CASSAVA AND TROPICAL FRUIT PATHOLOGY

Activity 1. DNA sequence analysis of specific regions of phytoplasma, *Glomerella*, *Sphaceloma*, *Ralstonia*, *Phytophthora*, *Pythium*, and cassava.

Objective

Report in GenBank the sequences of fungi, bacteria, and phytoplasmas that affect important crops in Colombia (*Manihot esculenta* Crantz*, Elaeis guineensis*, *Solanum quitoense*, *Coffea arabica, Anona muricata* and *Musa* AAB*)* and several resistance genes.

Methodology

DNA fragments of phytoplasma from *Elaeis guineensis*, *Manihot esculenta* Crantz, *Solanum quitoense* and *Coffea arabica* were obtained using the polymerase chain reaction (PCR). Fungi and bacteria affecting different crops—for example *Phytophthora* sp. (*Manihot esculenta* Crantz), *Sphaceloma* sp. (*Manihot esculenta* Crantz), *Glomerella* sp. (*Anona muricata*), and *Ralstonia solanacearum* (*Musa* AAB)—and resistance gene analogs of cassava (obtained from varieties resistant to *Phytophthora* sp. and *Xanthomonas axonopodis* pv *manihotis*) were purified and then ligated in pGEM-T Easy vector, which was introduced into the *Escherichia coli* strain DH5-a by electroporation at 2.4 kV/cm^2. Transformants were selected on blue/white color screening by plating on LB/ampicillin/IPTG/X-gal media. Positive inserts were observed by plasmid restriction with *Eco*RI 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. Using the DNAMAN software with the Assambly option, different fragments of each microorganism or gene were aligned to obtain complete sequences. To report the sequences in the GenBank (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov), the read bases and their taxonomic classification at both morphologic and molecular levels were analyzed for each species **(Table 1)**.

Table 1. Sequences submitted to the GenBank database.

Accession GenBank	Name	Size (bp)	Organism	Isolate/ Clone	Host/ Source Genotype	Location
AY525125	*Coffee crispiness phytoplasma* 16S rRNA gene	941	*Phytoplasma X-Disease group*		*Coffea arabica*	Caldas, Colombia
AY737646	*Cassava frogskin disease phytoplasm* (FSD)	1260	*Phytoplasma X-Disease group*	FSDY17	*Manihot esculenta*	Valle del Cauca, Colombia
AY737647	*Cassava frogskin disease phytoplasm* (FSD)	1298	*Phytoplasma X-Disease group*	FSDY29	*Manihot esculenta*	Valle del Cauca, Colombia
AY731819	*Solanum quitoense machorreo phytoplasma* 16S rRNA gene	1567	*Phytoplasma X-Disease group*		*Solanum quitoense*	Valle del Cauca, Colombia
AY739023	*Lethal decline oil palm phytoplasma* strain PO8.90OilCol 16S rRNA gene	1235	*Phytoplasma*	PO8.90 OilCol	*Elaeis guineensis*	Villanueva, Colombia
AY739024	*Lethal decline oil palm phytoplasma* strain PC2.1014R 16S rRNA gene	1424	*Phytoplasma Aster Yellows*	PC2.1014R	*Elaeis guineensis*	Villanueva, Colombia
AY737648	*Colletotrichum acutatum* isolate CA15 5.8S rRNA gene ITS1 ITS2	438	*Glomerella acutata*	CA 15	*Annona muricata*	Valle del Cauca, Colombia
AY739025	*Colletotrichum gloesporioides* isolate CG5 18S rRNA gene ITS1 ITS2	524	*Glomerella cingulata*	CG 5	*Annona muricata*	Valle del Cauca, Colombia
AY739018	*Sphaceloma manihoticola* 18S rRNA gene ITS1 ITS2	644	*Sphaceloma manihoticola*	S2	*Manihot esculenta*	Brazil
AY739019	*Sphaceloma manihoticola* 18S rRNA gene ITS1 ITS2	625	*Sphaceloma manihoticola*	S47	*Manihot esculenta*	Colombia
AY739020	*Sphaceloma krugii* 18S rRNA gene ITS1 ITS2	629	*Sphaceloma krugii*	S1	*Euphorbia heterophylla*	Brazil
AY737489	*Ralstonia solanacearum* isolate G175 16S rRNA gene fragment	255	*Ralstonia solanacearum*	G175	*Solanum melongena*	Kenya
AY745758	*Ralstonia solanacearum* isolate CIAT 1017 16S rRNA gene fragment	225	*Ralstonia solanacearum*	CIAT 1017	*Canna indica* (Indian shot)	La Dorada, Colombia
AY745759	*Ralstonia solanacearum* isolate G 218 16S rRNA gene fragment	223	*Ralstonia solanacearum*	G 218	*Capsicum sp.*	Philippines
AY745760	*Ralstonia solanacearum* isolate G 217 16S rRNA gene fragment	216	*Ralstonia solanacearum*	G 217	*Heliconia sp.*	Costa Rica
AY745757	*Ralstonia solanacearum* isolate CIAT 1016 16S rRNA gene fragment	235	*Ralstonia solanacearum*	CIAT 1016	*Solanum tuberosum*	Popayán, Colombia
AY745755	*Ralstonia solanacearum* isolate Urabá 6 16S rRNA gene fragment	203	*Ralstonia solanacearum*	Urabá 6	*Musa* sp.	Urabá, Colombia

Accession GenBank	Name	Size (bp)	Organism	Isolate/ Clone	Host/ Source Genotype	Location
AY745761	*Ralstonia solanacearum* isolate Quindío 1 16S rRNA gene fragment	223	*Ralstonia solanacearum*	Quindío 1	*Musa* AAB	Montenegro, Colombia
AY737486	*Ralstonia solanacearum* isolate Jamundí soil 16S rRNA gene fragment	235	*Ralstonia solanacearum*	Jamundí a	Soil – plantain	Jamundí, Colombia
AY737487	*Ralstonia solanacearum* isolate 16a soil 16S rRNA gene fragment	244	*Ralstonia solanacearum*	16a	Soil – plantain	Montenegro, Colombia
AY737488	*Ralstonia solanacearum* isolate CIAT 1043 16S rRNA gene fragment	220	*Ralstonia solanacearum*	CIAT 1043	*Nicotiana tabacum*	Socorro, Colombia
AY745756	*Ralstonia solanacearum* isolate CIAT 1077 16S rRNA gene fragment	225	*Ralstonia solanacearum*	CIAT 1077	*Lycopersicon Esculentum*	North Carolina USA
AY730038	*Manihot esculenta* resistance gene analog clone N37 NBS-LRR	325	*Manihot esculenta*	N37	M Bra 1045	Palmira, Colombia
AY730040	*Manihot esculenta* resistance gene analog clone N38 NBS-LRR	474	*Manihot esculenta*	N38	M Bra 532	Palmira, Colombia
AY730041	*Manihot esculenta* resistance gene analog clone K1 NBS-LRR	496	*Manihot esculenta*	K1	M Bra 532	Palmira, Colombia
AY737490	*Manihot esculenta* resistance gene analog clone N33 NBS-LRR	342	*Manihot esculenta*	N33	M Bra 1045	Palmira, Colombia
AY745762	*Manihot esculenta* resistance gene analog clone N31 NBS-LRR	210	*Manihot esculenta*	N31	CM6438-14	Palmira, Colombia
AY745763	*Manihot esculenta* resistance gene analog clone P32 kinase	449	*Manihot esculenta*	P32	CM 3311-4	Palmira, Colombia
AY745764	*Manihot esculenta* resistance gene analog clone W5 kinase	487	*Manihot esculenta*	W5	CM 7772-13	Palmira, Colombia
AY745765	*Manihot esculenta* resistance gene analog clone X1 kinase	441	*Manihot esculenta*	X1	CM 3311-4	Palmira, Colombia
AY745766	*Manihot esculenta* resistance gene analog clone X5 kinase	535	*Manihot esculenta*	X5	CM 7772-13	Palmira, Colombia
AY745767	*Manihot esculenta* resistance gene analog clone X9 kinase	336	*Manihot esculenta*	X9	CM 6438-14	Palmira, Colombia
AY745768	*Manihot esculenta* resistance gene analog clone W6 kinase	117	*Manihot esculenta*	W6	CM 6438-14	Palmira, Colombia
AY745769	*Manihot esculenta* resistance gene analog clone W10 kinase	442	*Manihot esculenta*	W10	CBB resistant bulk	Villavicencio, Colombia
AY745770	*Manihot esculenta* resistance gene analog clone P36 kinase	336	*Manihot esculenta*	P36	CM 3311-4	Palmira, Colombia
AY745771	*Manihot esculenta* resistance gene analog clone P41 kinase	599	*Manihot esculenta*	P41	M Nga 19	Palmira, Colombia

Accession GenBank	Name	Size (bp)	Organism	Isolate/ Clone	Host/ Source Genotype	Location
AY745752	*Phytophthora cinnamomi* 5.8S and 28S rRNA gene ITS1-ITS4	883	*Phytophthora cinnamomi*	CLT	*Calathea* sp.	The Netherlands
AY745749	*Phytophthora cryptogea* 5.8S and 28S rRNA gene ITS1-ITS4	860	*Phytophthora cryptogea*	HMA	*Heliconia* sp.	Palmira, Colombia
AY739022	*Phytophthora tropicalis* 18S rRNA gene ITS1 ITS2	747	*Phytophthora tropicalis*	P71	*Manihot esculenta*	Quindío, Colombia
AY739021	*Phytophthora melonis* 18S rRNA gene ITS1 ITS2	920	*Phytophthora melonis*	P12	*Manihot esculenta*	Brazil
AY745754	*Phytophthora nicotianae* 18S, 5.8S and 28S rRNA gene ITS4-ITS5	839	*Phytophthora nicotianae*	STD3	*Manihot esculenta*	Santander de Quilichao, Colombia
AY745753	*Phytophthora melonis* 18S, 5.8S and 28S rRNA gene ITS4-ITS5	869	*Phytophthora melonis*	B10	*Manihot esculenta*	Brazil
AY745750	*Phytophthora palmivora* 5.8S and 28S rRNA gene ITS1-ITS4	657	*Phytophthora palmivora*	PP7	*Theobroma cacao*	Caldas, Colombia
AY745751	*Phytophthora palmivora* 5.8S and 28S rRNA gene ITS1-ITS4	791	*Phytophthora palmivora*	FLMA1	*Theobroma cacao*	Caldas, Colombia
AY745748	*Pythium chamaehyphon* 5.8S and 28S rRNA gene ITS1-ITS4	773	*Pythium chamaehyphon*	MTR4	*Manihot esculenta*	Mitú, Colombia

Activity 2. Sample collection and isolation of the bacterium *Ralstonia solanacearum* obtained from plantain, its conservation, identification by PCR, DNA sequencing, and determination of races, biovars, and pathogenicity.

Objectives

1. To isolate *R. solanacearum* from soil and diseased plants of plantain and banana by culturing in semi-selective medium, and identifying through the PCR technique
2. To evaluate the pathogenicity of *R. solanacearum* isolates
3. To biochemically characterize the isolates to determine biovars
4. To identify the isolates, using DNA sequencing

Methodology

Obtaining R. solanacearum isolates

We processed samples collected from 14 farms suffering from problems of moko, a bacterial wilt that attacks various crops of economic importance. Samples were obtained from plants of plantain (31) and banana (4) infected with the wilt, soil (193), weeds (2), and water (1) in the production regions of the Colombian Departments of Quindío, Antioquia, Valle del Cauca, Caquetá, and Meta.

From these samples, we obtained and selected, according to their growth in the SMSA semi-selective culture medium (which reduces growth of saprophytic bacteria) and to their positive reaction to the oxidase and KOH tests, bacterial isolates that seemed to be *R. solanacearum*. These were later identified as such by the PCR technique, using specific primers to amplify a sole fragment of *R. solanacearum* DNA.

DNA extraction and PCR analysis

Genomic DNA was extracted from bacterial cells, using pure colonies of isolates that had had 36 h of growth in nutritive agar (Seal et al. 1992). A colony was suspended in 100 µL of sterilized distilled water, heated to 96°C for 5 min, and centrifuged at 12,000 rpm for 2 min. We took 2.5 µL of the supernatant as DNA mold for the PCR reaction. The volume of the reaction was 12.48 µL and, moreover, contained 1X *Taq* polymerase buffer, 0.16 mM of each dNTP, 1.5 mM $MgCl_2$, 0.25 U *Taq* polymerase, and 0.16 µM of each of the specific primers Oli1 (5'-GGGGGTAGCTTGCTACCTGCC-3') and Y2 ((5'-CCCACTGCTGCCTCCCGTAGGAGT-3'), previously reported by Martins (2000).

DNA amplification was carried out in a thermocycler (MSJ-Research PTC-100), using the following program: 2 min at 96°C, 50 cycles of denaturation for 20 s at 94°C, annealing for 20 s at 62°C, extension for 30 s at 72°C, and another final extension for 5 min at 72°C. The PCR products were separated on 1.5% agarose gels, stained with ethidium bromide, and visualized under ultraviolet light.

Determining biovars

Isolates belonging to *R. solanacearum* could be classified into different biovars by their acid production, using three disaccharides (cellobiose, lactose, and maltose), and their oxidation of

three hexose alcohols or polyols (sorbitol, dulcitol, and mannitol) in a base medium for determining biovars as according to Hayward (1964).

The disaccharides and polyols were sterilized through filtration, and the sterilized base medium then added. To inoculate with bacteria, we used a colony from a culture grown in nutritive agar over 24 h. We then observed the reactions over 1, 3, and 7 days of incubation at 28°C. A change of color from olive green to yellow, from top to bottom, indicated the production of acids from the disaccharides, whereas a color change from purple to yellow indicated oxidation of the polyols.

The reaction to 3% KOH was assessed by placing 1 drop of reagent on a glass slide and then dissolving in it a colony from a pure and metabolically active culture with a 24-h growth. The reaction was regarded as positive if it gave rise to a viscous strand 15 to 30 s afterwards.

For the oxidase test, 2 drops of 1% aqueous solution of tetramethyl-*p*-phenylenediamine dihydrochloride (Kovács' oxidase reagent) were placed on a piece of filter paper and a colony rubbed in the drops. A positive reaction was determined by appearing purple color 10 sec later.

Testing for pathogenicity on plantain

The pathogenicity test was carried out by inoculating different isolates identified by PCR as *R. solanacearum* on plants of plantain (*Musa* sp.) derived from in vitro meristem culture. Before inoculation, these plants had been transplanted to plastic bags containing sterilized soil and left for 15 days in a humid chamber to ensure the plants' development.

