Activity 10. Designing specific primers for high-specificity detection of a phytoplasma associated with frogskin disease (FSD) of cassava.

Specific objective

To obtain high specificity in the technique and improve it for detecting phytoplasmas in cassava plants with symptoms of FSD, weeds, and potential insect vectors

Methodology

Sequencing and analyzing phytoplasma rDNA. We previously described obtaining complete sequences of DNA fragments through PCR from samples of two cassava varieties. They were reported to GenBank, which gave them accession numbers AY737646 and AY737647 (CIAT 2004). We conducted analyses of homology with these sequences against 24 sequences of the 16SrIII group and accessions of phytoplasmas representing at least 14 primary phytoplasma groups, using multiple alignments among the sequences (DNAMAN, version 5.2.2, Lynnon BioSoft). Specific differences in nucleotides were sought, seeking a series of bases that would be specific to the cassava phytoplasma. The homology of the sequences was calculated (in %) by taking the identical number of bases over the difference of aligned sequences and total size of gaps (in %). "Gap (%)" is the number of gaps of all sequences over the size of aligned sequences.

We used the option "Quick Alignment" to perform pairwise alignment with all sequences, using the method developed by Wilbur and Lipman (1983). With this method, DNAMAN aligns each pair of sequences, constructs a homology tree from the results of pairwise alignment, and finally builds up alignment based on the homology tree with the previously established alignment. This tree is set up with the distance matrix, using the UPGMA method (Sneath and Sokal 1973). The matrix can be built up only with Observed Divergence (this method uses directly unmatched residues divided by compared length between two sequences. No correction is applied to distances). After the tree is constructed, dynamic programming is finally used to optimize group alignment (Feng and Doolittle 1987; Thompson et al. 1994).

The phylogenetic analysis was constructed with the distance matrix, using the neighbor-joining method (Saitou and Nei 1987). Bootstrapping statistically shows typical variations (Felsenstein 1985). It involves creating a new data set by sampling randomly with replacements, so that the resulting data set has the same size as the original, but some characters have been left out and others are duplicated. The method assumes that the characters evolve independently. Phylogenetic analysis of the 16Sr RNA sequences was resolved, using the PAUP Software Program, version 3.1.

Results

Designing primers. The results of the phylogenetic and homology analyses show that the FSD phytoplasma clustered closely with other known X-disease (16SrIII) group strains, thus supporting its assignment to this group. We found multiple differences among the sequences of the FSD phytoplasma and the group 16SrIII phytoplasmas **(Figure 1)**, generating sufficient

information to design primers. The primers for the specific amplification of the phytoplasma associated with FSD were designed with the assistance of the program PRIMER 3.0 (www-genome.wi.mit.edu/cgi-bin/primer/primer3-www.cgi [2004]), taking into account certain criteria such as the contents of G + C and A + T, close to 50%, a minimum of nitrogenous bases, absence of extensive palindrome sequences within the primers, and that mating among their pairs was minimum. The primers obtained were synthesized by Integrated DNA Technologies, Inc.

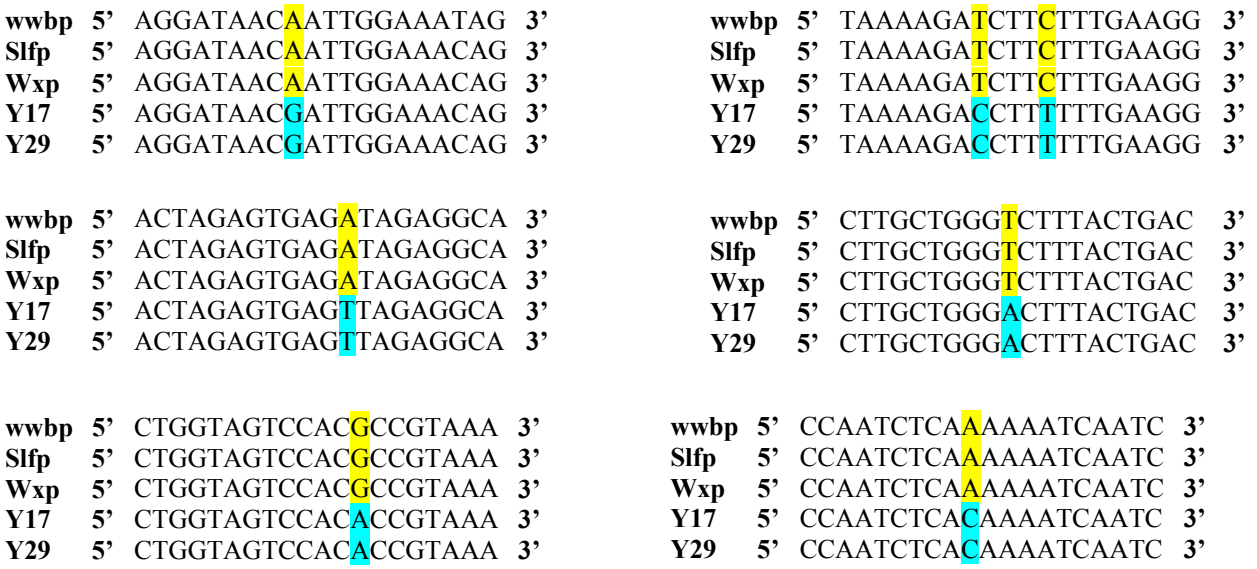

Figure 1. Some differences found in region 16Sr DNA between phytoplasmas of the 16SrIII groupand the cassava frogskin disease phytoplasma. Wwbp (Walnut witches'-broom phytoplasma), Slfp (Strawberry leafy fruit phytoplasma), Wxp (Western X phytoplasma), and Y17 indicate the cassava frogskin disease phytoplasma AY737646 (1260 bp). Y29 indicates the cassavafrogskin disease phytoplasma AY737647 (1298 bp).

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Acknowledgments

We thank the following people for their support: Tom Harrington and Joe Steimel of the Plant Pathology Department, and Gary Polking of the DNA Sequencing and Synthesis Facility, Iowa State University.

Activity 11. Detecting phytoplasmas by electron microscopy.

Specific objective

To detect, through electron microscopy, phytoplasma structures in tissues infected with FSD that was positive to nested PCR

Methodology

Tissues. Portions of roots exhibiting typical FSD symptoms were chosen from four different cassava genotypes. Typical symptoms are small longitudinal fissures distributed all over the root. On healing, these fissures develop "lips". The root portions for each variety comprised different cuts directed mainly at the phloem. Healthy plant samples were also processed to act as control **(Table 1)**.

Table 1. Processed samples of insects (*Scaphytopius marginelineatus*) and cassava plant tissues for detecting phytoplasmas by electron microscopy.

Tissue sample	Insect's developmental stage	Cassava genotype	Electron microscopy
Insect SE1	Adult	M Col 2063	In process
Insect Ss1[a]	Adult	M Col 2063	In process
Insect 383 (1)	Male nymph	M Bra 383	In process
Insect 383 (2)	Female nymph	M Bra 383	In process
Insect 383 (3)	Adult	M Bra 383	In process
Roots-petiols	-	CM 9582-64	+
Roots-petiols	-	CM 9582-65	+
Roots-petiols	-	CM 9582-24	+
Roots-petiols	-	M CR 81	+

a. Tissues in this sample were healthy, whereas tissues in the other samples were infected.

We also processed Homopteran insects (*S. marginelineatus*) collected from cassava crops infected with FSD and bred them in cages with different susceptible cassava genotypes that would show severe symptoms of the disease. Three individuals per developmental stage of the insect were taken as samples **(Table 1)** (CIAT Cassava Entomology, personal communication, 2004). The tissue fragments were cut into 1 × 2 mm pieces to be prefixed in 2%–3% glutaraldehyde (0.1 M phosphate buffer, pH 7.3). Complete insects were also fixed in the same buffer.

Electron microscopy. The samples for electron microscopy were prepared by making ultra-thin (60–90 nm) sections with a Reichert Ultracut S ultramicrotome (North Central Instruments, Plymouth, MN). After post-fixation and precontrasting in uranyl acetate, they were dehydrated in an acetone series 50, 70, 90 (15 min each) and 100% (15 min, three times), and were embedded in Spurr's resin. A previous 18-h infiltration with acetone-Spurr (1:1) was done to facilitate the entry of resin into the tissues. The ultra-thin sections were mounted on copper grills, and images taken, using a Megaview III digital camera system with SIS software (Soft Imaging System Corp., Lakewood, CO) on a JEOL 1200EX Woburn, MA scanning/transmission electron microscope (Japan Electron Optics Laboratory, Peabody, MA).

Results

In the previous studies, diverse tissues (stem, leaf midrib, petioles, and roots) were evaluated for numerous cassava plants, but only some could be compared with the results obtained for nested PCR. In this study, guided by the results of the nested PCR, root tissues of four cassava genotypes susceptible to FSD were first examined. These showed severe symptoms of the disease. Cells characteristic of phytoplasmas were detected in root phloem. The phytoplasma structures observed were pleomorphic, comprising round, elongate, dumbbell, and ring-shaped elements, mostly 150 to 250 nm wide and 1000 nm long **(Figure 1)**. The phytoplasma structures were limited only to phloem tubes and were never seen in large quantities (Andersen *et al.* 2001). The insect-tissue samples are still being processed.

