds from other genotypes.

Collection of Phytophtora strains. The objective of having a broad collection of Phytophtora is to test rootstocks againgsof isolates with different degree of pathogenecity and to be able to associate tolerance to particular strains. For this purpose, on similar sites (but sometimes on differentes dates) small roots of trees showing symptoms of the disease are collected to isolate the pathogen. A protocol for collecting, shipping and handling of the roots was established and it is currently used in the project. Samples arrived to the pathology lab were are processed to isolate the fungus and develop single spore colonies. This pseudo-fungus is rather difficult to grow in vitro and it requires “living traps”using apples or potatoes to isolate the pathogen from soils. When isolating the organism from root, roots are thinly sliced and placed on selective media PDA and CMA, which have the antibiotics rifampicin, penicillin, and ampicillin, and fungicides previously added. The tissue is maintained under darkness at room temperature (19∀C) for 3 to 4 days. Samples are examined for the presence of pseudo-fungus and treated to induce sporangia from which monosporic culturing is carried out to multiply and conserve the organism. To avoid loosing pathogenecity from subculting, small pieces of media are stored in 15% glycerol and bidestilled water at 10C . Mycelia is collected for DNA analysis. So far, 5 isolates of *Phytophtora* have been rescued from infected trees.

Establishment of Experimental Plots. At least two experimental plots where collected material, grafted plants and closely related species will be established. One plot will be established in CIAT and at least one more in Corpoica Experimental station. Land preparation, irrigation and propagation of collected material is an ongoing process. Plots in

CIAT will be planted with commercial rootstocks, commercial varieties grafted onto phytophtora tolerant stocks, progenie of Duke 7.