Four plantain plants per *R. solanacearum* isolate were inoculated with an injection of 1 mL of bacterial suspension over two sites on the pseudostem. The suspension was prepared with cultures of each bacterium identified as *R. solanacearum* grown over 24 h in nutritive agar at an absorbance of 0.6 to 600 nm wavelength, corresponding to an approximate concentration of 1×10^8 cfu/mL. As positive check, inoculations were also carried out with a strain of *R. solanacearum* (CIAT 1008) collected from Ibagué, Tolima, and now part of the strain bank at CIAT. The negative check was inoculation with sterilized water.

The inoculated plants were incubated in a humid chamber at temperatures between 24°C and 29°C, relative humidity between 80% and 91%, and about 13 h of light. At day 4, the plants were evaluated for symptoms such as leaf flaccidity; between days 8 and 10, signs of yellowing appeared and growth declined; and at day 15 onwards, lodging and wilting occurred.

Testing for hypersensitivity

The isolates grown in nutritive agar over 24 h were inoculated onto tobacco plants, infiltrating a bacterial suspension of 1×10^8 cfu/mL through the intercellular spaces of two leaves per plant, and three plants per isolate. Tissue collapse as reaction was observed at 24 h.

DNA sequencing

Fragments measuring 288 bp, amplified by PCR, were sequenced at the DNA sequencing laboratory of the Iowa State University. The sequences were then cleaned and homologized with reported sequences in the GenBank database. A phylogenetic tree that included sequences

reported in the database was constructed with Phylogenetic Analysis Using Parsimony (PAUP) and bootstrapping of 1000 replications.

Results

Obtaining R. solanacearum isolates

The samples yielded 52 isolates of bacteria that were tentatively considered as *R. solanacearum* for their growth in the SMSA semi-selective medium. At 48 h after incubating at 28°C, their growth was similar to that of check strain CIAT 1008.

PCR analysis

In a 1.5% agarose gel, a band, indicating a fragment located at gene 16S rRNA and with a molecular weight of 288 base pairs, was detected in 21 of the 52 isolates, identifying the isolates as *R. solanacearum*. **Figure 1** shows 6 of the isolates eventually identified.

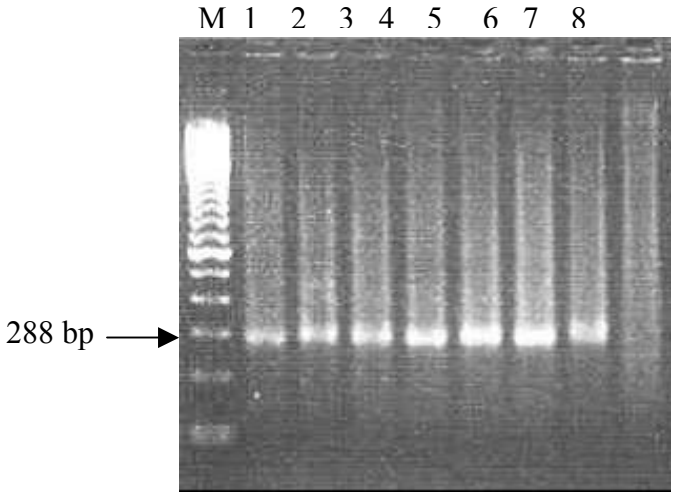

Figure 1. A 288-bp-long product amplified in the 16S rRNA region of DNA from the bacterium *Ralstonia solanacearum*. Six of the 22 isolates eventually identified are shown here: M = 100-bp marker; lanes 1–3 = isolates from Quindío, Colombia; lanes 4–6 = isolates from Urabá, Colombia; lane 7 = *R. solanacearum* strain CIAT 1008; lane 8 = negative control.

Determining biovars

Table 1 lists the reaction of each of the 21 isolates to the sugars and alcohols evaluated, indicating that all the isolates characterized belonged to biovar I. That is, none of the isolates used the three sugars, nor oxidized the three alcohols, in the biochemical tests made. Moreover, the 21 isolates reacted positively to the oxidase and KOH tests.

Table 1. Characteristics of 22 isolates of the bacterium *Ralstonia solanacearum* from Colombia as determined by biochemical tests, PCR amplification, and pathogenicity.

Isolate	Origin	Source/Treatment	SMSA[a]	Oxidase[b]	3% KOH[c]	PCR	HR tobacco[d]	Pathogenicity	Biovar[e]
1	Quindío	Plantain/Flower	(+)	(+)	(+)	(+)	(+)	(+)	1
2	Quindío	Plantain/Petiole	(+)	(+)	(+)	(+)	(+)	(+)	1
3	Quindío	Plantain/Petiole	(+)	(+)	(+)	(+)	(+)	(+)	1
4	Antioquia	Banana/Pseudostem	(+)	(+)	(+)	(+)	(-)	(+)	1
5	Antioquia	Plantain/Strain	(+)	(+)	(+)	(+)	(-)	(+)	1
6	Antioquia	Plantain/Fruit	(+)	(+)	(+)	(+)	(-)	(+)	1
7	Antioquia	Plantain/Fruit	(+)	(+)	(+)	(+)	(-)	(+)	1
15	Quindío	Soil/Farmer	(+)	(+)	(+)	(+)	(+)	(+)	1
16a	Quindío	Soil/Mucuna mulch	(+)	(+)	(+)	(+)	(+/-)	(+)	1
16b	Quindío	Soil/Mucuna mulch	(+)	(+)	(+)	(+)	(+/-)	(+)	1
17	Valle	Soil	(+)	(+)	(+)	(+)	(+)	(+)	1
18	Valle	Plantain/Sucker	(+)	(+)	(+)	(+)	(+)	(+)	1
32	Caquetá	Plantain/Pseudostem	(+)	(+)	(+)	(+)	(+)	(+)	1
33	Caquetá	Plantain/Pseudostem	(+)	(+)	(+)	(+)	(+)	(+)	1
34	Caquetá	Plantain/Raceme rachis	(+)	(+)	(+)	(+)	(+)	(+)	1
38	Quindío	Soil/Agroplus® + coffee pulp	(+)	(+)	(+)	(+)	(+)	(+)	1
39	Quindío	Soil/Agroplus® + coffee pulp	(+)	(+)	(+)	(+)	(+)	(+)	1
40	Quindío	Soil/Center of disease pressure	(+)	(+)	(+)	(+)	(+)	(+)	1
41	Quindío	Soil/Center of disease pressure	(+)	(+)	(+)	(+)	(+)	(+)	1
42	Meta	Plantain/Pseudostem	(+)	(+)	(+)	(+)	(+)	(+)	1
43	Meta	Plantain/Pseudostem	(+)	(+)	(+)	(+)	(+)	(+)	1
48	Quindío	Plantain/Pseudostem	(+)	(+)	(+)	(+)	(+)	(+)	n.d.
CIAT 1008[f]	Tolima	Plantain	(+)	(+)	(+)	(+)	(+)	(+)	1

a. Spizizen's minimal salts, a semi-selective medium.
b. Oxidase test, using Kovács oxidase reagent.
c. Reaction to 3% KOH.
d. Test for hypersensitivity to *R. solanacearum* in tobacco.
e. n.d.= Not determined.
f. Check strain from CIAT collection.

Testing for pathogenicity, and confirming "Race 2"

The *R. solanacearum* isolates obtained from infected plantain tissue were pathogenic on inoculating plantain plants, confirming that they belonged to Race 2. This was further confirmed by the typical hypersensitivity reaction obtained with tobacco leaves to 15 isolates **(Figure 2)**. The soil isolates, coded 16a and 16b, caused leaf yellowing on tobacco, which is possibly related to differences in pathogenicity.

Likewise, isolates 4, 5, 6, and 7 from banana samples, reacted negatively to the hypersensitivity test **(Table 1)**. These last four isolates were pathogenic to banana and plantain.

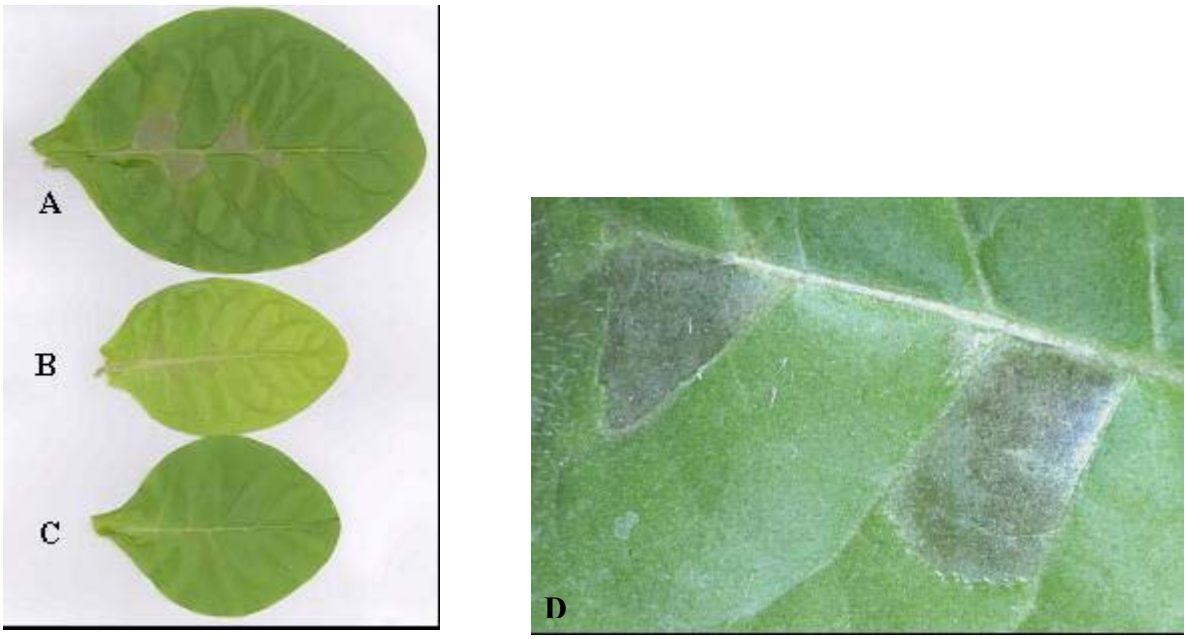

Figure 2. Reaction of hypersensitivity in tobacco to isolates of the bacterium *Ralstonia solanacearum* Race 2. Tobacco leaves were inoculated by infiltration. (A) and (D) Typical reactions of hypersensitivity; (B) an atypical reaction, induced by isolates from soil and banana; and (C) control infiltrated with sterilized distilled water.

DNA sequencing

Figure 3 shows a certain degree of similarity between isolates collected from different production regions. Isolate Caquetá 32, obtained from plantain, separated into a cluster next to isolate Urabá 6, obtained from banana. The latter isolate, in its turn, showed phylogenetic differences with isolate Urabá 4, also from banana. Isolates from the soil at Quindío also separated among themselves, except for 38 and 16a, which formed a cluster. Meanwhile, isolates from Meta, Quindío soils, and Tolima (1008) showed greater similarities with isolates from potato and tobacco than with either the three sequences reported in GenBank (AY 216796, obtained from soil; PS01716SR, and AY642432) or with the sequences of different bacterial species (*R. mannitolilytica*, *R. picketii*, *R. thomasii*, and *Burkholderia solanacearum*).

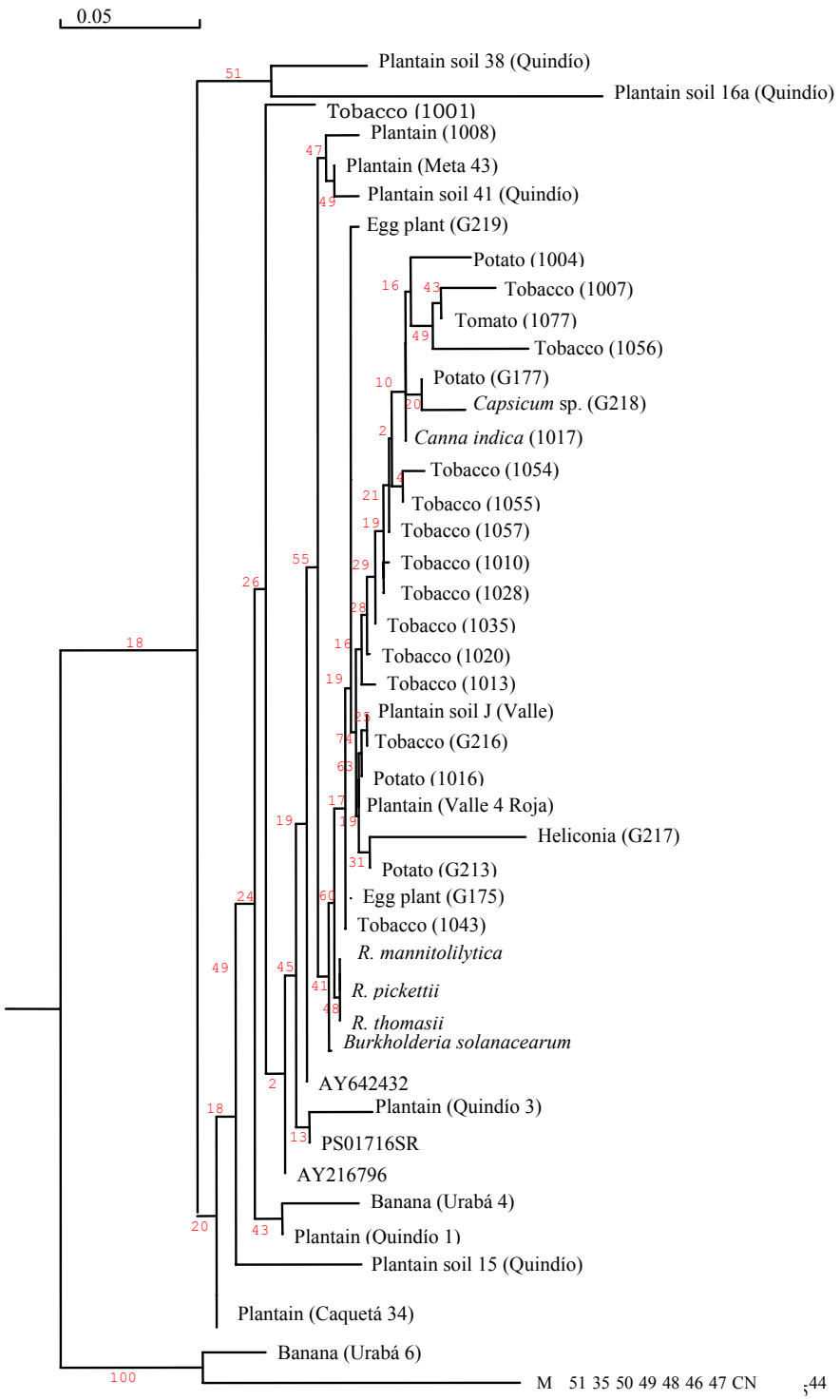

Figure 3. Phylogenetic tree constructed with PAUP and bootstrapping of 1000 replications from 44 isolates of the bacterium *Ralstonia solanacearum* from plantain in Tolima (1008), Valle del Cauca, Quindío, Meta, Caquetá; banana from Urabá; and soil from Quindío and Valle del Cauca, Colombia. The tree compares the isolates with those from several crops and with accessions in GenBank (AY 216796, AY642432, and PS01716SR of *R. solanacearum, R. picketii, R. thomasii, R. mannitolilytica*, and *Burkholderia solanacearum*).