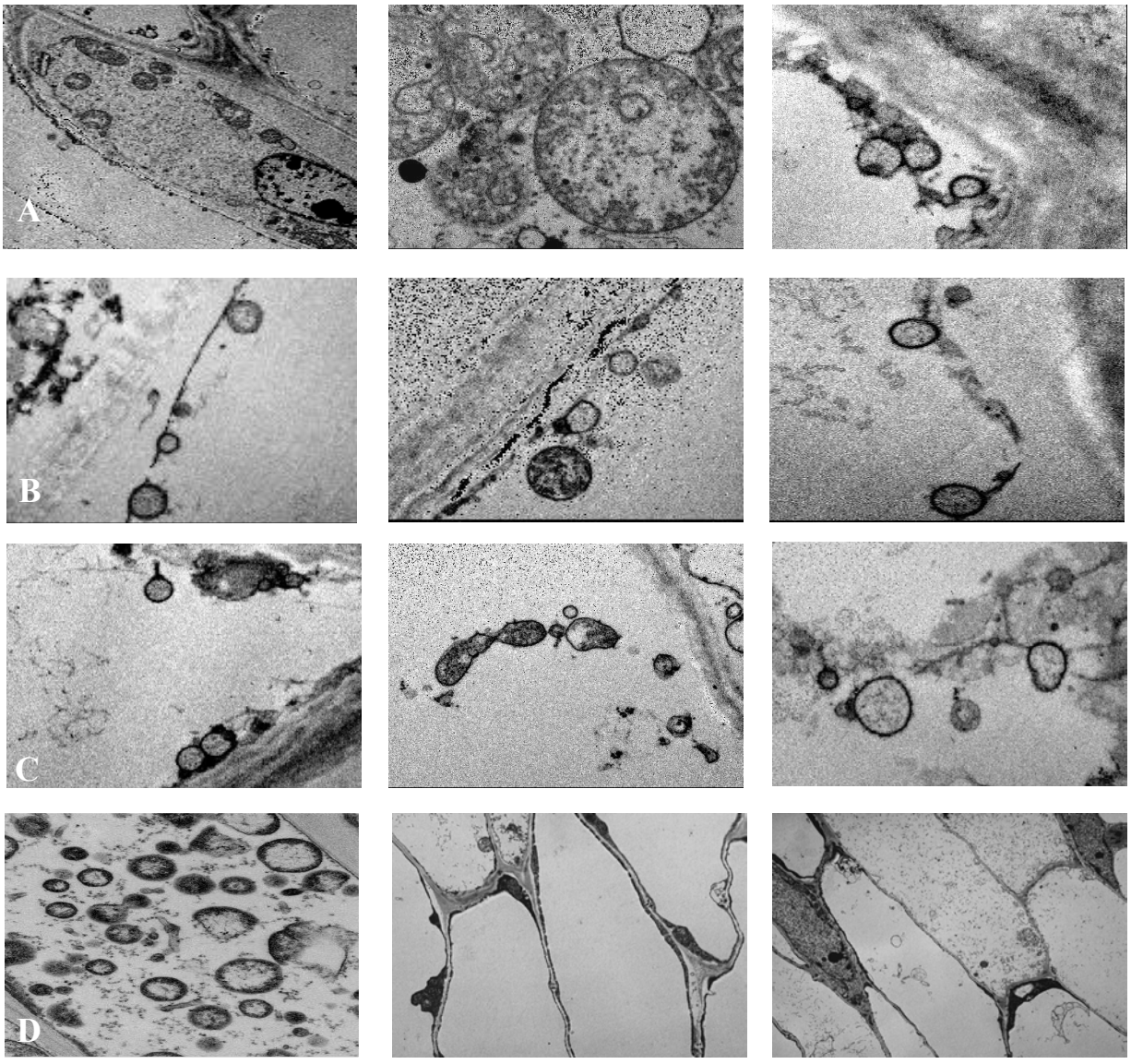

Figure 1. Micrographs, taken by cell transmission microscopy, of phytoplasmas FSD. (A) and (B) Infected cassava petiols. (C) Infected cassava roots and (D) Positive control (Periwinkle) and healthy cassava petiols. Photos (Alvarez, 2004).

Reference

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Acknowledgments

We thank the following people for their support: Tracey Pepper, Bessey Microscopy Facility, Iowa State University; and Bernardo Arias, Pilar Hernández, and Claudia Holguín, CIAT Cassava Entomology.

Activity 12. The detection and molecular characterization of a phytoplasma associated with *Machorreo* of Lulo (*Solanum quitoense*) in Colombia.

Objective

To detect presence of phytoplasmas in lulo attacked by *machorreo*.

Introduction

Phytoplasmas are microorganisms that lack cell walls. They were first observed under the electron microscope in 1967. Currently classified within the class Mollicutes, phytoplasmas are associated with plant diseases, causing more than 600 diseases in several hundreds of plant species, usually concentrating in phloem sieve tubes (Oshima et al. 2001). The affected plants show yellowing or reddening of leaves, reduced leaf size, stunting with shortened internodes, and loss of apical dominance. This leads to poor development of the producing plant, proliferation of shoots and roots, witches' broom, necrosis of phloem tissues, branch death in woody plants, reduced production, plant decline, and, occasionally, death. Several symptoms unique to diseases related to phytoplasmas involve flowers: virescence (green coloring in flowers), phyllody (conversion of petals and sepals into leaves), and flower sterility causing floral abortion (Agrios 1997; Oshima et al. 2001).

Phytoplasmas are commonly transmitted between plants by homopterous insects. The microorganism first multiplies in the intestinal cells of their insect vectors and subsequently in the hemolymph after passing through the salivary glands. They infect internal organs such as the thoracic ganglion and fatty bodies (lipids) (Kawakita et al. 2000).

These Mollicutes are detected through electron-microscopy, using both light and immunofluorescence with 4', 6-diamidino-2-phenylindole (DAPI). These techniques are limited by the concentration of the microorganism used (Ahrens and Seemüller 1992). Recently, the polymerase chain reaction (PCR) method was used to detect phytoplasmas by amplifying, with universal and specific primers located in the 16S rDNA region, the intergenic spacer (IS) and the 23S rDNA of a given phytoplasma's genome (Ahrens and Seemüller 1992). Sequence analysis and restriction fragment length polymorphisms (RFLPs) (Lee et al. 1993) are also used to determine and classify phytoplasmas.

Most authors who use PCR to identify phytoplasmas also use, as positive control, *Catharanthus roseus* (syn. *Vinca rosea*; also periwinkle). This indicator plant is highly susceptible to infection by phytoplasmas from different crops. It enables the conservation of live phytoplasmas isolated from different species of affected plants (Ahrens and Seemuller 1992; Davis and Lee 1982; Deng and Hiruki 1991; Firrao et al. 1993; Prince et al. 1993).

Lulo or naranjilla (*Solanum quitoense*) is a fruit with commercial potential for food processing in Colombia. It is attacked by a disease known as *machorreo*, the causal agent of which has yet to be determined. The disease is a serious constraint to lulo production in the country, with reports of production having been reduced by as much as 70%. The disease characteristically stunts the plant and causes floral abortion, which symptoms suggest the presence of a phytoplasma.

For our study, to ascertain the presence of phytoplasmas in lulo attacked by *machorreo*, we used molecular techniques, particularly those based on PCR, RFLPs, and sequencing of the 16S rDNA region, as being the most likely to detect this type of microorganism in plant crops.

Materials and Methods

Tissue samples

Lulo plants showing stunting, phyllody, and floral abortion were collected from the municipalities of Manizales and Dosquebradas in the Departments of Caldas and Risaralda, respectively **(Table 1)**. In vitro lulo plants were included as negative controls. Positive controls were samples of *C. roseus* of the type 'besito' or 'vinca' that were clearly stunted or showed reddening in their terminal buds—typical symptoms of phytoplasma infection. Also used was DNA from the coffee crispiness phytoplasma of the X-disease group (NCBI's GenBank accession no. AY525125), provided by the Colombian Centro Nacional de Investigaciones de Café (*National Coffee Research Center*; CENICAFE).

Table 1. List of lulo (*Solanum quitoense*) materials used to study the possibility of phytoplasmas being the causal agent of the Colombian phylody and virescense disease *machorreo*.

No. of Samples[a]	Variety	Department[b]	Municipality	Village District	Positive samples from PCR per processed sample (%)
3	Lulo hybrid 'La Selva'	Caldas	Manizales	La Trinidad	100
3	Lulo hybrid 'La Selva'	Caldas	Manizales	Alto Bonito	100
3	Lulo hybrid 'La Selva'	Risaralda	Dosquebradas	Chaquiro	100
1	–	Valle del Cauca	El Cerrito	Los Cuchos	100
1	Lulo 'Castilla'	Valle del Cauca	Buga	El Janeiro	100

a. Total number of samples received from each farm.
b. An administrative and political division of Colombia.

Transmission by grafting

We conducted tests for transmission by grafting, using infected lulo plants showing symptoms of phyllody, viresecence, and flower abortion. The plants were collected from the municipalities of Manizales (Caldas), Dosquebradas (Risaralda), and El Cerrito and Buga (Valle del Cauca). We grafted buds and petioles from both diseased and healthy lulo plants onto plants of *C. roseus*. The grafted plants were then kept in the greenhouses at ICA's Palmira Experiment Station until symptoms appeared.

DNA extraction

DNA was extracted according to the protocol of Gilbertson and Dellaporta, 1983. Tissues from leaf veins, stems, and petioles, which had been conserved at -80°C, were macerated with liquid nitrogen, using a porcelain mortar. About 0.4 g of the pulverized tissue were mixed with 0.51 mL of extraction buffer (50 mM EDTA, pH 8.0; 500 mM NaCl; and 10 mM 2-mercaptoethanol) and agitated for 2 min at speed 8 in a blender (Vortex-Genie 2, model G560, Scientific Industries, Bohemia, NY). Then 90 µL of SDS at 10% were added and the whole agitated for 2

min before being incubated at 65°C for 10 min. Subsequently, 150 µL of potassium acetate at 5 M, pH 5.5, were added and the whole agitated for 2 min. The mixture was centrifuged at 14,000 rpm for 10 min and the supernatant (about 600 µL) collected. Then, 0.5 volumes of isopropanol at 100% (300 µL) were added and left to precipitate at -20°C for 30 min. The whole was then centrifuged at 14,000 rpm for 10 min, the supernatant eliminated, and the pellet washed with 500 µL of ethanol at 70%, centrifuging at 10,000 rpm for 5 min. Finally, the supernatant was eliminated and the pellet re-suspended in TE at 30 and 50 µL and 50°C. It was then incubated overnight with 2 µL of RNase A (10 mg/mL) at 4°C.

Detection by PCR

Seven primer pairs were used for amplification of the 16S rRNA gene, 23S rRNA gene, rp genes, and 16S/23S spacer region, to detect phytoplasmas in lulo **(Table 2)**. The locations of these primers are shown in **Figure 1**.

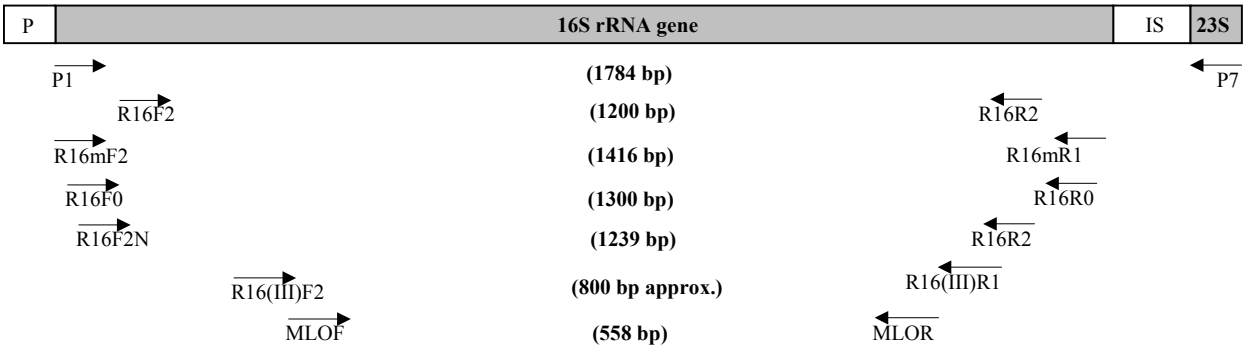

Figure 1. Location of primers in the rRNA operon. P = promoter; IS = intergenic spacer; 23S = 23S rRNA gene (From Guo et al. 2000; Heinrich et al. 2001).

Table 2. Primers used for PCR amplification and sequencing of genes 16S and 23S rRNA from phytoplasma-infected plants of lulo (*Solanum quitoense*).

Primers[a]	Sequence (5' → 3')	Amplified region[b]
[(C)]R16F2/[(E)]R16R2	ACG ACT GCT GCT AAG ACT GG TGA CGG GCG GTG TGT ACA CCC G	***16S (universal)***
[(A)]R16F0/R16R0	CTGGCTCAGGATTAACGCTGGCGGC GGATACCTTGTTACGACTTAACCCC	16S (universal)
[(B)]R16mF2/R16mR1	CAT GCA AGT CGA ACG GA CTT AAC CCC AAT CAT CGA C	16S
[(D)]R16F2N	GAA ACG GCG GTG TGT ACA AAC CCC G	16S
[(B)]P1/[(F)]P7	AAG AGT TTG ATC CTG GCT CAG GAT T CGT CCT TCA TCG GCT CTT	16S 23S
[(B)]LD16-1/23S	CGG AAA ACC TTC GGG TTT TAG TCT TTT CCT GCG GTT ACT TAG AT	16S 23S
[(D)]P4	GAA GTC TGC AAC TCG ACT TC	16S
[(D)]R16(III)F2/R16(III)R1	AAGAGTGGAAAAACTCCC TCCGAACTGAGATTGA	16S (X-disease group)
[(A)]MLOF/MLOR	ACGAAAGCGTGGGGAGCAAA GAAGTCGAGTTGCAGACTTC	16S

a. (A) Primers used in direct PCR; (B) primers used in nested PCR for the first cycle; (C) primer used in direct and nested PCR for the first cycle; (D) primers used in nested PCR for the second cycle; (E) primer used in direct PCR, and nested PCR for the first and second cycles; (F) primer used in nested PCR for the first and second cycles.

b. Location of primer within rRNA operon, that is, within each of the genes 16S rRNA and 23S rRNA.

Samples of DNA from healthy and diseased lulo and the controls *C. roseus* and coffee were evaluated with direct and nested PCR based on dilutions made at 20 ng/µL. For direct and nested amplification in the first cycle, we used primers that had amplified a broad region of the 16S rRNA and 23S rRNA genes **(Table 2)**. Conditions were as follows: 100 ng DNA, 1X buffer, 3 mM $MgCl_2$, 0.8 mM dNTPs, 0.1 µM of each primer, and 1 U *Taq* polymerase.

For the primers R16F0/R16R0 and R16F2/R16R2, we carried out 35 cycles in a MJR PTC-100 thermocycler (MJ Research), using the following conditions: 1 min of denaturation (2 min for the first cycle) at 94°C, annealing for 2 min at 50°C, and primer extension for 3 min (10 min in the final cycle) at 72°C.

For the primers LD16-1/23S, we used 29 cycles, reducing annealing and extension time by 1 min.

For the P1/P7 primers, we carried out 35 cycles: denaturation for 30 s (90 s for the first cycle) at 94°C, annealing for 50 s at 55°C, and primer extension for 80 s (10 min in the final cycle) at 72°C. We used the same conditions for the primers R16mF2/R16mR1 (Gundersen and Lee 1996; Schneider et al. 1995), but carried out 28 cycles.

For the MLOF/MLOR primers, we used 24 cycles: denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and primer extension for 30 s (4 min in the final cycle) at 72°C.

For the nested PCR, the amplified product of the first pair of primers was diluted to 1:50 with sterilized distilled water to use it as DNA mold, in quantities of 1 µL **(Table 3)**. The four pairs of the second-cycle primers were evaluated under the same conditions as for the first-cycle primers, except for R16F2N/R16R2 for which the annealing temperature was lowered to 50°C.

Table 3. Primers used in nested PCR, conducted to detect the presence of phytoplasmas in lulo attacked by the phylody and virescense disease *machorreo*.

First Cycle	Second Cycle	Approx. Fragment Size (Base Pairs)
P1/P7	R16F2N/R16R2	1200
R16mF2/R16mR1	R16F2N/R16R2	1200
LD16-1/23S	P4/P7	550
R16F2/R16R2	R16(III)F2/R16(III)R1	800

Restriction fragment length polymorphisms (RFLPs)

To classify the phytoplasma in terms of the 15 groups so far reported, we amplified—using nested PCR, with the universal primers P1/P7 and R16F2N/R16R2, and the specific primers for the 16Sr III group R16F2/R16R2 and R16(III)F2/R16(III)R1—the sequence of gene 16Sr RNA (1.2 kb and 0.8 kb). The nested PCR product of universal and specific primers was then analyzed by digestion with restriction enzymes *Rsa*I, *Alu*I and *Mse*I (Invitrogen Life Technologies, Carlsbad, CA). The enzyme *Taq* I was evaluated only with the PCR product obtained with specific primers. , as controls, phytoplasmas from the 16Sr I group (aster yellows, as represented by palm phytoplasma), 16Sr III group (X-disease, as represented by coffee phytoplasma), and 16Sr IX group (pigeonpea witches' broom, as represented by phytoplasma from periwinkle), were used.

15 µL of the PCR product and added 2 µL of 10X buffer enzyme and 1 µL of the restriction enzyme (500 units/µL), were taken. This mixture was incubated for 16 h at 37°C (except for enzyme *Taq*I, which was incubated at 65°C). We then added 3 µL loading buffer (bromophenol blue at 0.25%, glycerol in water at 30%) and ran it in acrylamide gel at 5% for 1 h at 100 V, 24 mA, in TBE 1X buffer and stained with ethidium bromide at 10 mg/mL. The PCR product obtained with the specific primers for the X-disease group, was also digested.

Sequence analysis

The product amplified from direct and nested PCR was purified, following the protocol described for the QIAquick PCR Purification Kit, and sequenced. Another analysis was carried out with cloned DNA. The purified PCR products were ligated to the vector (pGEM®-T Easy Vector). Competent cells were transformed by electroporation and planted in selective culture medium (LB agar, Xgal, IPTG, and ampicillin). Colonies presenting white coloring were selected. The plasmid's DNA was purified for the clones that had inserts of the expected size. These clones were sequenced with primers T7 and Sp6, using the BigDye Terminator Kit (Applied Biosystems) in an ABI PRISM® 377 DNA Sequencer. Sequence analysis was done with the programs Sequencher 4.1 and DNAMAN 4.13. Homology was sought in the NCBI's GenBank (www.ncbi.nlm.nih.gov), using the tool BLAST®n.

Results

DNA extraction

The method of extracting nucleic acids showed satisfactory results for detecting phytoplasmas. High quality and concentration (between 300 and 600 ng/µL) were obtained, sufficient to conduct molecular tests. The highest yields were observed when extraction was based on a mixture of leaf-petiole and leaf-stem tissues. On visualization in agar gel at 0.8%, a strong band was observed in all the samples examined, applying 50 V consistently over 1 h.

Transmission by grafting

Results of the tests for transmission by grafting indicated that the disease is contagious. The C. roseus cv. Periwinkle plants, grafted with buds and petioles from diseased lulo plants, showed yellowing of leaves, noticeably reduced leaf size, and diminished plant development. These symptoms are similar to those described for diseases caused by phytoplasmas (Agrios 1977; Oshima et al. 2001). The Periwinkle plants grafted with tissues from healthy lulo plants had no symptoms.

Detection by PCR

To conduct the PCR, we had to carry out nested PCR, as one PCR only was insufficient to detect this phytoplasma, given its specificity (many bands) and low sensitivity **(Table 4)**. Primers that gave the best results were P1/P7–R16F2N/R16R2, R16mF2/R16mR1–R16F2N/R16R2, LD16-1/23S–P4/P7 as universal, and R16F2/R16R2–R16(III)F2/R16(III)R1 as specific **(Table 3)**. These last primers were specific to all the phytoplasmas of the 16Sr III group (X-disease). They detected with greater sensitivity and specificity several positive samples than did the universal pairs. The expected sizes in base pairs were obtained for each primer in the lulo samples and the periwinkle and coffee controls **(Figure 2)**.