Activity 3. DNA sequence analysis of *Ralstonia solanacearum* obtained from banana, *Heliconia* sp., eggplant, potato, tomato, tobacco, and Indian shot (*Canna indica* L.)

Specific objectives

1. To confirm the identity of strains of *R. solanacearum* isolated from tomato, tobacco, potato, plantain, *Heliconia* sp., Indian shot (*C. indica*), banana, and eggplant through PCR with the specific primers Oli1/Y2 and sequencing
2. To identify, by sequencing, 18 bacterial strains isolated from plantain and now part of the bacterial collection held at CIAT's Cassava Pathology
3. To characterize, through biochemical tests, strains of *R. solanacearum*

Methodology

DNA extraction and PCR analysis

We wanted to confirm the identity of 41 isolates of *R. solanacearum* belonging to the bacterial collection at Cassava Pathology, CIAT, obtained from different crops **(Table 1**, codes 1 to 41), and to discover the identity of 18 strains more, collected from plantain suckers from a farm located in Jamundí (Valle del Cauca), both before and after thermotherapy **(Table 1**, codes 42 to 59).

Table 1. Characteristics of isolates of the bacterium *Ralstonia solanacearum* from Colombia as determined by PCR amplification[a].

Code	Number in Collection[b]	Race	Source	Origin	Host[c]	PCR
1	1001		CIAT	Floridablanca, Colombia	Tobacco	+
2	1003	1	CIAT	Trinidad	Tomato	-
3	1004	1	CIAT	Nambour, Australia	Potato	+
4	1005	3	CIAT	Atherton, Australia	Potato	-
5	1006	1	CIAT	Worthi Co., Georgia, USA	Tomato	-
6	1007	1	CIAT	Quency, Florida, USA	Tobacco	+
7	1008	2	CIAT	Ibagué, Tolima	Plantain	+
8	1010		CIAT	Floridablanca, Colombia	Tobacco	+
9	1011	3	CIAT	Toowoomba, Australia	Potato	-
10	1012	3	CIAT	Las Palmas, Colombia	Potato	-
11	1013	1	CIAT	North Carolina, USA	Tobacco	+
12	1014	3	CIAT	Africa	Potato	-
13	1015		CIAT			-
14	1016	3	CIAT	Popayán, Colombia	Potato	+
15	1017	2	CIAT	Dorada, Colombia	Indian shot	+
16	1018	3	CIAT	Popayán, Colombia	Potato	-
17	1020		CIAT	Girón, Colombia	Tobacco, Col. var. 37	+
18	1028		CIAT	Girón, Colombia	Tobacco, Col. var. 37	+
19	1035		CIAT	Socorro, Colombia	Tobacco, Col. var. 37	+
20	1043		CIAT	Socorro, Colombia	Tobacco, Col. var. 37	+
21	1051		CIAT	Villanueva, Colombia	T, woolly var.	-
22	1052		CIAT	Girón, ICA, Colombia	Tobacco, Col. var. 37	-
23	1053		CIAT	Girón, ICA, Colombia	Tobacco, Col. var. 37	-
24	1054		CIAT	San Gil, La Flora, Colombia	Tobacco	+
25	1055		CIAT	San Gil, El Comunismo, Colombia	Tobacco	+

Code	Number in Collection[b]	Race	Source	Origin	Host[c]	PCR
26	1056		CIAT	Floridablanca, Colombia	Tobacco	+
27	1057		CIAT	Floridablanca, Colombia	Tobacco	+
28	1077	1	CIAT	Wake Co., North Carolina	Tomato	+
29	G212	3	Dinamarca		Potato	-
30	G213	3	Dinamarca		Potato	+
31	G175		Dinamarca	Kenya	Eggplant	+
32	G176	2	Dinamarca	Peru	Banana	-
33	G177	3	Dinamarca	Australia	Potato	+
34	G215	3	Dinamarca	French Réunion	Potato	-
35	G216	1	Dinamarca	Japan	Tobacco	+
36	G217	2	Dinamarca	Costa Rica	*Heliconia* sp.	+
37	G218	1	Dinamarca	Philippines	*Capsicum* sp.	+
38	G219	1	Dinamarca	Sri Lanka	Eggplant	+
39	R3		CIAT	Quindío	Soil	-
40	R18		CIAT	Quindío	Plantain	-
41	R22		CIAT	Quindío	Plantain	-
42	1		CIAT	Jamundí, Colombia, no. 1	Pl w. th.	+
43	2		CIAT	Jamundí, Col., no. 3	Pl w. th.	+
44	3		CIAT	Jamundí, Col., no. 4	Pl w. th.	+
45	4		CIAT	Jamundí, Col., no. 9	Pl w. th.	-
46	5		CIAT	Jamundí, Col., no. 10	Pl w. th.	+
47	6		CIAT	Jamundí, Col., no. 11	Pl w. th.	+
48	7		CIAT	Jamundí, Col., no. 1, red	Pl bef. th.	+
49	8		CIAT	Jamundí, Col., no. 1, pink	Pl bef. th.	+
50	9		CIAT	Jamundí, Col., no. 4, red	Pl bef. th.	+
51	10		CIAT	Jamundí, Col., no. 4, clear	Pl bef. th.	+
52	11		CIAT	Jamundí, Col., no. 11a	Pl bef. th.	+
53	12		CIAT	Jamundí, Col., no. 11b	Pl bef. th.	-
54	13		CIAT	Jamundí, Col., Colony a	Soil	+
55	14		CIAT	Jamundí, Col., Colony b	Soil	+
56	Termoterapia 1 roja		CIAT	Jamundí, Col., no. 1, red	Pl bef. th.	+
57	Termoterapia 1 clara		CIAT	Jamundí, Col., no. 1, pink	Pl bef. th.	+
58	Termoterapia 4		CIAT	Jamundí, Col., no. 4	Pl bef. th.	+
59	Termoterapia 11		CIAT	Jamundí, Col., no. 11	Pl bef. th.	+

[a] The first 41 are from CIAT collection. Codes 42 to 59 corresponds to plantain suckers from Jamundí (Valle del Cauca).

[b] Th. = thermotherapy.

[c] Pl bef. th = plantain before thermotherapy; Pl w. th. = plantain with thermotherapy; T, woolly var. = tobacco, woolly variety; Tobacco, Col. var. 37 = tobacco, Colombian variety 37.

We evaluated a band of 288 base pairs generated by amplification with the specific primers Oli1 (5'-GGGGGTAGCTTGCTACCTG CC-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAG GAGT-3'), previously reported by Martins (2000). To do this, we conducted a direct PCR, using 24-h-old cultures grown in nutritive agar (Seal et al. 1992). We resuspended a colony in 100 µL sterilized distilled water and heated it to 95°C for 5 min. It was then centrifuged at 2800 rpm for 5 min, and 5 µL of the supernatant was taken as mold for the PCR reaction.

Each PCR reaction was carried out in a final volume of 25 µL, taking into account the following final concentrations: 0.1 mM of each of dATP, dCTP, dGTP, and dTTP; 1X *Taq* polymerase buffer; 1.5 mM $MgCl_2$; 0.5 U *Taq* polymerase; and 0.16 µM of each primer. Amplification was carried out in an MSJ-Research PTC-100 thermocycler with the following program: 2 min at 96°C, 50 cycles of denaturation for 20 s at 94°C, annealing for 20 s at 68°C, extension for 30 s at 72°C, and another final extension for 10 min at 72°C. The amplified product was visualized in 1.5% agarose gels, stained with ethidium bromide.

DNA sequencing

The products that had been amplified with primers Oli1 and Y2 (fragments of 288 bp) were sequenced. The generated sequences were then homologized with sequences of *R. solanacearum* reported in the NCBI's GenBank database (www.ncbi.nlm.nih.gov), using the application BLAST®n.

Using the program DNAMAN 4.13 and the sequences homologized with *R. solanacearum* and some sequences previously reported in the GenBank, we generated a phylogenetic tree with PAUP and bootstrap statistical analysis with 1000 replications. We took the strains presenting high homology, while taking into account that they may represent the same isolate if they came from the same host and place of origin.

Biochemical testing

When the presence of the 288-bp band was confirmed in the strains of *R. solanacearum* held at Cassava Pathology, We then determined their macroscopic morphology and biochemically characterized each strain to determine its biovar, using eight different tests on 24-h-old pure cultures.

We evaluated reaction to 3% KOH by placing 1 drop of reagent on a glass slide and then dissolving in it a colony from a pure and metabolically active culture with a 24-h growth. A reaction was considered positive if it gave rise to a viscous strand 15 to 30 s later.

The oxidase test was carried out by placing 2 drops of 1% aqueous solution of tetramethyl-*p*-phenylenediamine dihydrochloride (Kovács' oxidase reagent) on a piece of filter paper and rubbing a colony in the drops. A positive reaction was determined by appearing purple color 10 sec later.

Tests were also conducted with the disaccharides cellobiose, lactose, and maltose; and with the oxidation of hexose alcohols (or polyols) dulcitol, mannitol, and sorbitol. The methodology described by Hayward (1964) and Schaad (1988) was followed. The disaccharides and alcohols were sterilized by filtering. Three milliliters of culture medium were placed in test tubes and inoculated with a colony from a 24-h-old culture. They were then incubated at 30°C and reactions were observed at days 3, 7, and 14 after inoculation. The change of color from olive green to yellow, from top to bottom, indicated the production of acids from the disaccharides. When the color changed from purple to yellow, it indicated oxidation of the polyols. Biovars were determined according to the classification proposed by García et al. (1999) and Gunawan et al. (2002).

Results

PCR analysis

The presence of a 288-bp band generated by amplifying with primers Oli1 and Y2 enabled us to confirm the identity of 24 of the 41 strains from the Cassava Pathology collection **(Table 1**, codes 1 to 41). We could also identify 16 of the 18 isolates from plantain as *R. solanacearum* **(Table 1**, codes 42 to 59), 14 of which are shown in **Figure 1**.

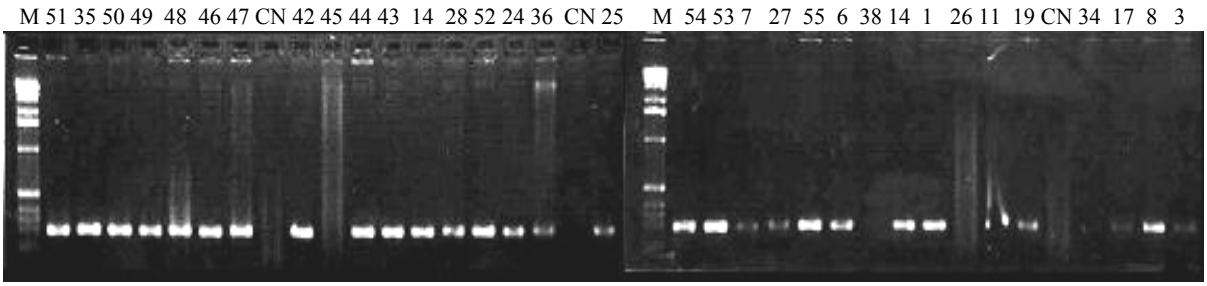

Figure 1. Product amplified from DNA of the bacterium *Ralstonia solanacearum* with primers Oli1/Y2. M = 1-kb-long marker; NC = negative control. The number above each lane corresponds to the strain code number described in Table 2.

DNA sequencing

The DNA sequences of the 288-bp amplified fragment presented high homology with *R. solanacearum*, with a statistical significance (e-value) between 4^{-88} and 1^{-128}. We also saw the presence of identical sequences when bacteria were isolated from different samples of a common origin. For strains isolated from plantain from Jamundí (Valle del Cauca), strain 59 presented a homology of 99% and 100% with strains 50 and 49, respectively. Strains 1, 27, and 8, isolated from tobacco in Floridablanca (Santander), behaved in a similar fashion.

Likewise, identical sequences were isolated from the same host, but of different geographical origins. The sequences of strains 31 and 38, isolated from eggplant, presented a homology of 100%, even though they had come from Kenya and Sri Lanka, respectively.

The results of the sequencing indicated that primers Oli1 and Y2 enabled us to detect a region of the genome of *R. solanacearum* that was being conserved among different races and biovars isolated from different crop species and even among different bacteria species.

The phylogenetic tree **(Figure 1)** was constructed with 44 sequences, including those of strains isolated from plantain, potato, tobacco, tomato, eggplant, Indian shot, and *Heliconia* sp, and selected from GenBank (7, i.e., *R. mannitolilytica*, *R. picketii*, *R. thomasii*, *Burkholderia solanacearum*; and AY 216796, PS01716SR, and AY642432 of *R. solanacearum*). The tree showed that some of the sequenced isolates formed a homogeneous cluster together with isolates from *R. solanacearum, R. mannitolilytica*, *R. picketii*, *R. thomasii*, and *Burkholderia solanacearum* from GenBank. However, they presented minor differences with isolate CIAT 1001 from tobacco and G219 from eggplant, which tended to separate from the rest.

On generating the phylogenetic tree with the sequenced strains, we could not separate them on an evolutionary basis. Nor could we find clusters according to geographical origin or type of host, which indicates that the sequenced region is highly conserved, even among strains of different species and isolated from different hosts.

Biochemical testing

The biochemical tests enabled us to classify some of the strains from the Cassava Pathology collection into different biovars. All the strains tested as Gram negative, and reacted positively to both the oxidase and 3% KOH tests. The results of other tests are described in **Table 2**.

Table 2. Results of biochemical tests carried out with isolates of the bacterium *Ralstonia solanacearum* that had amplified in the specific PCR.