Table 4. Identifying phytoplasmas in samples of lulo (*Solanum quitoense*) with symptoms of the phylody and virescense disease *machorreo*, using universal and specific primers.

Primers	Positive Samples per Symptomatic Sample (%)	Reproducibility (%)
Nested PCR (P1/P7 and R16F2N/R16R2)	100	75
Nested PCR (R16mF2/R16mR1 and R16F2N/R16R2)	100	75
Nested PCR (LD16-1/23S and P4/P7)	100	80
Nested PCR (R16F2/R16R2 and R16(III)F2/R16(III)R1)	100	100
MLOF/MLOR	Amplification of healthy samples	
R16F2/R16R2	Non-specific	
R16F0/R16R0	Non-specific	

The primers of the X-disease group amplified only for the phytoplasmas obtained from lulo and the coffee control. The periwinkle controls did not amplify for this region, suggesting an approximation to the group to which the lulo phytoplasma would belong **(Figure 2D)**.

Phytoplasmas are found exclusively in phloem vessels. Normally, they have a heterogeneous distribution in the plant, and are found in low concentrations. These characteristics make their detection and identification difficult (Seemüller et al. 1998). Hence, specific primers are needed to increase sensitivity for detecting the pathogen in plants with very low levels of inoculum (i.e., with initial or intermediate symptoms).

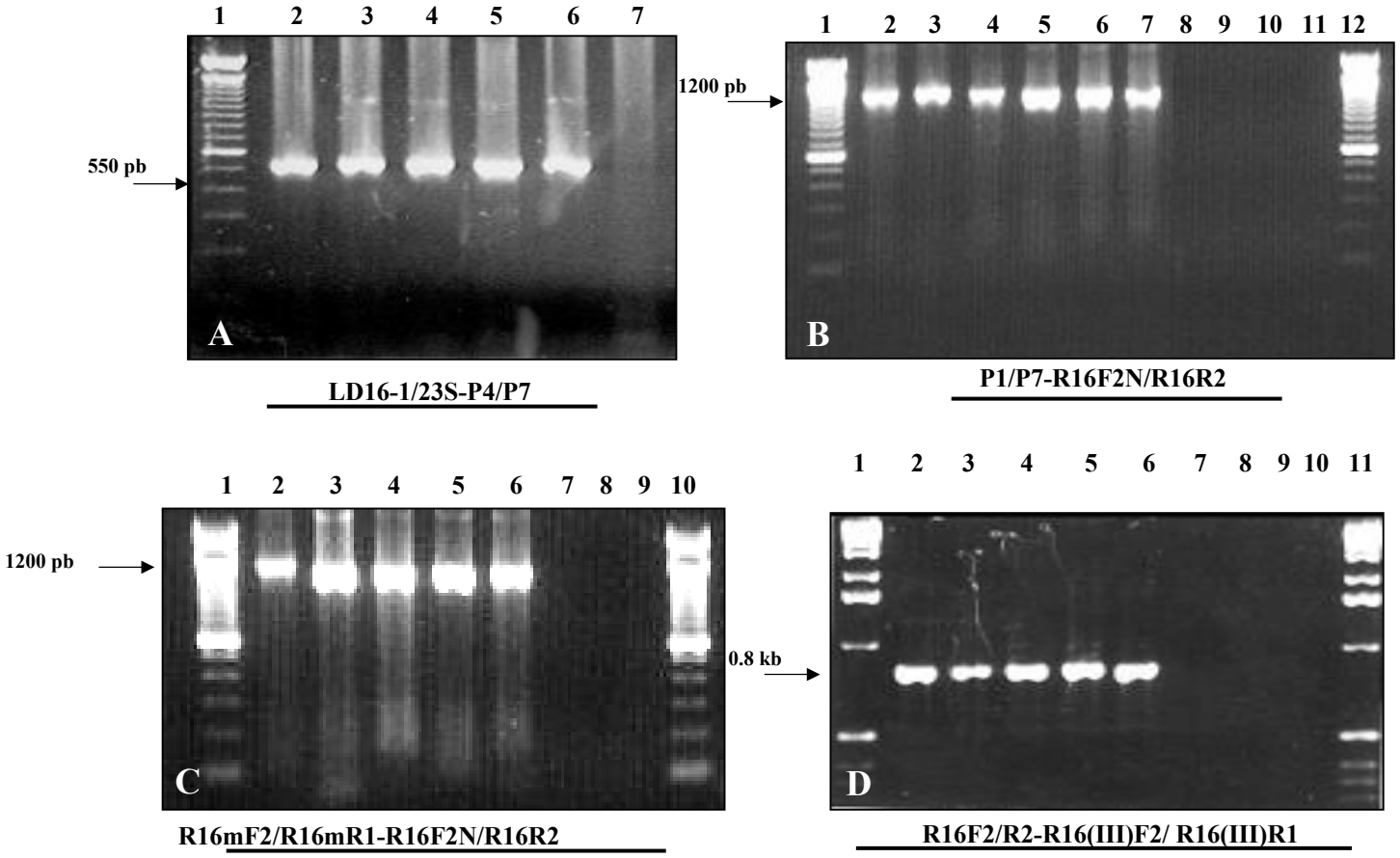

Figure 2. **Amplifications with nested PCR, using universal and specific primers, for detecting phytoplasmas. (A) Lane 1 = 100-bp marker; lanes 2–4 = lulo phytoplasma; lane 5 = phytoplasma from periwinkle; lane 6 = phytoplasma from coffee; lane 7 = healthy lulo plant. (B) Lanes 2–5 = lulo phytoplasma; lane 6 = phytoplasma from periwinkle; lane 7 = phytoplasma from coffee; lane 8 = healthy lulo plant; lane 9 = negative control. (C) Lanes 2–4 = lulo phytoplasma; lane 5 = phytoplasma from periwinkle; lane 6 = phytoplasma from coffee; lane 7 = healthy lulo plant; lane 8 = negative control. (D) Lanes 1 and 11 = 1-kb marker; lanes 2–5 = lulo phytoplasma sampled from different village districts, Colombia: lane 2 = La Trinidad, lane 3 = Alto Bonito, lane 4 = Chaquiro, and lane 5 = Los Cuchos; lane 6 = phytoplasma from coffee; lane 7 = healthy lulo plant; lane 8 = phytoplasma from periwinkle; lane 9 = negative control.**

Restriction fragment length polymorphisms (RFLPs)

The positive samples were amplified with primers R16F2N/R16R2 and digested with the four enzymes. Their band patterns were compared with phytoplasmas of the groups 16Sr I (aster yellows, as represented by palm phytoplasma), 16Sr III (X-disease, as represented by phytoplasma from coffee), and 16Sr IX (pigeonpea witches' broom, as represented by

phytoplasma from periwinkle) **(Figure 3)**. The lulo phytoplasma was placed in the 16Sr III group (Lee et al. 1993). For each enzyme evaluated, the band patterns were different, clearly differentiating among the phytoplasma groups. The amplified products were digested, using specific primers for the 16Sr III group, and compared with the phytoplasmas from coffee and cassava (*Manihot esculenta* Crantz) (Alvarez et al. 2003) **(Figure 4)**. The same band pattern was observed for all isolates, suggesting the presence of only one phytoplasma in the set of samples evaluated.

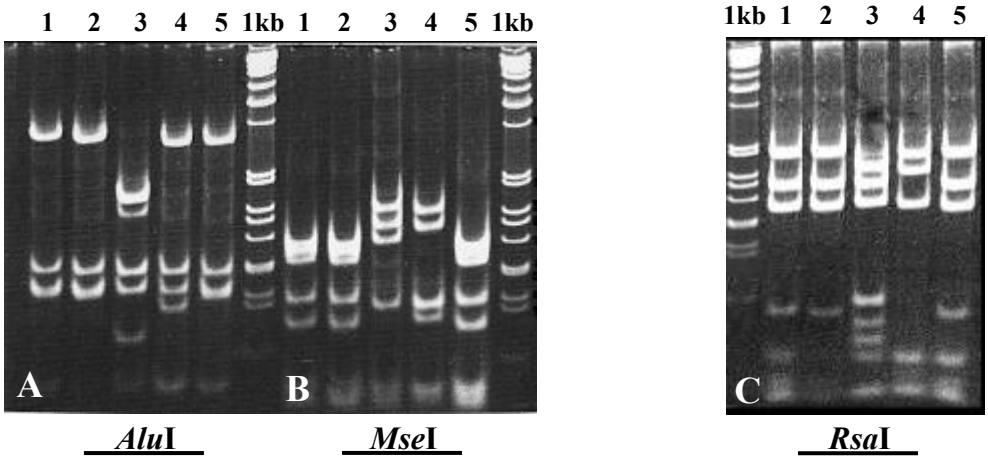

Figure 3. Restriction fragment length polymorphisms (RFLPs) compared across three restriction enzymes (*Alu*I, *Mse*I, and *Rsa*I) for the fragments amplified by nested PCR with primers R16F2N/R16R2. Lanes 1 and 2 = lulo phytoplasma; lane 3 = 16Sr I group (palm phytoplasma); lane 4 = 16Sr IX group (phytoplasma from periwinkle); lane 5 = 16Sr III group (phytoplasma from coffee).

Sequence analysis

The determination and comparison of the phytoplasma sequence in the 16S rRNA region involved about 1050 nucleotides of the sequence of each PCR fragment determined by direct sequencing and cloning, using the primers R16F2N/R16R2. The sequenced samples were from leaf-petiole and leaf-stem tissues. An extensive analysis was performed, based on the results of the sequencing (maximum pairing and higher percentages of homology), using the tool BLAST®n in GenBank.

The phytoplasma analyzed in this study presented high levels (97%) of homology with the sequences of the 16Sr III group (X-disease) **(Table 5)**. The annealing of the computer-assisted sequences indicated the sequence of the nucleotides from the 16S rRNA region of the lulo phytoplasma was very similar to that of 16S rRNA of the GenBank accessions AF147706, AF510724, and AY034090. The lulo phytoplasma differed from the other phytoplasmas of the 16Sr III group by its nitrogen bases varying in the position of 4 cytosines and 1 thymine throughout the sequence of 16Sr RNA.