Number in Collection	Crop	Maltose	Lactose	Cellobiose	Mannitol	Sorbitol	Dulcitol	Biovar[a]
1001	Tobacco	-	-	-	-	-	-	1
1004	Potato	-	-	-	+	+	+	4
1007	Tobacco	-	-	-	-	-	-	1
1008	Plantain	-	-	-	+	+	-	D
1010	Tobacco	-	-	-	-	-	-	1
1013	Tobacco	-	-	-	-	-	-	1
1016	Potato	-	-	+	-	-	-	2
1017	Indian shot	-	-	-	-	-	-	1
1020	Tobacco	-	-	-	-	-	-	1
1035	Tobacco	-	-	-	-	-	-	1
1043	Tobacco	-	-	-	+	-	-	D
1054	Tobacco	-	-	-	-	-	-	1
1055	Tobacco	-	-	-	-	-	-	1
1056	Tobacco	+	+	-	+	+	-	3
1057	Tobacco	-	-	-	-	-	-	1
1077	Tomato	-	-	-	-	-	-	1
G213	Potato	-	-	-	-	-	-	1
G175	Egg plant	-	-	-	+	+	+	4
G177	Potato	-	-	-	+	-	-	D
G216	Tobacco	-	-	-	+	+	+	4
G217	*Heliconia* sp.	-	-	-	-	-	-	1
G218	*Capsicum* sp.	-	-	-	-	-	+	D
G219	Egg plant	-	-	-	+	+	+	4

a. D = strain that had biochemical reactions that were outside the expected for the five classes of biovar. No biovar 5 was detected.

Most of the strains isolated from tobacco, together with strains from potato, Indian shot, tomato, and *Heliconia* sp., were classified as Biovar 1. One strain from potato was classified as Biovar 2, another strain from tobacco as Biovar 3, and strains from eggplant, potato, and tobacco as Biovar 4. No strains were classified as Biovar 5, and 4 strains had biochemical reactions that were outside the five classes (marked as "D" in **Table 2**).

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Activity 4. Isolation of *Fusarium oxysporum* f. sp. *cubense*, causal agent of Panama disease of banana, and evaluating its pathogenicity.

Specific objective

To isolate *Fusarium oxysporum* f. sp. *cubense*, causal agent of Panama disease of banana, and evaluate its pathogenicity

Methodology

From processed samples of wilted banana that came from the Department of Quindío, three fungi were isolated and identified by microscope as *Fusarium* spp. They were also selected for their growth in PDA and a selective medium with 0.1% pentachloronitrobenzene (PCNB), developed by Nash and Snyder (1962) to isolate *Fusarium* species from plant tissue. Microscopic observation (40X) showed morphological characteristics of the fungus grown in the above-mentioned selective medium and in PDA.

Isolates identified as *Fusarium* and previously grown for 6 days in PDA were evaluated for their pathogenicity in plants of the banana known as 'Cocos' and plantain under the controlled conditions of the greenhouse. Inoculation was carried out by infiltration in the pseudostem with 3 mL of a suspension with 1×10^6 conidia per milliliter, as calculated in the Neubauer chamber. For an absolute check, pseudostems were also inoculated with sterilized water.

Results

Microscopic examination showed abundant proliferation of microconidia, usually loose and oval, the presence of chlamydospores formed in the hyphae, branched and unbranched monophialids, and scarce presence of macroconidia. In the PDA medium, the colony showed white aerial mycelium and the presence of red pigment, whereas in the Nash and Snyder culture, the mycelium was white and grew slowly.

The isolates were not pathogenic to two genotypes of banana from the FHIA collection, whereas they did infect the regional variety Cocos, cultivated in Quindío.

Reference

Nash SM; Snyder WC. 1962. Quantitative estimations by plate counts of propagules of the bean root rot *Fusarium* in field soils. Phytopathology 52:567–572.

Activity 5. DNA sequence analysis of the 16S rRNA region of phytoplasmas obtained from oil palm, insect vectors, and weeds in Casanare, Colombia.

Specific objectives

1. To identify the causal agent of lethal wilt in oil palm by using PCR and sequencing.
2. To determine possible insect vectors of the causal agent of lethal wilt in oil palm.

Methodology

Sample collection

We collected 268 samples of tissue from oil palm showing early, intermediate, and advanced symptoms (as evaluated by each plantation) of the disease known as "lethal wilt". The causal agent had been found to be a phytoplasma (CIAT 2002). The collection included 42 samples from healthy plants and 7 from weeds collected at affected lots. The samples originated from three plantations **(Table 1)**, located in the Department of Casanare.

Table 1. Detecting phytoplasmas, using nested PCR and primers Fu5/Ru3 and R16F2N/R16R2, in samples of oil palm affected by lethal wilt. The samples were taken from three plantations at Casanare, Colombia.

Sample Code Number	Plot	Tree Line	Palm Tree Number	Plantation	Plant Part	Dev't Dtage of Lethal Wilt	PCR (+)	
							Fu5/ Ru3	R16F2N/ R16R2
P7	9H	162	14	1	Inflorescence	Intermediate		+
P8	9H	162	14	1	Leaf spear	Intermediate		+
P9	9H	162	14	1	Base of leaf spear	Intermediate		+
P10	9H	162	14	1	Meristem	Intermediate		+
P24	9H	17	6	2	Flower primordia	Intermediate		+
P73	8E	16	28	3	Base of leaf spear	Advanced		+
P108	10H	239	2	2	Flower primordia	Advanced		+
P109	10H	88	4	2	Meristem	Advanced		
P110	23H	126	7	2	Flower primordia	Intermediate		+
P114	10H	88	4	2	Forming inflorescence	Advanced		+
P115	23H	126	7	2	Forming inflorescence	Intermediate		+
P120	23H	126	7	2	Base of leaf spear	Intermediate		+
P125	8G	79	11	2	Base of leaf spear	Advanced		+
P130	8G	79	11	2	Base of inflorescence	Advanced		+
P131	G16 (88)	62	P6	1	Inflorescences	Advanced		+
P138	G16 (88)	80	P5	1	Inflorescences	Advanced		+
P143	G17 (88)	65	P1	1	Leaf spears	Advanced		+
P144	G17 (88)	65	P1	1	Meristem	Advanced		+
P 147	10H	99	2	2	Forming inflorescence	Intermediate	+	+
P 148	10H	99	2	2	Leaf spears	Intermediate	+	
P153	10H	99	2	2	Peduncle of inflorescence	Intermediate	+	
P155	9H	3	3	2	Forming inflorescence	Advanced	+	+
P156	9H	3	3	2	Leaf spears	Advanced	+	
P157	9H	3	3	2	Base of leaf spears	Advanced	+	
P158	9H	3	3	2	Isolated meristem	Advanced	+	
P159	9H	3	3	2	Base of meristem	Advanced	+	
P161	9H	3	3	2	Peduncle of inflorescence	Advanced	+	
P174	G18	39	3	1	Base of meristem	Advanced	+	+
P175	G18	39	3	1	Forming inflorescence	Advanced	+	+
P 178	G18	39	3	1	Isolated meristem	Advanced		+
P 185	G17	21	16	1	Lower stipe	Intermediate		+
P 201	10H	96	3	2	Cylinder of stipe	Intermediate		+
P 202	6H	21	9	2	Cylinder of stipe	Intermediate		+
P 203	6H	48	1	2	Cylinder of stipe	Intermediate		+
P 207	6H	20	6	2	Cylinder of stipe	Intermediate		+
P 209	10H	96	2	2	Cylinder of stipe	Intermediate		+
P 213	6H			2	Grass	Infected plot		+
P 214	10H			2	Grass	Infected plot		+

Sample Code Number	Plot	Tree Line	Palm Tree Number	Plantation	Plant Part	Dev't Dtage of Lethal Wilt	PCR (+)	
							Fu5/ Ru3	R16F2N/ R16R2
P 220	MP1C	4	28	1	Base of leaf spear	Affected by Bud Rot		
P 224	9A	10	3	3	Base of meristem	Intermediate		+
P 225	9A	10	3	3	Isolated meristem	Intermediate		+
P 226	9A	10	3	3	Upper meristem	Intermediate		+
P 227	9A	10	3	3	Base of leaf spear	Intermediate		+
P 228	9A	10	3	3	Forming inflorescence	Intermediate		+
P 229	9A	10	3	3	Flower primordia	Intermediate		+
P 230	9A	10	3	3	Fruits	Intermediate		+
P 231	9A	10	3	3	Roots	Intermediate		+
P 233	G16	62	10	1	Roots	Advanced		+
P 234	10H	67	7	2	Leaf spears	Advanced		+
P 235	10H	67	7	2	Base of leaf spear	Advanced		+
P 236	10H	67	7	2	Flower primordia	Advanced		+
P 237	10H	67	7	2	Base of inflorescence	Advanced		+
P 238	10H	67	7	2	Forming inflorescence	Advanced		+
P 239	10H	67	7	2	Base of meristem	Advanced		+
P 240	10H	67	7	2	Meristem	Advanced		+
P 241	10H	67	7	2	Roots	Advanced		+
P 243	9G	35	11	2	Adventitious roots	LW in Bud Rot plot[a]		+
P 245	10H	190	3	2	Adventitious roots	Early		+
P 246	10H	190	3	2	Roots	Early		+
P 247	10H	190	3	2	Forming male inflorescence	Early		+
P 248	10H	190	3	2	Base of meristem	Early		+
P 249	10H	190	3	2	Leaf spears	Early		+
P 250	10H	190	3	2	Base of leaf spears	Early		+
P 251	10H	190	3	2	Flower primordia	Early		+
P 252	10H	190	3	2	Base of inflorescence	Early		+
P 253	10H	190	3	2	Meristem	Early		+
P 254	10H	237	8	2	Roots	Intermediate		+
P 255	10H	237	8	2	Meristem	Intermediate		+
P 256	10H	237	8	2	Base of meristem	Intermediate		+
P 257	10H	237	8	2	Leaf spears	Intermediate		+
P 258	10H	237	8	2	Base of leaf spear	Intermediate		+
P 259	10H	237	8	2	Base of inflorescence	Intermediate		+
P 260	10H	237	8	2	Flower primordia	Intermediate		+
P 261	10H	237	8	2	Forming inflorescence	Intermediate		+
P 262	10H	237	8	2	Fruit	Intermediate		+
P 266	1OH	190	3	2	Leaves	Early		+
P 267	9A	10	3	3	Base of meristem with yellowing	Intermediate		+
P 268	10H	67	7	2	Base of meristem with yellowing	Advanced		+

a. LW = lethal wilt in a site affected by Bud Rot.

DNA extraction

The DNA from palm tissues was extracted as described by Gilbertson et al. (1993) from samples of leaf spear, base of leaf spear, inflorescence, meristem, base of meristem, flower primordium, raceme peduncle, inflorescence peduncle, fruit, stipe, and roots. As positive controls, we used DNA from lulo (*Solanum quitoense*) and periwinkle (*Catharantus roseus*) plants, and samples of palm tissue in which the phytoplasma was originally detected in extractions made in July 2002. The DNA was diluted in sterilized distilled water at a final concentration of 20 µg/µL.

Six collections of insects were made in three plantations at Casanare (Colombia) from November to December 2002, and September 2003 to March 2004. A total of 170 samples were processed, covering different homopterous species of the families Cicadellidae, Nogodinidae, and Membracidae **(Figure 1)**. The insects were collected from the lower parts of infected palms located in plots under the highest disease pressure. The samples were sent either in 70% alcohol or NaCl solution, or dehydrated and wrapped in cotton wool. A total of 24 species of Homoptera were received. From some species, we extracted the following organs: the ovariole or eggs, parts

of the intestine, and glands (female), and intestine and glands (male). To prevent the organs dehydrating, they were extracted with the insects submerged in serum (NaCl at 8.5 g/L).

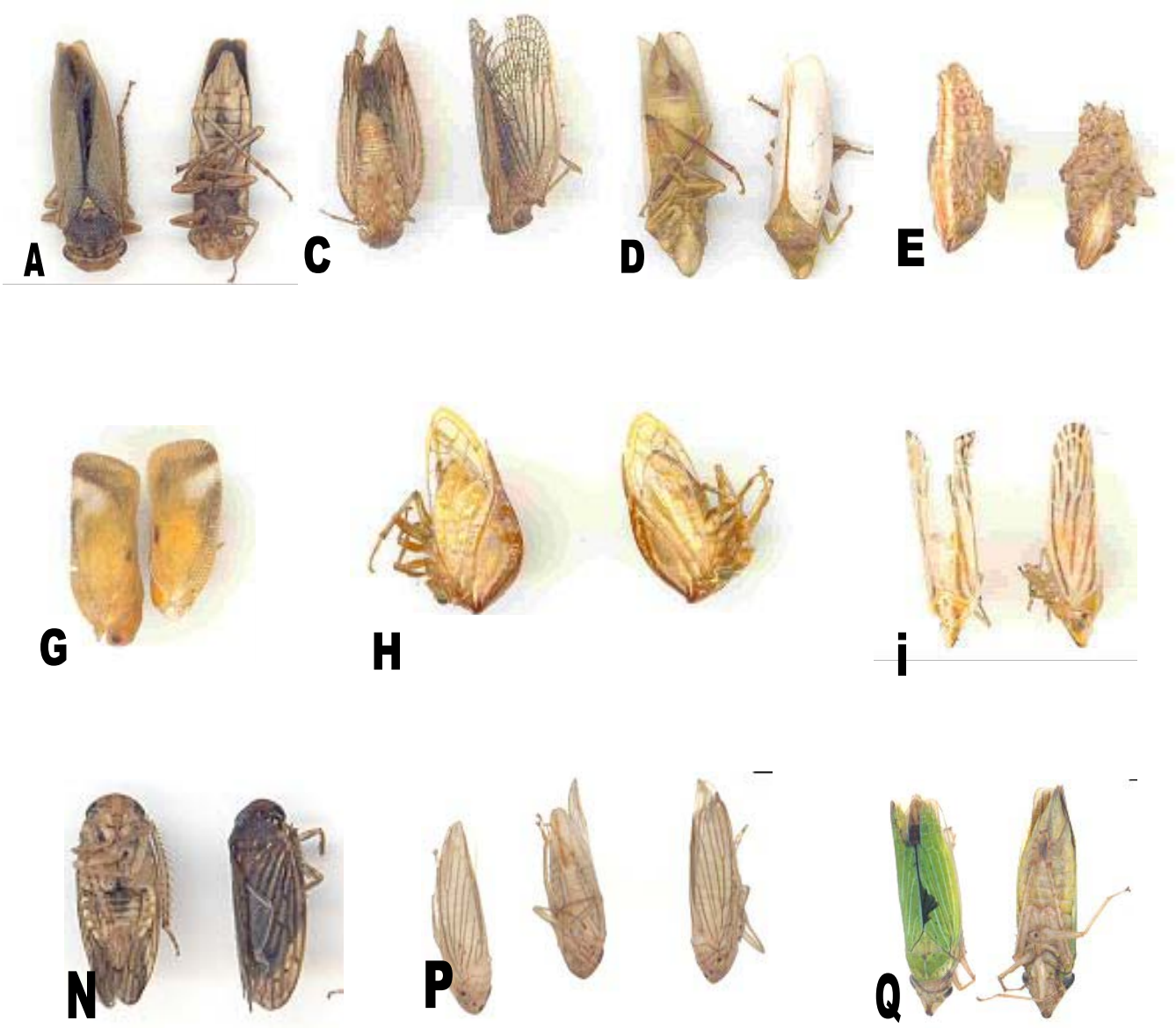

Figure 1. Insect species collected from three oil-palm plantations at Casanare, Colombia, where phytoplasmas were detected. A, E, G, I, N, P, Q = Cicadellidae family; C = Nogodinidae family; H = Membracidae family.

Detection by PCR in plant or insect tissues

Samples of DNA from healthy and infected palms, and from weeds from infected plots were amplified, using direct and nested PCR. For the first amplification we used the primer pairs P1/P7 or R16mF2/R16mR1 under the following conditions: 100 ng DNA, 1X buffer, 3 mM $MgCl_2$, 0.8 mM dNTPs, 0.1 µM of each primer, and 1 U *Taq* polymerase (CIAT). For the primers P1/P7, 35 cycles were conducted in a thermocycler (PTC-100), with the following

conditions: 30 s (90 s for the first cycle) of denaturation at 94°C, annealing for 50 s at 55°C, extension for 80 s at 72°C, and another final extension for 10 min at 72°C. For the primers R16mF2/R16mR1, 28 cycles were conducted under the same conditions as for the previous primers. The PCR products were diluted to 1:50 with sterilized distilled water for use as 1-µL DNA molds in nested PCR. This PCR was amplified with primer pairs R16F2N/R16R2 and Fu5/Ru3, with an annealing temperature of 50°C and 53°C, respectively. The PCR products were analyzed by electrophoresis in 1.5% agarose gel to visualize the bands.

PCR-RFLP

To classify them into phytoplasma groups, the amplified fragments were digested with restriction enzymes *Msp*I, *Alu*I, and *Taq*I. We took 5.4 µL of PCR product and added 2 µL of 10X buffer enzyme and 0.6 µL of restriction enzyme (500 units/µL), and completed the final volume with water to 20 µL. The suspension was incubated for 16 h at 37°C (the enzyme *Taq*I at 65°C). Then 3 µL of loading buffer (0.25% bromphenol blue and glycerol in water at 30%) was added and the whole was run under electrophoresis in Synergel for 6 h at 100 V, 24 mA, in 1X TBE buffer, staining the gel with ethidium bromide at 10 mg/mL.

Electron microscopy

From each sample of plant tissue, 1–3 mm fragments were cut out and pre-fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.2). Post-fixing was done in 1% OsO_4 buffer. The fragments were then dehydrated by immersing for 20 min in pure isopropanol alcohol for each of the following concentrations: 25%, 50%, 70%, 80%, 95%, and 100%. They were then washed three times in pure acetone. The samples were embedded, using 100% and different mixtures of epoxy resin (Spurr's medium) and acetone, all polymerized at 60°C for 8 h.

The embedded samples were cut with a microtome, using a diamond knife, to obtain ultra-thin sections 60 to 90 nm thick. The dispersion of atoms and staining were done with uranyl acetate and Reynold's lead citrate. Observations were then made with a transmission electron microscope. The fixed fragments from a total of 37 tissue samples were evaluated by Dr. Tracey Pepper, specialist in electron microscopy, at the Iowa State University.

Results

DNA extraction

With the Gilbertson extraction method, high quality and high concentration DNA (between 50 and 600 ng/µL) was obtained both from plant tissues and insect organs. However, smaller quantities of DNA (10 to 30 ng/µL) were normally obtained from roots, tending to increase with young, unlignified roots.

Detection by PCR in plant or insect tissues

We obtained amplifications from 40% of processed samples with intermediate or advanced symptoms, 17.5% with early symptoms, and none from healthy samples. Meristem bases presented the highest percentage of amplifications (78%), followed by flower primordia (75%), leaf spears (60%), and stipes (33%). Amplifications were also obtained from all four samples from inflorescence bases. The positive checks from lulo, periwinkle, and palm indicated that the

band obtained from the samples with lethal wilt of palm corresponded to a phytoplasma. **Figures 2 and 3** show the results obtained for the amplifications. For sequencing, we selected samples on the basis of different tissues.

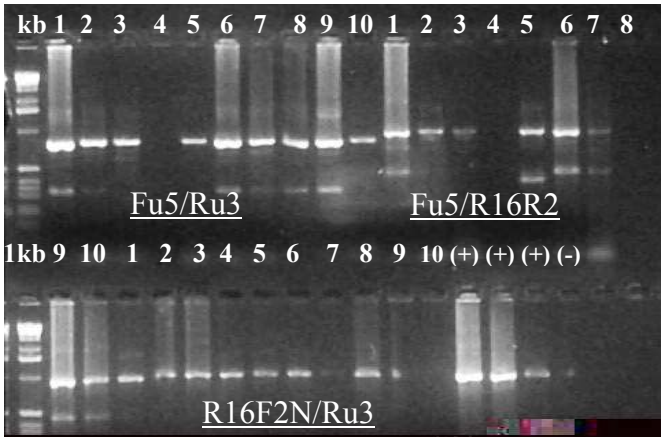

Figure 2. Amplifications with universal primers for phytoplasmas (P1/P7 nested with R16F2N/R16R2–Fu5/Ru3) from oil palms with early symptoms of lethal wilt. M = marker. Samples are lane 1 = p229; 2 = p233; 3 = p236; 4 = p256; 5 = p257; 6 = p258; 7 = p259; 8 = P8; 9 = lulo; 10 = periwinkle. Positive controls for phytoplasmas are lulo and periwinkle; negative control is water.

From crop weeds sampled in infected plots, we obtained PCR amplifications from a grass known as *pasto negro* (black grass), which harbored a high population of insects of the Nogodinidae family. A phytoplasma was also detected by PCR in this insect's tissues.

Phytoplasmas appear exclusively in phloem vessels, have a normally heterogeneous distribution in the plant, and are found in low concentrations. These characteristics make their detection and identification difficult (Seemüller et al. 1998). Hence, we had to design specific primers from the sequences obtained in our study to increase sensitivity for detecting the pathogen in plants with very low levels of inoculum (i.e., early symptoms).

Of the 23 species of insects collected at points of highest disease pressure, 11 presented the highest percentages of amplification, between 45% and 100%, regardless of the organ DNA amplified, indicating the samples as positive (**Figure 3**). Detection by PCR in Nogodinidae, Cicadellidae, and Membracidae was 40%, 57.1%, and 60%, respectively, on preparing a mixture of DNA of different organs extracted from the insects.

According to insect organ, detection for glands was 51.2%; intestines, 68.3%; and eggs, 37.5%, with no significant differences between them. This indicates that, in future evaluations of new species, fragments of the abdomen and entire thoraxes can be taken, without having to dissect each insect, thus making detection more efficient. According to the species of insect, 100% amplifications were obtained from individuals coded as A, E, I, N, and Q; and 50% from individuals coded as G and P, both groups from the Cicadellidae family. Moreover,

amplifications were observed for insects coded as C (Nogodinidae family; 40%) and H (Membracidae family, 60%) **(Figure 1**).

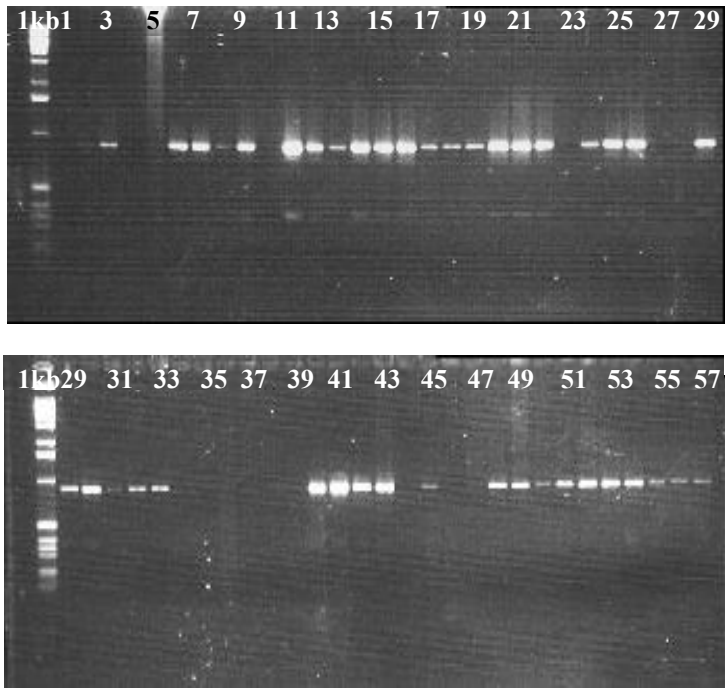

Figure 3. DNA from insect tissues amplified with primers P1/P7 nested with Fu5/Ru3. Lanes 1–24 = Membracidae family; lanes 25–37 = Nogodinidae family; lanes 38–57 = Cicadellidae family.

PCR-RFLP

Taking into account the polymorphism indicated in the patterns of bands (**Figure 4**), we observed that at least two groups of phytoplasmas existed. One corresponded to samples from plantation number 1 (lane 7) and the other to samples from plantation number 2 (lane 8). These results were similar to those obtained with samples from both plantations sequenced at the Iowa State University in 2001 by Elizabeth Álvarez and Thomas Harrington. Lanes 6 and 9 correspond to *Acholeplasma.*

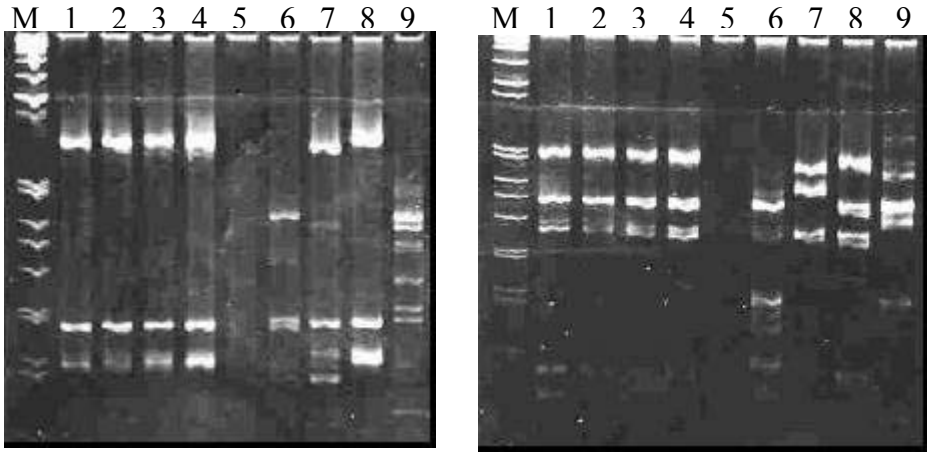

Figure 4. Restriction patterns of DNA amplified and later digested with enzymes *Alu*I and *Rsa*I. M = 1-kb marker; lanes 1–5 = cassava controls; lane 6 = *Acholeplasma* from leaf-base tissue from the oil-palm plantation number 1; lane 7 = phytoplasma from leaf-base tissue from plantation number 1; lane 8 = phytoplasma from inflorescence tissue from the oil-palm plantation number 2; lane 9 = *Acholeplasma* from leaf-base tissue from plantation number 2, Colombia.

Sequencing analysis

The sequence of the DNA band, measuring 1200 nucleotides, was determined, using primers R16F2N/R16R2 and Fu5/Ru3. It was then compared with sequences of phytoplasmas reported in GenBank, using the tool BLAST®n. Some of the amplified fragments were cloned with *Escherichia coli* and sequenced using primers T7 and Sp6.

The phytoplasma sequences analyzed in this study showed homology with sequences of the 16Sr I group (aster yellows; 92%), the 16Sr IV group (coconut lethal yellowing; 93%), and of *Acholeplasma palmae* (94%). All three groups belong to the Acholeplasmataceae family. The computer-assisted aligning of sequences revealed that the sequence of nucleotides of the 16S rRNA region of the some oil-palm phytoplasma was similar to the GenBank accessions AY180932.1, AY249248, and AF 487779, corresponding to Aster Yellows group. Other phytoplasma isolates were similar to the GenBank accessions AF 105316 and AF361019, corresponding to Pigeonpea Witches' Broom group (**Figure 5**).