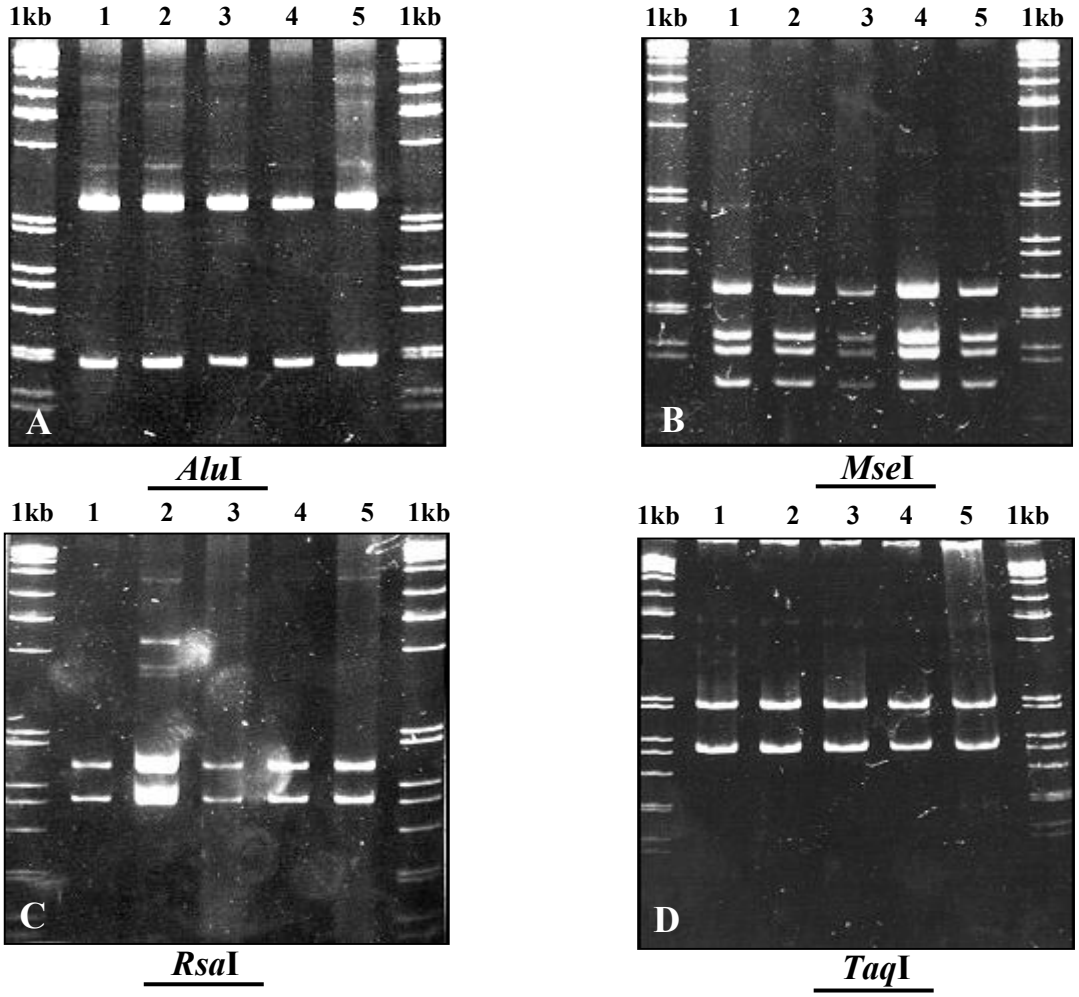

Figure 4. **Restriction fragment length polymorphisms (RFLPs) compared across four restriction enzymes (*Alu*I, *Mse*I, *Rsa*I, and *Taq*I) for fragments amplified by nested PCR with primers R16(III)F2/R16(III)R1. Lanes 1–3 = lulo phytoplasma; lane 4 = cassava phytoplasma; lane 5 = 16Sr III group (phytoplasma from coffee).**

Table 5. The degree of homology found between sequences of phytoplasma DNA obtained, through nested PCR, from leaf-petiole and leaf-stem tissues of lulo (*Solanum quitoense*) attacked by the phylody and virescense disease *machorreo* and sequences of phytoplasmas reported in NCBI's GenBank.

Pairing with GenBank[a]	GenBank Code	Probability of Favorable Homology[b]	Homologized Bases[c]	Homology (%)
Chayote witches'-broom phytoplasma (ChWBIII), strain ChWBIII(Ch10), 16S ribosomal RNA gene; 16S-23S ribosomal RNA intergenic (1813)	AF147706	0.0	1004/1031	97
Milkweed yellows phytoplasma 16S ribosomal RNA gene, partial sequence; 16S-23S intergenic spacer region and tRNA-Ile gene, complete sequence; and 23S ribosomal RNA gene, partial sequence (1812)	AF510724	0.0	1003/1031	97
Blueberry proliferation phytoplasma 16S ribosomal RNA gene, partial sequence; tRNA-Ile gene, complete sequence; and 23S ribosomal RNA gene, partial sequence (1770)	AY034090	0.0	1002/1031	97

a. Values in italics refer to the total number of bases reported in GenBank.
b. The value 0 is expected for high percentages of homology.
c. Number of homologized bases in different regions of the sequence reported in GenBank.

When several accessions of the 15 groups of phytoplasmas reported in the GenBank were homologized with the lulo sequences, using the DNAMAN 4.13 program (Homology Tree), the high homology already found with the 16Sr III group **(Figure 5)** was confirmed.

Conclusions

We detected the presence of a phytoplasma in association with *machorreo* in lulo, using phylogenetic analysis and homology with the sequence of the 16S rRNA region and the gene tRNA. Using the following techniques, we identified the phytoplasma as belonging to the 16Sr III group (X-disease; 97% homology): nested PCR to obtain, with universal primers, amplifications of fragments measuring about 1.2 kb and, with specific primers, fragments measuring 800 bp in plants affected by *machorreo*; restriction enzymes and partial sequencing of the phytoplasma's DNA to obtain cloned fragments; amplification of these with primers SP6 and T7, using pGEM®-T Easy Vector and PCR; and direct sequencing with internal primers.

For future studies, we recommend identifying possible vectors through samplings of crop insects, particularly of the order Homoptera. Attempts should be made to reproduce symptoms of the disease in healthy plants of lulo to confirm the insects' association with the disease. Through molecular techniques, crop weeds should also be evaluated for their potential as hosts to the phytoplasma. Finally, as a possible management practice, resistance of promising lulo hybrids to *machorreo* should also be evaluated.

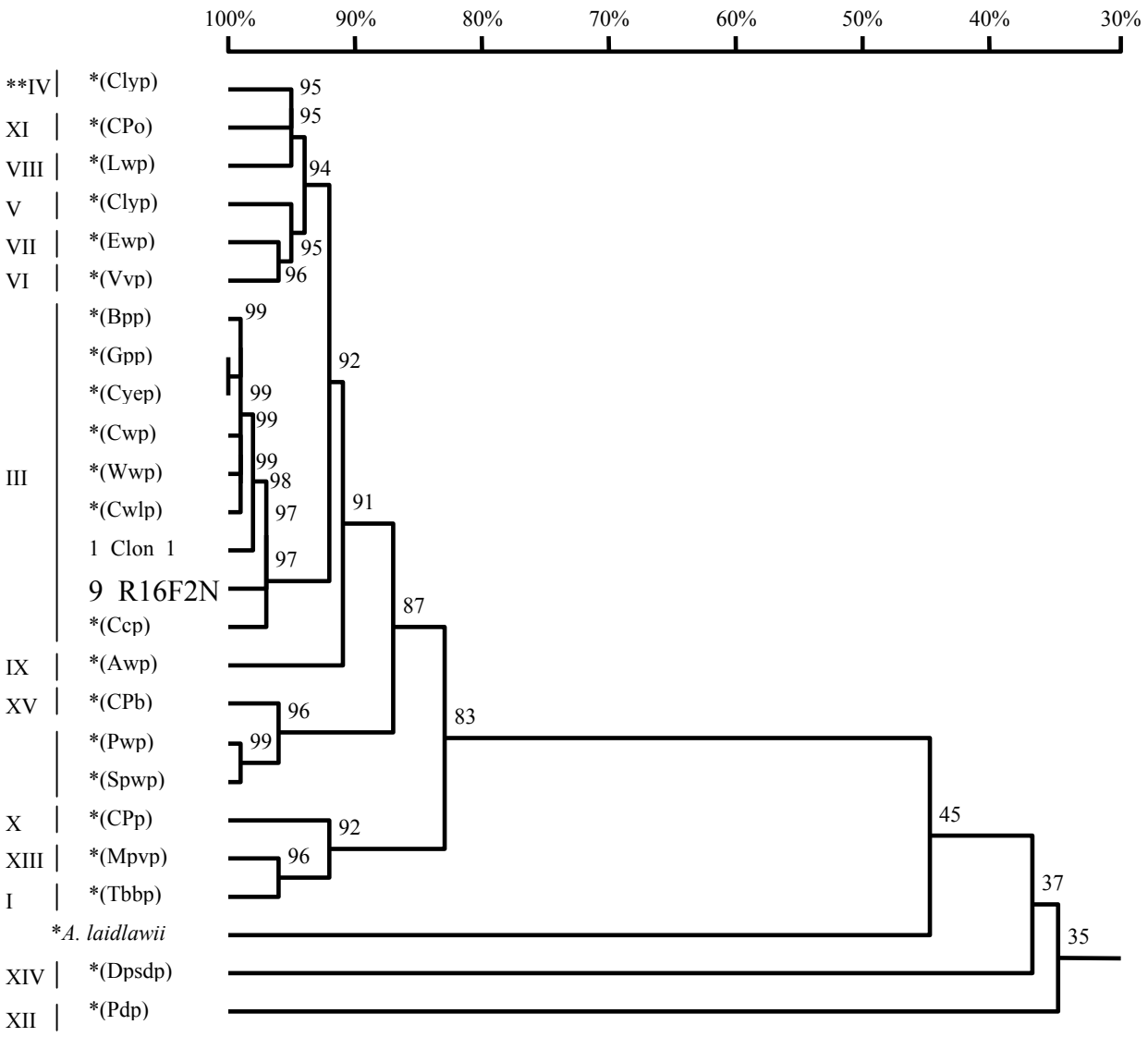

Figure 5. 16S rRNA homology tree of sequences of 22 phytoplasmas and *Acholeplasma laidlawii* (*A. laidlawii*) belonging to the same family of phytoplasmas. It includes the sequences of fragments of direct PCR (9_R16F2N) and one of the cloned fragments (1_Clon_1) obtained from lulo. * = GenBank accessions; ** = phytoplasma group number.