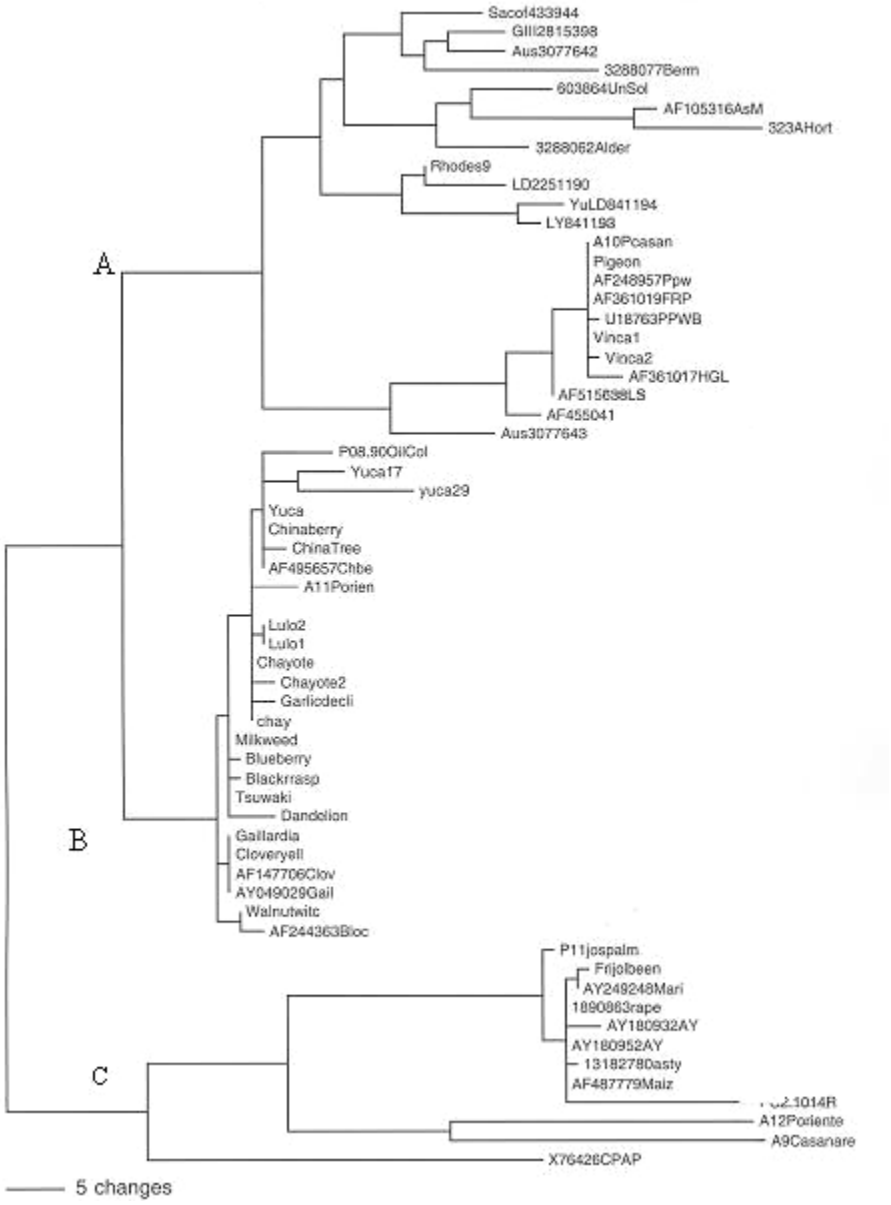

Figure 5. Phylogenetic tree constructed for oil-palm samples, using PAUP and bootstrapping of 1000 replications. Cluster A = 16Sr IX (pigeonpea witches' broom); cluster B = 16Sr III (x-disease); cluster C = 16Sr I (aster yellows). A10Pcasan = plantation number 1 (base of leaf spear); P08.90OilCol = plantation number 2 (inflorescence); A11Porien = plantation number 2 (inflorescence); P11jospalm = plantation number 1 (base of leaf spear); A12Poriente = plantation number 2 (leaf spear); A9Casanare = plantation number 1 (base of leaf spear).

The homology tree (**Figure 6**) presented two genetic groups that separated at 75%. One relatively uniform group was made up of phytoplasmas detected in palm or insect tissues from the three plantations. The other group comprised phytoplasmas from various groups registered at the GenBank database.

The differences between the homologies resulted when, in the first case, comparisons were made between two sequences—one from a palm and the other from GenBank—indicating their similarity; whereas, in the second case, many sequences were compared, thus accentuating their differences, even though these are small. Moreover, the primers used by each researcher reporting sequences to GenBank are different, such that different subregions of the 16S rRNA region are amplified, thus influencing the generation of the homology tree. The GenBank group of phytoplasmas, however, included two sequences of oil-palm phytoplasma corresponding to this study (A9 and FP 2001).

The sequence of phytoplasma DNA obtained from insects and the grass *pasto negro* showed high homology with oil-palm phytoplasmas. Diversity was also found among phytoplasmas detected in palms, insects, and the grass, thus confirming the polymorphism observed with the PCR-RFLP. However, transmission tests must be made to verify that the insect species involved is capable of transmitting the phytoplasma, as an insect may carry the phytoplasma without necessarily being able to transmit it.

The homology analysis of the sequence from the 16S rRNA region and the tRNA gene confirmed the presence of a phytoplasma in association with lethal wilt of oil palm. To produce a more specific diagnosis when detecting the phytoplasma, DNA fragments obtained in this study could be cloned for use as specific probes for pathogenic phytoplasmas in oil palm.

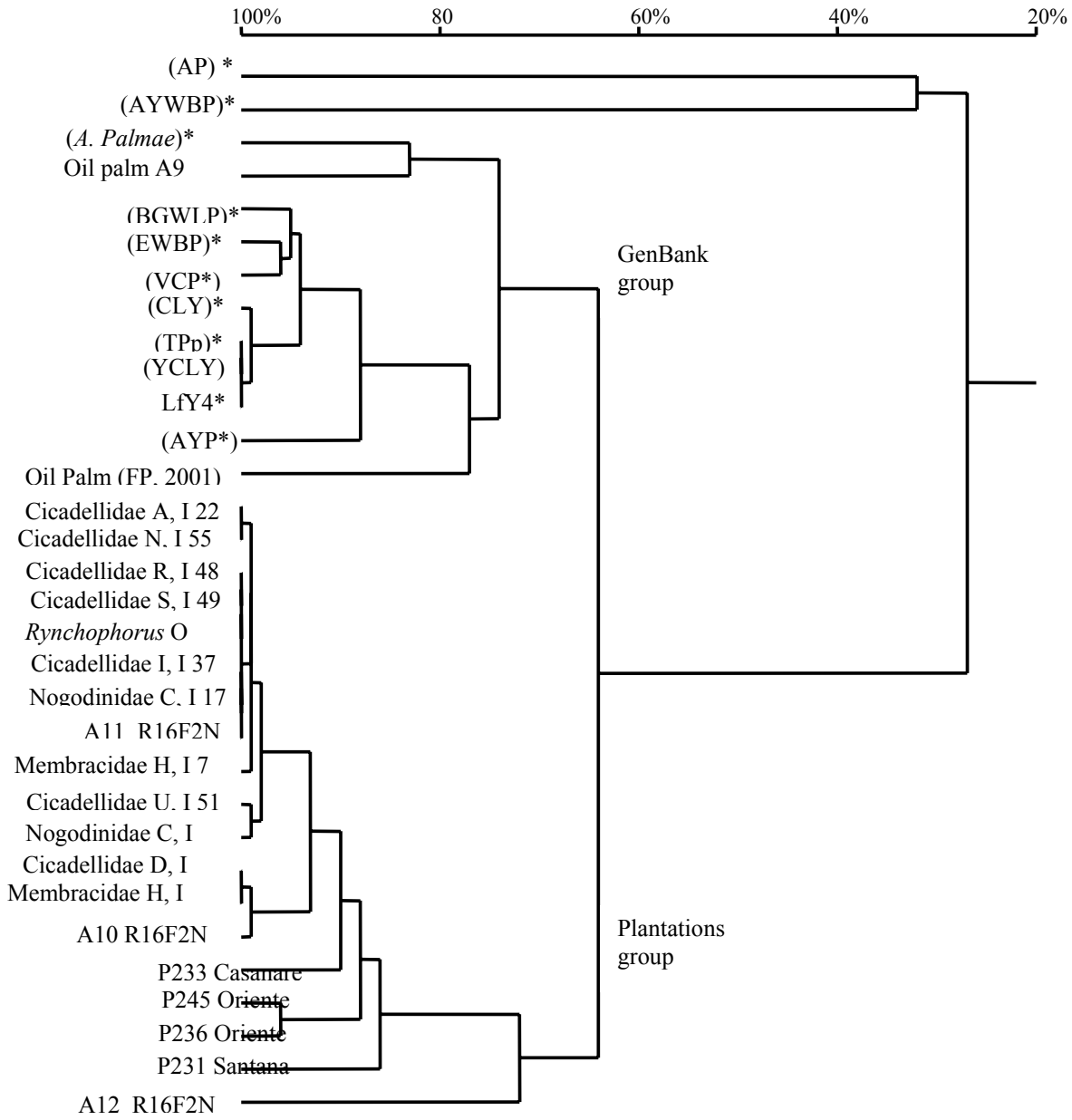

Figure 6. Homology tree of sequences from the 16S rRNA region of phytoplasmas detected in oil palm and insects. * = GenBank accessions: Apple proliferation (AP), Aster yellows witches'-broom phytoplasma (AYWBP), *Acholeplasma palmae* (A. palmae), Bermuda-grass white-leaf phytoplasma (BGWLP), Erigeron witches'-broom phytoplasma (EWBP), Virginia creeper phytoplasma (VCP), Coconut lethal-yellowing phytoplasma (CLY), Texas Phoenix palm phytoplasma (TPP), Yucatan coconut lethal-decline phytoplasma (YCLY), Phytoplasma sp. LfY4 (MA5)-Oaxaca (LfY4), Aster yellows phytoplasma (AYP), and Phytoplasma palma (FP). The codes for the insect ranks correspond to those in Figure 1.

Electron microscopy

In Dr Tracey Pepper's laboratory at the Iowa State University, phytoplasmas were observed in sieve tubes in samples from plantation number 1 and 2, compared with *Catharanthus roseus*, using as a control (**Figure 7**).

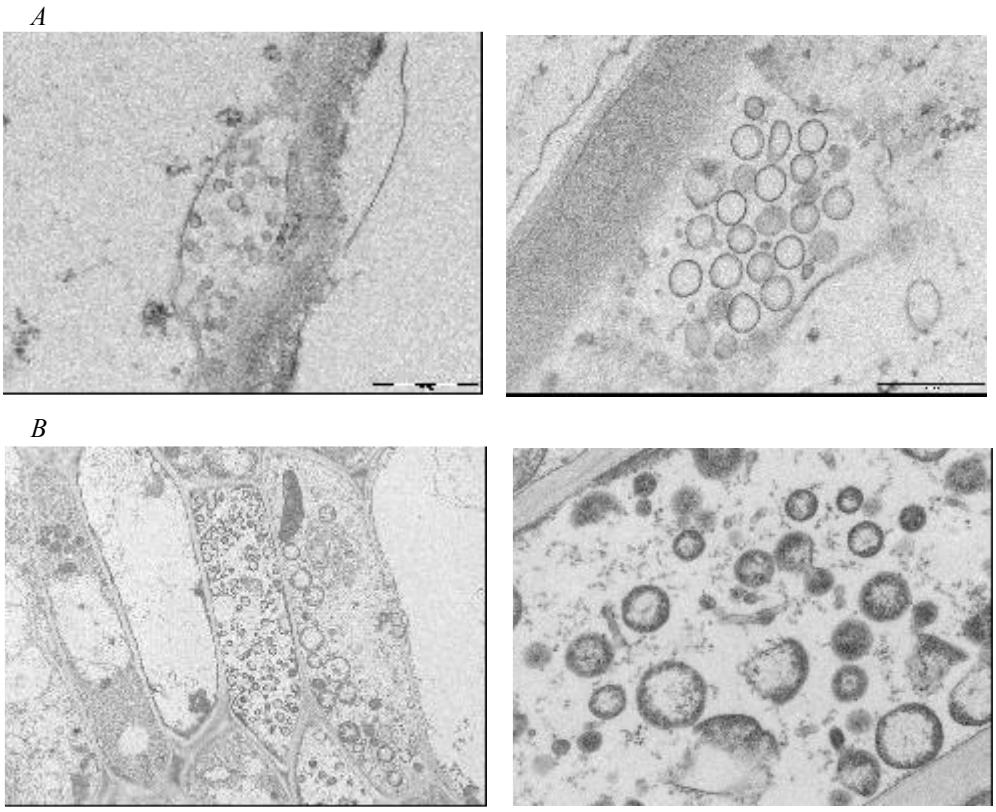

Figure 7. Phytoplasmas found in samples of (A) oil palm infected by lethal wilt, and (B) periwinkle (*Catharanthus roseus*), using transmission electron microscopy.

Conclusions

From our study, we arrive at the following conclusions:

1. We developed a method for detecting, through nested PCR and using primers P1/P7 and re-amplifying with primers R16F2N/R16R2 and Fu5/Ru3, phytoplasmas in (a) 40% of samples of palm tissue showing intermediate and advanced stages of lethal wilt, and (b) in 17.5% of samples of palm tissue showing early symptoms.
2. Phytoplasmas were detected in infected palms, principally in tissues from meristem bases, flower primordia, and leaf spears.
3. Phytoplasmas were detected in insects of different species of the following families: Cicadellidae, Membracidae, and Nogodinidae.

4. Phytoplasmas detected in insects were found principally in glands and intestines.
5. By DNA sequencing, we identified different phytoplasmas in plant and insect tissues.
6. The homology found between sequences of phytoplasmas from plant and insect tissues was almost 100%.
7. A phytoplasma amplified from a grass that grew in an infected plot at plantation number 2 showed high homology with phytoplasmas from palms and insects.

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Seemuller, E.C., U. Marcone, A. Lauer, A. Ragozzino, and M. Goschl. 1998. Current status of molecular classification of the phytoplasma. J. Plant Pathol. 80:3-26.

Activity 6. Collecting oomycetes and bacteria from oil palm, and evaluating their pathogenicity.

Objectives

1. To isolate and conserve bacteria and Pythiaceae microorganisms obtained from oil palm infected with lethal wilt
2. To evaluate their pathogenicity and establish their association with the disease

Introduction

Lethal wilt is considered as an economically significant disease of oil palm. It spreads very quickly, although only over short distances, and causes plant death within 4 to 6 months after the first symptoms appear. To date, the disease cannot be controlled. Activity 5 presented the results of research on the association between phytoplasmas and lethal wilt. In this Activity, we present results of evaluations of not only their pathogenicity but also of bacteria found in association

Methodology

Isolating and conserving bacteria. We took 42 samples from palms infected with lethal wilt and growing in two plantations at Villa Nueva, Department of Casanare, Colombia. The sampled tissues were from roots, leaf spear and base, cylinder of stipe, meristems and base, forming inflorescence, peduncle of inflorescence, and flower primordium. The samples were washed with deionized water for 15 min. We then cut 3-mm fragments from each sample and placed them on nutritive agar medium. The petri dishes were incubated at 30°C for 24 to 48 h. The isolates were then purified, using the same culture medium. The isolates obtained were conserved in 60% glycerol in cryopreservation tubes at -80°C.

Pathogenicity of bacteria. Bacterial colonies isolated from tissues of infected oil palm were planted in nutritive agar and incubated at 30°C for 48 h. A bacterial suspension was then prepared to a concentration of about 1×10^9 cfu/mL. Inoculation with the suspension was done in two ways: the first was to inject 5 mL per point located diagonally on the base of shoots. The second way was to spread 50 mL of the suspension on the soil surface around each palm and cutting the palm's root apices.

The inoculated palms were 2–3 years old, of the genotype AR Malasia, and considered as susceptible to lethal wilt. They had been planted in 60-lb bags containing a mixture of 2 parts sand to 1 part soil and pasteurized by steaming for 5 h at about 80°C. Two palms per isolate were inoculated and incubated for 3 days at 20°C to 30°C in a humidity chamber. Relative humidity was at first 98%, then 60%, and raised again to 90%. Negative controls were palms or bags inoculated with sterilized distilled water.

Obtaining Pythiaceae isolates. Roots were washed with deionized water, and 3-mm fragments were cut out. These were disinfected first in 1% sodium hypochlorite and then in 50% ethanol for 1 min. They were then dried on sterilized paper toweling and five fragments were planted in

each of three selective media: oat agar (OA), potato dextrose agar (PDA), and V8-agar (V8A). The media contained benomyl at 15 ppm, rifampicin (10), penicillin (200), hymexazol (50), and PCNB (200). Tissue fragments were also planted in OA, PDA, and V8A without antibiotics or fungicides. The petri dishes were incubated at 20°C in the dark for 10–15 days. The cultures were checked every day for the presence of oomycetes. Tips of hyphae were also planted to ensure purity of the cultures.

Conserving Pythiaceae isolates. The isolates were conserved in 10 mL of nutritive broth and incubated at 26°C for 48 h and then at 15°C. Fragments of mycelia from PDA were also conserved in water at 15°C.

Extracting total DNA. We took about 0.1 g of fresh mycelia from pure isolates planted in OA and PDA and placed them in 1.5-mL Eppendorf tubes. We added a small quantity of beach sand, 50 mg polyvinylpolypyrrolidone (PVPP), and 750 µL extraction buffer (200 mM Tris HCl, pH 7.5; 250 mM NaCl; 25 mM EDTA; and 0.5% SDS). The mixture was homogenized with a plastic Eppendorf micro-pestle, centrifuged at 13,000 rpm for 5 min, and the supernatant removed to a fresh tube. We then added 500 µL of phenol:chloroform:isoamyl alcohol at 25:24:1 to the supernatant, and gently inverted and re-inverted the whole several times. It was then centrifuged at 13,000 rpm for 5 min, and the aqueous phase removed. To the remaining volume, we added cold isopropanol and vigorously inverted and re-inverted the whole several times. It was then centrifuged at 13,000 rpm for 10 min, after which the entire supernatant was discarded. The remaining pellet was washed with 1 mL 70% ethanol, mixed vigorously, and centrifuged for 2 min to later discard excess ethanol. The pellet was dried and resuspended in 30 µL sterilized bi-distilled water. We added 1 µL RNase at 10 mg/mL, and the whole was conserved at -20°C (http://www.phytid.org/methods.htm).

PCR-RFLP and sequencing. Total DNA was used at a concentration of 5 ng/µL to amplify the internal transcribed spacer regions (ITS 1 and ITS 2) and subunit 5.8S of rDNA with the universal primers ITS 4 and ITS 6. Conditions were as follows: 5 ng DNA, 1X buffer, 1 mg/mL bovine serum albumin (BSA), 3 mM $MgCl_2$, 0.8 mM dNTPs, 0.5 µM of each primer, and 1 U *Taq* polymerase. We conducted 35 cycles in a PTC-100 thermocycler, using the following conditions: 30 s (3 min for the first cycle) of denaturation at 94°C, annealing for 30 s at 55°C, extension of the primer for 60 s at 72°C, and a final extension for 10 min at 72°C. The range of molecular weight expected in the bands was 860 to 950 bp for Pythiaceae. As positive control, we used an isolate of *P. tropicalis*.

To determine the species and classify the isolates, the amplified fragments were digested with restriction enzymes *Msp*I, *Alu*I, and *Taq*I. We took 5.4 µL of the PCR product and added 2 µL 10X enzyme buffer and 0.6 µL restriction enzyme (500 U/µL), and completed the final volume of 20 µL with water. This suspension was incubated for 16 h at 37°C (that with the enzyme *Taq*I was incubated at 65°C). Then, 3 µL loading buffer (0.25% bromphenol blue and glycerol in water at 30%) were added. Electrophoresis was run in Synergel™ for 6 h at 100 V, 24 mA, in TBE 1X buffer and stained with 10 mg/mL ethidium bromide.

The DNA fragments, amplified in the ITS region, were purified by adding a volume of a 20% solution of polyethyleneglycol (PEG) with 2.5 M NaCl for later sequencing with BigDye

Terminator Kit (Applied Biosystems) in an ABI Prism® 377 sequencer. Sequencing analysis was conducted with the programs Sequencher 4.1 and DNAMAN 4.13. Homology was sought in GenBank (www.ncbi.nlm.nih.gov), using the tool Blast®n. Homology was determined on the basis of the region ITS 1, 5.8S and ITS 2.

Pythiaceae pathogenicity. The six Pythiaceae isolates obtained from planting in PDA culture medium were incubated for 8 to 12 days at 20°C, and then inoculated into 6 to 8-month-old palms of the variety AR Malasia. The inoculation method was to inject the base of shoots of 5-month-old plants, standing 125 to 150 cm high, through perforations 3 to 5 cm deep into the plant and with 4-mm diameters. The inoculum was 10 mL of a suspension of mycelia from PDA or OA had been colonized by the microorganism. Once the perforation was injected, the orifice was covered with cement. For controls, we had plants inoculated with sterilized water.

The inoculated plants were incubated at a relative humidity of 90%–100% and 22°C and 30°C for 3 days. The plants were then incubated at an RH between 50% and 80% and 19°C and 30°C.

Results

Isolating bacteria and evaluating their pathogenicity. From the processed plant samples, we obtained 102 bacterial isolates. Colonies of different colors and growth types were isolated. **Table 1** presents the origins of the isolates that are now being evaluated for their pathogenicity. The bacterial colonies were grouped according to morphological characteristics, and 66 isolates were selected for inoculating the palms. Four months after inoculation, the bacteria's pathogenicity was evaluated.

Table 1. Origins of the bacterial isolates obtained from tissue samples of oil palms infected with lethal wilt, Department of Casanare, Colombia.

Tissue	No. of Samples	No. of Isolates	
		Isolated	Inoculated
Base of leaf spear	5	10	6
Base of meristem	4	11	10
Cylinder of stipe	5	5	5
Leaf spear	2	5	5
Forming inflorescence	3	7	6
Meristem, including base	1	1	1
Meristem, not including base	2	6	5
Peduncle of inflorescence	3	10	5
Flower primordium	3	4	3
Root	14	43	20
Total	**42**	**102**	**66**

Isolating and identifying Pythiaceae, and evaluating their pathogenicity. From processed root samples we obtained four isolates belonging to the Pythiaceae family. **Table 2** indicates their origins.

Table 2. Origins of Pythiaceae isolates obtained from root tissues of oil palms infected with lethal wilt (LW), Department of Casanare, Colombia.

Isolate No.	Sample No.	Plot	Tree Line	Palm No.	Plantation	Severity of LW
P216	P216	G16	57	4	1	Healthy in disease pressure point
P217	P217	G16	62	10	1	Intermediate
P231	P231	9A	10	3	2	Intermediate
P241	P241	10H	67	7	3	Advanced

For the four isolates evaluated, on amplifying the primers ITS 4 and ITS 6, we obtained a band of about 950 bp, larger than that of the positive control, *P. tropicalis*, which was 900 bp. These fragments were then digested with restriction enzymes. Although enzymes *Alu*I and *Taq*I had different band patterns, they remained consistent for the four isolates. Enzyme *Msp*I digested only the positive control and isolate P241. The band patterns obtained with the three enzymes were compared with patterns reported for some *Phytophthora* species (Cooke and Duncan 1997). No similarities were found, indicating that either an unreported species or a different Pythiaceae such as *Pythium* sp. was found.

To confirm these results, we sequenced the region ITS 1, 5.8S, and ITS 2 (between 600 and 750 bp) of the four isolates and sought for homology in GenBank. Isolates P216, P217, P231, and P241 showed a homology of 97% with *Pythium chamaehyphon* and 96% with *Pythium vexans* **(Table 3)**.

Table 3. Homology found between sequences of DNA obtained from oomycetes by PCR and sequences of Pythiaceae reported in GenBank.

Pairing with GenBank[a]	GenBank Code	Probability of Greatest Homology[b]	Homologized Bases[c]	Homology (%)
Pythium chamaehyphon ITS 1, 5.8S rRNA gene, and ITS 2, strain MS6-10-8V (792)	PCH233440	0 1e-78	457/469 204/220	97 92
Pythium vexans ITS 1, 5.8S rRNA gene, and ITS 2, complete sequence (789)	AY269998.1	1e-139 1e-132	290/301 241/241	96 100

a. Italicized value in parentheses indicates total number of bases reported in GenBank.
b. The value 0 is expected for the highest percentages of homology.
c. Values indicate number of bases homologized in different regions of the sequence reported in GenBank.

The alignment of the sequences revealed that the sequence of the nucleotides of region ITS 1, 5.8S, and ITS 2 of GenBank the isolates was similar to that for the same region in the GenBank accessions AY269998.1 and PCH233440 belonging to the *Pythium* genus, thus confirming the results obtained for the restriction patterns.

Although no symptoms have appeared so far in the palms inoculated with the bacterial and oomycete isolates, we are still evaluating the trials established at CIAT.

References

Cooke D. E. L. & Duncan J. M. (1997) Phylogenetic analysis of *Phytophthora* species based on the ITS1 and ITS2 sequences of ribosomal DNA. *Myc. Res.* 101, 667-677.

NCBI (National Center for Biotechnology Information). GenBank overview. http://www.ncbi.nlm.nih.gov/Genbank/ (June, 2004).

PhytID - Identification of Plant Pathogenic *Phytophthora* Species by ITS Fingerprinting, http://www.phytid.org/methods.htm (September, 2003).

Activity 7. DNA sequence analysis of the ITS region of oomycete species obtained from oil palm.

From processed samples of roots we obtained four isolates (**Figure 1** shows two) belonging to the Pythiaceae family. The origins of the isolates are described in **Table 1**.

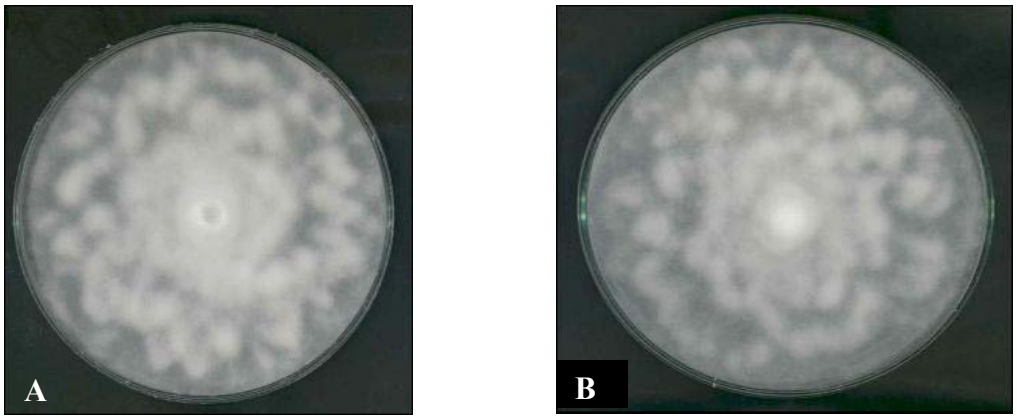

Figure 1. Isolates of oomycete species obtained from oil-palm roots in plot G16 of plantation number 1, Villanueva, Department of Casanare, Colombia. (A) Isolate P216 obtained from an oil palm infected with lethal wilt; (B) isolate P217 from a palm from the same plot but not showing lethal wilt.

Table 1. Origins of Pythiaceae isolates obtained from roots of oil palms infected with lethal wilt (LW) in the Department of Casanare, Colombia.

Isolate Code Number	Sample Code Number	Plot	Tree Line	Palm Tree Number	Plantation	Tissue	Dev't Stage of LW
P216	P216	G16	57	4	1	Roots	Healthy in site of highest disease pressure
P217	P217	G16	62	10	1	Roots	Intermediate
P231	P231	9A	10	3	2	Roots	Intermediate
P241	P241	10H	67	7	3	Roots	Advanced

Although no symptoms, bacteria, or oomycetes have yet been reproduced, we are continuing with the evaluations of the trials established at CIAT.

PCR-RFLP and sequencing

On amplifying with primers ITS 4 and ITS 6, we obtained, for the four isolates, a band size of about 950 bp, contrasting with the 900 bp of the positive control of *Phytophthora tropicalis* **(Figure 2A)**. When these fragments were digested with restriction enzymes, we saw that enzymes *Alu*I and *Taq*I had band patterns that differed among themselves but were exactly the same for the four isolates **(Figures 2B** and **2C)**. Enzyme *Msp*I only digested the positive control and isolate P241 **(Figure 2D)**. The band patterns obtained with the three enzymes were compared with patterns reported for some *Phytophthora* species (Cooke and Duncan 1997). No

similarities were found among them, indicating either an unreported species or another Pythiaceae such as *Pythium* sp.