Clyp = coconut lethal yellowing phytoplasma
CPo = *Candidatus* Phytoplasma oryzae
Lwp = loofah witches'-broom phytoplasma
Clyp = cherry lethal yellows phytoplasma
Ewp = Erigeron witches'-broom phytoplasma
Vvp = periwinkle virescence phytoplasma
Bpp = blueberry proliferation phytoplasma
Gpp = Gaillardia phyllody phytoplasma
Cyep = clover yellow-edge phytoplasma
Cwp = chayote witches'-broom phytoplasma
Wwp = walnut witches'-broom phytoplasma
Cwlp = Cirsium white-leaf phytoplasma
[a]Ccp = coffee crispiness phytoplasma
Awp = almond witches'-broom phytoplasma
CPb = *Candidatus* Phytoplasma brasiliense
Pwp = peanut witches'-broom phytoplasma
Spwp = sweetpotato witches'-broom phytoplasma
CPp = *Candidatus* Phytoplasma prunorum
Mpvp = Mexican periwinkle virescence phytoplasma
Tbbp = tomato big-bud phytoplasma
Dpsdp = date-palm slow-decline phytoplasma
Pdp = papaya dieback phytoplasma

a. Check used in this study.

Acknowledgements

We express our sincere thanks to Carlos Huertas, Francy Varón (Instituto Colombiano Agropecuario, ICA), Carlos Fergusson, Plant Disease Prevention and Eradication Group, Risaralda Branch; the farmers Javier Sarria, Department of Valle del Cauca, Jorge Hernán Rivas and Graciela de León, Department of Caldas, for the samples they sent; and Thomas Harrington and Gary Polking, University of Iowa (Ames), for their valuable help in obtaining primers and their advice on cloning and DNA sequencing.

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Activity 13. Evaluating the effects of various control practices on the incidence and severity of *Phytophthora* root rots under field conditions in Quindío, Colombia.

Objective
Reduce cassava root rot caused by *Phytophthora* by ecological practices.

Methodology
Different control practices for *Phytophthora* spp. were evaluated for disease incidence and severity, and for yield in a field trial conducted at "La Elena" Farm, Municipality of Montenegro, Department of Quindío. The trial was planted with the local variety Chiroza (M Col 2066). Treatments were as follows:

1. An integration of the following practices:
   a. Selection of high-quality stem cuttings, including for root yield per plant harvested.
   b. Thermotherapy: planting stakes were immersed for 49 min in water heated to 49°C over a wood fire.
   c. Biological control: strain 14 PDA-4 of *Trichoderma* spp. was used to make a suspension of 1×10^4 conidia/mL. Planting stakes were then inoculated by immersing them in the suspension for 10 min. The suspension was also applied to the soil in which the stakes were planted, using 100 mL/plant.
2. Traditional farmer's practice, including chemical control. Planting stakes were immersed for 5 min in a mixture of Orthocide® (captan) at 4 g/L and Ridomil® at 3 g/L of water.

All plots were fertilized 45 days after planting, using 500 kg/ha of the fertilizer mix Nitrax®, DAP, and KCl, applied at a rate of 1:2:2. The plots were planted according to a randomized complete block experimental design with six replications and 150 plants per treatment.

Results

The commercial variety Chiroza is highly susceptible to Phytophthora root rot. The disease affected cassava development, as only 82.7% of the plants (all treatments) were harvested **(Table 1)**. This percentage is relatively low for this region. In this trial, seed selection, heat treatment of stakes, and immersion of stakes in a suspension of conidia of the fungus *Trichoderma* and its application to the soil did not affect germination nor plant development, compared with the plants under farmer agronomic management, which involved using fungicides.

Table 1. The effects on yield of two different control strategies to manage root rots in cassava, Montenegro, Department of Quindío, Colombia. The local variety Chiroza was used and evaluated 12 months after planting.

Control strategy	Plants harvested	Roots harvested (t/ha)	Yield of Healthy Roots (t/ha), Type: Commercial	Non-com.	Com. + non-com.	Commercial and non-commercial roots affected by root rots (t/h)	(%)	Proximal	Distal
Integrated mgt based on ecological practices[a]	80%	26.3	15.1	4.3	19.4	6.9	26.2	14.1	44.9
Traditional farmer management[b]	85%	23.1	10.6	4.0	14.6	8.5	36.8	14.6	57.3
Average	82.7%	24.7	12.9	4.1	17.0	7.7	31.2	14.3	51.1

a. Planting stakes were selected for their quality. They were immersed for 49 min in water heated to 49°C over a wood fire. Strain 14 PDA-4 of the fungus *Trichoderma* sp., which attacks root-rot fungi (*Phytophthora* spp.), was used to make a suspension of 1×10^6 conidia/mL. The stakes were then inoculated by immersing them in the suspension for 10 min. The suspension was also applied to the soil near the base of each stake planted.

b. Planting stakes were immersed for 5 min in a mixture of Orthocide® (captan) at 4 g/L and Ridomil® at 3 g/L of water.

The package of ecological practices increased yield of commercial roots by 4.5 t/ha (42.5%), compared with conventional farmer practices.

From the plots under farmer agronomic management, 8.5 t/ha of roots with rot was obtained, as against 6.9 t/ha (i.e., 18.8% less) from the plots under ecological management. Of the harvest in each treatment, 36.8% of roots suffered from rot under traditional farmer management, whereas only 26.2% of the crop was attacked under integrated management based on ecological practices.

At harvest, distal parts of roots were observed to be more affected by rot than proximal parts. This observation was confirmed through trials in which roots were inoculated with *Phytophthora tropicalis*.

Conclusions

Ecological practices increased yield in commercial roots by 42.5% and reduced, by a smaller proportion, the number of roots with rot 18.8% compared with the chemical control used by farmers in the region. We therefore conclude that using a combination of different practices is essential for managing the disease, and that these practices must be strengthened by planting cassava varieties that are resistant to rot.

Acknowledgments

We thank the following for their support: Silverio González and Camilo López, Special, La Tebaida, Department of Quindío; and Rosy Santacruz, Universidad de Quindío, Armenia.

Activity 14. Developing and validating sustainable methods of preventing and controlling FSD and SED.

To determine the effect of sustainable methods of preventing and controlling FSD and SED, experiments are recently started or ongoing.

Objective

To examine the effect of heat treatment on stakes of cassava plants affected by superelongation disease (SED), their germination, and yield

Methodology

We used thermotherapy to treat six stakes from each of 168 cassava genotypes taken from a field affected by SED. The stakes were immersed in hot water at 49°C for 49 min and then planted at Santander de Quilichao. The percentage of germination and yield were estimated, and the data analyzed by T test.

Results

The group of genotypes treated with thermotherapy had a germination rate of 90.2%, and yield was 22.2 t/ha, whereas the untreated group had a germination rate of 98.5%, and yield was 22.9 t/ha. The T test for germination gave a value of 1.79 and a probability of 0.077, whereas for yield the value was 0.41 and the probability of 0.66. The results indicated that there were no significant differences between the two treatments.

Acknowledgments

Bernardo Arias, Cassava Entomology, CIAT.

Activity 15. Greenhouse and on-farm evaluations of the effects of amendments, cover crops, organic fertilizer, and green manure on *Ralstonia solanacearum*, with practices based on chemicals included as controls.

Objective

To evaluate the effect of non-contaminating alternatives to formol on a population of *R. solanacearum* Race 2, causal agent of bacterial wilt of plantain, using bacteria inoculated into soil under greenhouse conditions

Methodology

Greenhouse

In an arrangement of randomized complete blocks, with five replications, the following treatments were tested:

- Incorporation of marigold (*Tagetes patula*) at 1 kg/m^2
- Incorporation of calfos, a fertilizer for P-deficient acid soils, at 0.5 kg/m^2
- Liquid fertilizer Fulvan®, an organic fertilizer based on "first froth" from boiled sugarcane juice and enriched with microorganisms, at a dosage of 20 L/m^2
- Lixiviate of plantain compost, produced on an artisanal basis by farmers in Quindío, Colombia, at a dosage of 2.7 L/m^2

These treatments were compared with 20% formol at 9.3 L/m^2. The controls used were inoculated soil without treatment, and soil that was neither inoculated nor treated. The experimental unit consisted of 2 plant pots with 250 cc of soil. The bacterial population was evaluated weekly over 70 days.

Field

Trials were established on six farms located in the Department of Quindío to evaluate the following treatments:

- Fulvan®, an organic fertilizer
- Lixiviate of plantain compost
- A practice recommended by ICA, where glyphosate is injected into the infected plant and its healthy neighbors, followed by weeding and applying formol into holes in the soil and covering with plastic
- Agroplus®, an organic product
- Coffee pulp
- Basamid®, a soil fumigant
- Formol
- Covering the soil with *Mucuna* mulch
- Covering the soil with *Crotalaria* mulch

Results

Greenhouse

The formol reduced the bacterial population by 100%, according to the evaluation made 20 days after application to the soil. Marigold was the next most effective treatment, reducing the bacterial population by 84.8%, according to a count made 70 days after the treatment was applied. Differences between the two treatments were not significant (Tukey's test, $\alpha = 5\%$).

The bacterial populations in the soil were also reduced by lixiviate of plantain compost (55.8%), calfos (39.3%), and Fulvan® (36.7%), whereas the control with no application had a reduction of 50%.

The effect of marigold may be attributed to one or several of the following organic components: linalol, carvona, cineole, ocimene, phenol, eugenol, anethole, dextralinolene, quercetagine, quercetagetine, quercetagitrine, tagetiine, flavonoids, pyretrines, bi-thienylacetylene, heleniene, derivatives of selenophene, fatty acids (myristic, palmitic, stearic, lauric, and oleic), aterthienyl, lutein, campherol, campheritrine, (SIAMAZONIA 2003), and sulfur derivatives such as thiophene.

Figure 1 shows that the formol and marigold affected the bacterial population from application, whereas calfos required about 27 days to act, possibly because it dissolves slowly.

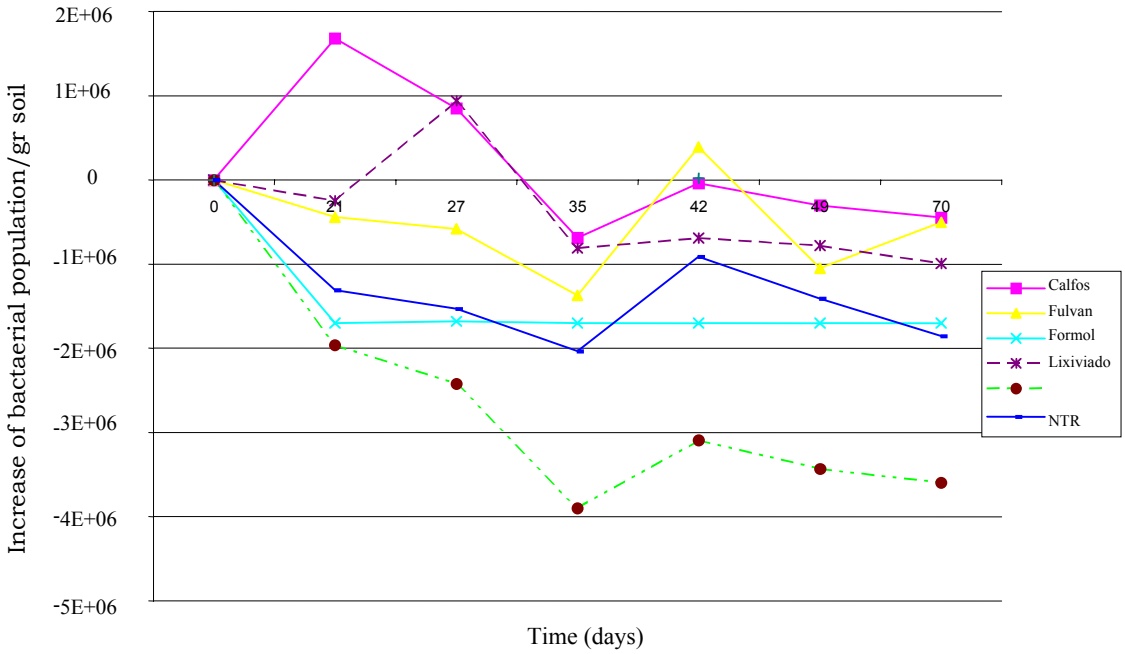

Figure 1. Changes in population sizes of the soil bacterium *Ralstonia solanacearum* after the application of different ecological alternatives to formol. Trials were carried out under greenhouse conditions. NTR = no treatment.

Field

In the field, the bacterial population in the soil was limited almost exclusively to the site where the infected plant was found in the point of highest disease pressure focuses where control treatments were carried out. The bacterium was isolated only from the Lusitania farms, from the check at 1.6×10^6 cfu/g of soil; La Habana, from the treatment Agroplus® + coffee pulp at 7.2×10^6 cfu/g of soil; and Belén Farm, from the treatments Basamid® and formol at 60,545 cfu/g.

Reference

SIAMAZONIA. 2003. "Rosa sisa". (http://www.siamazonia.org.pe/Publicaciones/2003/Enero Plantas_medicinales/ rosasisa.htm SIAMAZONIA.2003. "Rosa sisa".

Activity 16. Evaluating the resistance of banana FHIA 17 to *Ralstonia solanacearum.*

Objective

To evaluate the resistance of banana FHIA 17 to three isolates of the bacterium *R. solanacearum*

Methodology

A bacterial suspension of *R. solanacearum* was prepared for each of three isolates—CIAT 1008 (from Tolima), Rs2 (Quindío), and Jamundí-a (Valle del Cauca)—with an optical density of a 0.6 to 600 nm wavelength. We then injected 3 mL of the suspension into FHIA 17 banana plants, which are believed to be resistant to the bacterium. The inoculum was distributed across three points on the pseudostem. The inoculated plants were incubated in a chamber at 30°C for 10 days under constant moisture. They were then moistened for 1 h per day until evaluation, 20 days after inoculation.

Results

Twenty days after inoculation, the plants began showing leaf yellowing, and 5 to 8 days later, wilting was observed, which progressed until the plants died. The cuts on the plants' pseudostems showed necrosis of vascular bundles. The symptoms caused by isolate Rs2 were mild, whereas those of the other two isolates caused plant death.

Activity 17. Diagnosing plant diseases and technical assistance.

Bacteriological and fungal diagnoses were performed on different samples obtained from Colombian farmers and institutions (see **Table 1**).

Table 1. Bacteria and fungi isolated from different crops and identified at the CIAT Cassava Pathology Laboratory (Palmira, Colombia).

Location	Host plant	Disease	Detection method	Microorganism identified
Palmira, Valle del Cauca	Cassava	Wilting and root and stem root in seedlings	Isolation on selective media	*Fusarium sp.*
Valle del Cauca, Meta, Quindío	*Plantain*	Moko	Isolation, pathogenicity and DNA sequence analysis	*Ralstonia solanacearum*
Valle del Cauca, Putumayo	*Plantain*	Watery rot	Isolation	*Erwinia carotavora*
Antioquia	*Banana*	Moko	Isolation, pathogenicity and DNA sequence analysis	*Ralstonia solanacearum*
Quindío, Cauca	*Banana*	Fusarium wilt	Isolation and pathogenicity	*Fusarium oxysporum f.* sp. *cubense*
Palmira, Valle del Cauca	Dendrobium	Petals with brown-colored necrotic wounds	Isolation and direct observation under light microscope	*Colletotrichum* sp.
Palmira, Valle del Cauca	Dendrobium	Black stem rot	Isolation and direct observation under light microscope	*Fusarium sp*. associated with the stem borer *Xylosandrus mongenus*
La Unión, Valle del Cauca	Melon	Stems presenting internal orange-yellow dry rot	Isolation on selective media	*Fusarium sp.*
Cali, Valle del Cauca	*Tomato*	Powdery mildew	Direct observation under light microscope	*Oidium lycopersicum*

An *in vitro* bioassay to evaluate the effect of fungicides on *Colletotrichum* sp. isolates from *Dendrobium* showed that Mertect was the most effective of the six products tested.

Activity 18. Training researchers from Latin America, the Caribbean, and Africa on managing cassava diseases and research technology.

2003

- 23 October. Philipp Aerni, Senior Researcher, Center for Comparative and International Studies (CIS) of the Swiss Federal Institute of Technology (ETH) at Zürich. Disease diagnosis, molecular characterization of pathogens, molecular markers associated with resistance to cassava diseases, and management of cassava diseases.

2004

- 10 February. José Ventura and Ernesto Espinoza, INIVIT, Cuba. Disease detection and diagnosis in cassava.
- 15 February. Natali Cortés, Student, Tver State University, Moscow, Russia. Applied biotechnology to detect and control phytopathogenic agents.
- March. Okechukwu Eke-Okoro (Nigeria), Titus Alicai (Uganda), Christopher Omongo (Uganda), William Sserubombwe (Uganda), Mayanne Apok (Uganda), Steven Tumwesigye (Uganda). Disease diagnosis, molecular characterization of pathogens, molecular markers associated with resistance to cassava diseases, management of cassava diseases.
- 20 May. Colombo–Japanese Association (15 participants). Integrated management of diseases for cassava, plantain, palm, and flowers.
- 31 May to 12 June. CIAT (30 participants). International course on modern systems of cassava production, processing, and use. Organized by CLAYUCA.
- 9 September. Reinaldo Tovar. Universidad Nacional Experimental de Guayana. Puerto Ordaz, Venezuela. Thermotherapy for *in vitro* production of cassava plantlets.
- 30 September. Manuel Valdivié. Instituto de Ciencia Animal (ICA), Cuba. Management of cassava diseases.
- 1 October. Gustavo Córdova. Instituto Nicaragüense de Tecnología Agropecuaria. Managua, Nicaragua. Biological control of pathogens.

Training students, farmers, technicians, and researchers through field days and meetings on modern, sustainable, cassava production systems in different regions of Colombia to manage major cassava diseases, emphasizing selection of stem cuttings

2003

- 4 November. Five people from Chemonics International and farmers of Putumayo. Disease diagnosis and management for cassava and plantain.

2004

- February. Lorena Escobar. Universidad Nacional de Colombia. Isolation of *Phytophthora* species from chili pepper.
- March. Liliana Cadavid and Susana Mejía, Biology Students, Universidad del Valle, Cali, Colombia. Isolation, detection, and pathogenicity tests of pathogens.
- 29 February. 18 participants, including farmers and students from Pereira, Department of Risaralda. Disease diagnosis and management for cassava.

- 12 March. 12 participants, including Chemonics International, Fundación Futuro Ambiental, and Fundación Catatumbo, and farmers. Disease diagnosis and management for cassava, rubber, cacao, and vanilla.
- 15–17 March. Ana Claudia Gordillo, CORPOICA "La Libertad", Villavicencio. Diagnosis and identification of *Ralstonia solanacearum* in plantain, banana, soil, weeds, and water.
- 24 March. Meeting with Nicolás Cock Duque in Ecoflora. Integrated management of diseases through plant extracts.
- May. Meeting in Palmar del Oriente, with technicians from Palmar del Oriente, Palmas de Casanare, and Palmeras Santana oil-palm plantations; and with researchers from CENIPALMA. Discussion of progress in diagnosing and identifying the causal agent of lethal wilt of oil palm.
- 16 May. 30 participants, including farmers, functionaries from national bodies, and NGOs in Orito, Department of Putumayo. Diagnosis and management of frogskin and other cassava diseases.
- 18 May. Dr Octavio Vargas, Mitsui & Co., Ltd Natural products in disease management.
- 4 June. 37 Students, University of Caldas. Cassava and plantain diseases.
- 5 June. 350 participants, including farmers, functionaries from national bodies, and private enterprises in Montería, Department of Córdoba, at the release of new cassava varieties. Management of cassava diseases, with a presentation on the diagnosis and management of frogskin disease.
- 1 July. 6 farmers, Pescador, Department of Cauca. Diagnosis and management of cassava diseases.
- 22 July. Visitors from the Colombian Association of Banana Growers (AUGURA) and CENIBANANO, including Luis Fernando Patiño, León Toné Gaviria, and Ramiro Jaramillo Sosa of the Board of Directors of CENIBANANO. Integrated management of diseases for plantain, cassava, and oil palm.
- 26 July to 6 August. Hernán Zapata, Agrobiológicos SAFER (Natural Control). Isolation and conservation of pathogens and biocontrollers, inoculum preparation, and pathogenicity tests.
- 3 September. 3 farmers, Department of Quindío. Progress on the management of bacterial wilt (*moko*) of plantain.
- 9 to 10 August. Workshop. Diagnosis of diseases caused by viruses and phytoplasmas. CIAT, Cali.

Attendance at Meetings in 2004

8–14 March. Sixth International Scientific Meeting of the Cassava Biotechnology Network, Cali, Colombia

31 July to 4 August. Annual Meeting of the American Phytopathological Society (APS), Anaheim, CA

13–15 August. XXV National Congress of Phytopathology, Cali, Colombia (held by ASCOLFI)

Awards

13 August. Premio Nacional de Fitopatología "Rafael Obregón" categoría profesional, for the paper "Detección de marcadores microsatélites asociados con la resistencia al Añublo Bacterial de la yuca (*Manihot esculenta* Crantz) en Colombia". PX Hurtado, E Alvarez, M Fregene and GA Llano. Presented at XXIV Congress of ASCOLFI. June 25 – 27, 2003.

Feria de innovaciones, México 2004. Desarrollo de medidas de manejo del Moko (*Ralstonia solanacearum*) de plátano (*Musa AAB*) en Colombia, mediante investigación participativa con agricultores. Grupo Consultivo para la Investigación Agrícola Internacional (CGIAR).

Publications

Alvarez E; Mejía JF; Huertas C; Varón F. Detección y caracterización molecular de un fitoplasma, asociado con el "machorreo" del lulo (*Solanum quitoense*) en Colombia. Fitopatol Colomb 27(2):71–76.

Hurtado PX; Alvarez E. Búsqueda de genes análogos de resistencia asociados con la resistencia al añublo bacterial de la yuca. Fitopatol Colomb 27(2):59–64.

Loke JB; Pérez JC; Alvarez E; Cuervo M; Mejía JF; Llano G.; Pineda B. Cuero de Sapo: *Una Enfermedad de la Yuca* -Once Preguntas Muy Interesantes de Agricultores-. Poster presented June 5 during the release of new cassava varieties.

Extension Brochures

Alvarez, E., Llano, G. 2004. Añublo Bacterial de la yuca.

Alvarez, E., Mejia, J.M. 2004 Superalargamiento de la yuca.

Submitted

Alvarez E. and Ospina C.A. 2004. Morphological, genetic, and pathogenic characterization of *Colletotrichum gloeosporioides,* causal agent of anthracnose in soursop (*Annona muricata*) in the production areas of Valle del Cauca, Colombia. Plant. Dis.

Loke JB; Alvarez E; Vallejo FA; Marín J; Fregene M; Rivera S; Llano GA. Análisis de QTLs de la resistencia a pudrición de raíz causada por *Phytophthora tropicalis* en una población segregante de yuca (*Manihot esculenta* Crantz). Acta Agron.

Llano GA; Alvarez E; Muñoz JE; Fregene M. Identificación de genes análogos de resistencia a enfermedades en yuca (*Manihot esculenta* Crantz), y su relación con la resistencia a tres especies de *Phytophthora*. Acta Agron 53(1/2).

Calle F; Pérez JC; Gaitán W; Morante N; Ceballos H; Llano GA; Alvarez E. Genetics of relevant traits in cassava (*Manihot esculenta* Crantz) adapted to acid-soil savannas. Euphytica.

Hurtado PX; Alvarez E; Fregene M; Llano GA. Detección de marcadores microsatélites asociados con la resistencia a *Xanthomonas axonopodis* pv. *manihotis* en una familia de yuca (bc1). Rev Fitopatol Colomb.

Concept Notes and Proposal

Identification of insect vectors and alternative hosts of phytoplasmas causing cassava frogskin disease. Presented to USAID. Funds requested: US$ 12.000 for 1 year. Approved.

Desarrollo de estrategias de manejo de cuero de sapo y superalargamiento en yuca, mediante investigación participativa. Presented to Ministerio de Agricultura y Desarrollo de Colombia. Funds requested: US$ 14.546 for 1 year. Approved.

Combating Hidden Hunger in Latin America: Biofortified crops with improved Vitamina A, Essential Minerals and Quality Protein (English). Presented to CIDA. Funds requested: US$ 122.880 for 6 years. Approved.

Manejo integrado de la enfermedad del Moko en plátano. Presented to Ministerio de Agricultura y Desarrollo de Colombia. Funds requested: US$ 37.469. Approved.

Pest and disease resistance, drought tolerance and increased shelf life genes from wild relatives of cassava and the development of low-cost technologies to pyramid them into elite progenitors. Presented to The generation challenge programme. Funds requested: US$ 289.200 per year, for 3 years. Submitted.

Desarrollo de métodos rápidos de detección de *Ralstonia solanacearum*, agente causante de Moko de plátano, en plantas, malezas, agua y suelo. Presented to ASOHOFRUCOL. Funds requested: US$ 47.235. Submitted.

Manejo Integrado de Enfermedades del Cultivo de Yuca. Presented to Ministerio de Agricultura y Desarrollo de Colombia and IICA. Funds requested: US$ 77.700. Submitted.

Desarrollo de prácticas de manejo de Pudrición de Raíz de yuca mediante la detección molecular de *Phytophthora* en zonas semi áridas en Brazil y Colombia. Presented to Cassava Biotechnology Network for Latin America and the Caribean (CBN-LAC). Funds requested: US$ 77.700. Submitted.

Confirmación de fitoplasma, como agente causante de la Marchitez Letal en palma de aceite. Presented to Palmar del Oriente, Palmas de Casanare y Pameras Santana. Funds requested: US$72.288. Submitted.

Presentations at Meetings

25 Febrero. Hurtado PX. Internal seminar at CIAT. Detección de marcadores microsatélites con la resistencia al añublo bacterial de la yuca.

8–14 March. Llano GA; Alvarez E; Fregene M; Muñoz JE. Identification of resistance-gene analogs in cassava (*Manihot esculenta*), and their relationship to three *Phytophthora* species. Poster presented at the Sixth International Scientific Meeting of the Cassava Biotechnology Network, Cali, Colombia. Page 121.

8-14 March. Loke JB; Alvarez E; Corredor JA; Folgueras M; Jaramillo G; Ceballos H. Preliminary evidence between foliar and root resistance to root rot caused by *Phytophthora tropicalis* in cassava. Poster presented at the Sixth International Scientific Meeting of the Cassava Biotechnology Network, Cali, Colombia. Page.79.

8–14 March. Loke JB; Alvarez E; Fregene M; Marín J; Rivera S; Llano GA; Mejía JF. QTL mapping for resistance to root rot caused by *Phytophthora tropicalis* in cassava. Poster presented at the Sixth International Scientific Meeting of the Cassava Biotechnology Network, Cali, Colombia. Page 158.

11–13 August. Alvarez E; Mejía JF; Llano GA; Loke JB. Detección de un fitoplasma asociado a cuero de sapo de yuca (*Manihot esculenta* Crantz) en Colombia. Paper presented at the XXV ASCOLFI Congress, Cali.

11–13 August. Arenas A; López D; Llano GA; Alvarez E; Loke JB. Efecto de prácticas ecológicas sobre la población de *Ralstonia solanacearum* Smith, causante de moko de plátano. Paper presented at the XXV ASCOLFI Congress, Cali.

Thesis

Postgraduate Theses in Cassava

Paula X. Hurtado. Evaluación de marcadores microsatélites y genes análogos, asociados a la resistencia de yuca a *Xanthomonas axonopodis* pv. *manihotis*. Universidad de los Andes—Bogotá. For a Master's in Biology, emphasizing Plant Molecular Biology.

John B. Loke. Análisis genético de la resistencia de yuca (*Manihot esculenta* Crantz) a *Phytophthora tropicalis*, causante de pudrición radical. Universidad Nacional de Colombia—Palmira. For a Master's in Plant Breeding.

Undergraduate Theses in Cassava

Alejandro Corredor. Evaluación de la asociación de Marcadores bioquímicos y morfológicos con la resistencia a pudrición de raíz (Phytophthora tropicalis) y el deterioro fisiológico en yuca (Manihot esculenta Crantz). Universidad de Caldas, Manizales. For a degree in Agronomy.

Eduardo Gómez. Identificación y caracterización de aislamientos de Ralstonia solanacearum, obtenidos de zonas afectadas por moko de plátano y banano en Colombia. Universidad Pontificia Javeriana, Bogotá. For a degree in Microbiology.

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