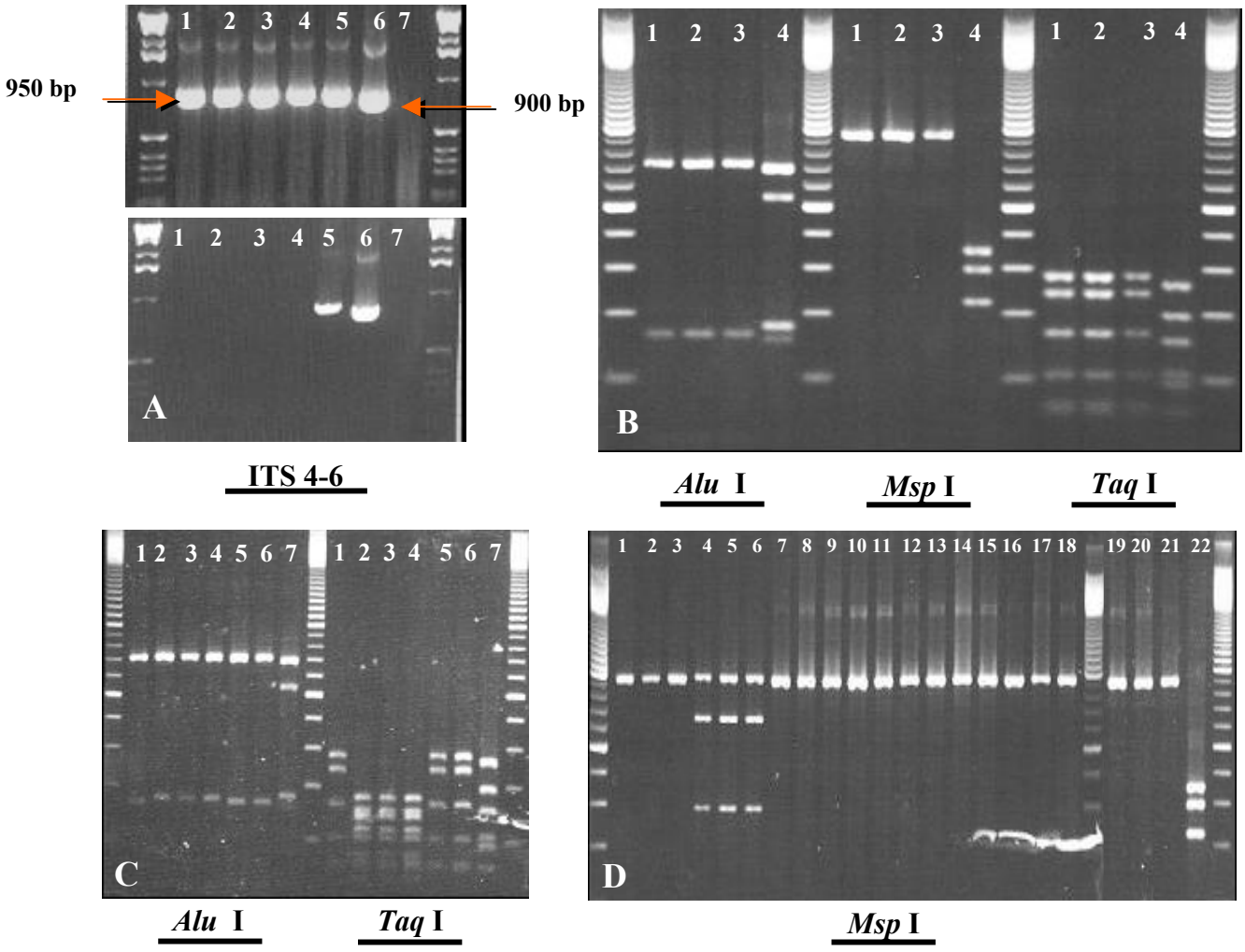

Figure 2. **(A) Amplification of an oomycete species with primers ITS 4 and ITS 6: lanes 1 and 2 = P216; lane 3 = P217; lane 4 = P231; lane 5 = P241; lane 6 = *Phytophthora tropicalis*; lane 7 = negative control; end lanes = 1-kb marker. (B) Cuts of the amplified product by restriction enzymes *Alu*I, *Msp*I, and *Taq*I: lane 1 = P216; lane 2 = P217; lane 3 = P231; lane 4 = *P. tropicalis.* (C) Cuts of the amplified product by restriction enzymes *Alu*I and *Taq*I: lane 1 = P231; lanes 2–4 = P241; lane 5 = P217; lane 6 = P216; lane 7 = *P. tropicalis.* (D) Cuts of the amplified product by restriction enzyme *Msp*I: lanes 1–3 = P231; lanes 4–6 = P241; lanes 7–12 = P217; lanes 13–18 = P216; lanes 19–21 = P231; lane 22 = *P. tropicalis.***

To confirm these results, the four isolates were sequenced and homologized according the whole region ITS 1, 5.8S, and ITS 2 at GenBank. Between 600 and 750 bases could be sequenced for each isolate. The isolates P216, P217, P231, and P241 showed a homology of 97% with *Pythium*

chamaehyphon and 96% with *Pythium vexans* **(Table 2)**. Alignment of the sequences showed that the sequence of the nucleotides in the region ITS 1, 5.8S, and ITS 2 in GenBank was similar to that same region of the GenBank accessions AY269998.1 and PCH233440, belonging to the *Pythium* genus, thus confirming the results obtained with the restriction patterns.

Table 2. Homology found between sequences of DNA from oomycete species obtained by PCR and sequences of Pythiaceae reported in the GenBank database. Homology report refers to two-fragment sequence.

Pairing with GenBank[a]	GenBank Code Number	Probability of high Homology[b]	Homologized Bases[c]	Homology (%)
Pythium chamaehyphon ITS 1, 5.8S rRNA gene and ITS 2, strain MS6-10-8V (792)	PCH233440	0	457/469	97
		1e-78	204/220	92
Pythium vexans ITS 1, 5.8S rRNA and ITS 2, complete sequence (789)	AY269998.1	e-139	290/301	96
		e-132	241/241	100

a. Italicized value in parentheses indicates total number of bases reported in GenBank; ITS = internal transcribed spacer; rRNA = ribosomal RNA.
b. The value 0 is expected for the highest percentages of homology.
c. Values indicate number of bases showing homology in different regions of the sequence reported in GenBank.

Reference

Cooke D. E. L. and Duncan J. M. 1997. Phylogenetic analysis of *Phytophthora* species based on the ITS1 and ITS2 sequences of ribosomal DNA. Mycological. 101, 667-677.

Activity 8. Detecting phytoplasmas in cassava affected by frogskin disease (FSD), using nested PCR.

Specific objective

To detect phytoplasmas in cassava plants affected by frogskin disease (FSD)

Methodology

Plant tissues. Asexual planting materials (stakes) from 40 plants of the commercial cassava varieties Catumare and Manzana were obtained. Twenty of the plants came from Rozo and Palmira, Department of Valle del Cauca, Colombia, and were either moderately infected (Catumare) or severely affected (Manzana) by FSD. The other 20 plants were disease-free and came from Montenegro, Department of Quindío, Colombia.

The stakes, 20 cm long, were planted in plastic bags containing pasteurized soil that was free of FSD. The bags were placed on plates to prevent contamination during watering.

All the plants were fertilized periodically and left in anti-aphid cages. The plants and cages were fumigated periodically, rotating the following products: Vertimec® 1.8% CE (abamectin at 0.5 cc/L of the commercial product), Malathion® (malathion at 1 cc/L), and Sistemin® (dimethoate at 3 cc/L).

As control we used healthy 'Secundina' from in vitro plants, placing them in the cages with the varieties being evaluated. They also functioned as monitors for the presence of insect vectors.

The trials were established in a greenhouse and screen house under different conditions of relative humidity and temperature. The greenhouse had an RH of 31% to 98%, and temperatures varied from 19°C to 28°C. Four replications of 10 plants were used per variety in each of the greenhouse and screen house, and placed in the same cages of their respective varieties.

Healthy plants from Armenia, Quindío, were also established under equal conditions in the same greenhouse and screen house but in separate cages.

Insects. In a separate experiment, Homopterans (*Scaphytopius marginelineatus*) were collected from cassava crops infected with FSD, and breeding was established in cages containing diseased plants. After a couple of generations, adult insects were transferred to healthy plants to test for transmission of disease (CIAT Cassava Entomology Section, personal communication, 2004).

DNA extraction. Total DNA was extracted as described by Gilbertson et al. (1983).

Nested-PCR analysis. We amplified 50 ng of genomic DNA, using nested PCR with the universal primers R16F2/R16R2 and the primers specific to the 16SrIII group (X-disease), R16(III)F2/R16(III)R1. The cocktail was prepared with 2 mM dNTPs, 1X Taq buffer, 2.5 mM $MgCl_2$, 1 U Taq polymerase, and 10 μM of each primer. The conditions for amplification were

94°C for the initial denaturation for 2 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 2 min, extension at 72°C for 3 min, and a final extension at 72°C for 10 min. PCR products were analyzed by electrophoresis on 1.5% agarose gel.

RFLP analyses. All amplified PCR products were digested with the restriction endonucleases *Alu*I, *Rsa*I, and *Taq*I to confirm the presence of a single group of phytoplasmas associated with the disease. The restriction products were analyzed by electrophoresis on 5% polyacrylamide gel. These enzymes had been used previously to classify FSD phytoplasmas.

DNA sequencing. The amplified PCR products were cleaned, using a purification kit (QIAGEN) and then sequenced by automated dideoxy sequencing (ABI PRISM® 377-96 DNA Sequencer, Applied Biosystems). The sequences obtained were homologized with the sequences reported in GenBank to identify the organism detected in the evaluated samples.

Results

DNA extraction. A total of 320 DNA samples were obtained from infected plant tissues —160 from roots and 160 from leaf midribs and petioles—and another 80 from healthy tissues. All samples were from the varieties Catumare and Manzana **(Table 1)**. In addition, 17 samples were extracted from tissues at different developmental stages of the insect *S. marginelineatus*, processing 1 to 2 individuals per sample **(Table 2)**.

Table 1. Tissues evaluated, using nested PCR and sequencing, to determine the incidence of phytoplasmas in cassava plants infected with frogskin disease.

Tissue	Variety	Samples processed[a]		No. of Positive Samples	% Nested PCR	PCR[b] sequencing	
		Roots	P and MR			Roots	P and MR
Infected[c]	Catumare[d]	80	80	124	77	8	10
Infected	Manzana[e]	80	80	138	86	10	12
Healthy[f]	Catumare	20	20	0	0	-	-
Healthy	Manzana	20	20	0	0	-	-

a. Total number of samples processed in the greenhouse and screen house; P = leaf petioles; MR = leaf midribs.
b. The same number of samples was taken for both greenhouse and screen house. P = leaf petioles; MR = leaf midribs.
c. Seed came from plots infected with frogskin disease in Rozo and Palmira, Valle del Cauca.
d. Moderately infected.
e. Severely infected.
f. Seed came from plots free of frogskin disease in Montenegro, Quindío.

Nested-PCR analysis. Of the 320 infected-plant-tissue samples evaluated, 262 were detected as having a phytoplasma (82%); of the 17 samples from insects fed on diseased plants, 50% showed amplification; and of the 80 healthy plant tissues, no amplifications were obtained.

The presence of a phytoplasma was shown by visualization in agarose gels. Bands of about 800 bp—typical of the 16SrIII group—appeared when the primer pair R16(III)F2/R16(III)R1 was used. The rates of detecting the presence of phytoplasmas in plants (82%) **(Figure 1A)** and insects (50%) **(Figure 1B)** are high, considering that a rate of no detection of phytoplasmas is possible in plants presenting symptoms typically associated with them. Lack of detection could

be attributed to substances in plant-tissue extracts inhibiting amplification, irregular distribution of phytoplasmas in the plant, or low concentrations of the microorganism in either plant or insect tissues (Chen and Liao 1975; Lee et al. 1994; Bianchini 2001).

Table 2. Identifying phytoplasmas in Homopterans (*Scaphytopius marginelineatus*) as evaluated by nested PCR with primers R16F2/R16R2 and R16(III)F2/ R16(III)R1.

	Sample	Genotype[a]	Stage	Nested PCR[b]
1	1[a]	M Col 2063 (I)	Adult	+(S)
2	1B	M Col 2063 (I)	Nymph	+
3	1C	M Col 2063 (I)	Nymph	-
4	2[a]	M Col 2063 (I)	Adult	+
5	2B	M Col 2063 (I)	Nymph	-
6	2C	M Col 2063 (I)	Nymph	+
7	3B	M Col 2063 (I)	Nymph	-
8	3C	M Col 2063 (I)	Nymph	+
9	4[a]	M Col 2063 (H?)	Adult	-
10	4B	M Col 2063 (H?)	Nymph	-
11	4C	M Col 2063 (H?)	Nymph	-
12	SE1	M Col 2063 (I)	Adult	-
13	Ss1	M Col 2063 (H?)	Adult	-
14	F1	Bean(H)	Adult	-
15	383 (1)	M Bra 383(I)	Male nymph	-
16	383 (2)	M Bra 383(I)	Female nymph	-
17	383 (3)	M Bra 383(I)	Adult	+(S)

a. Cassava germplasm materials facilitated and qualified as healthy or diseased by the CIAT Virology Unit. (I) = infected; (H) = healthy.

b. (S) Samples sequencing.

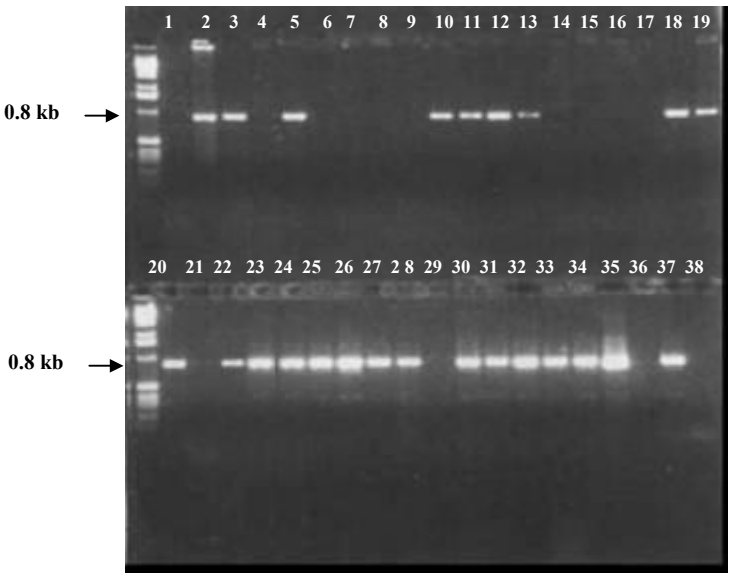

Figure 1A. DNA from infected tissues amplified with primers R16F2/R16R2 nested with R16(III)F2/R16(III)R1. Lanes 1–19 = Screenhouse tissues; lanes 20–38 = Glasshouse tissues.

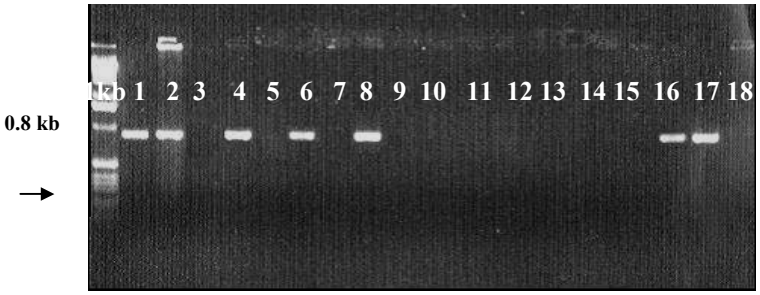

Figure 1B. Presence of phytoplasm typical of the group 16Sr III for *S. marginelineatus*, fed on infected plants, line 1,2,4,6,8 and 17; line 18 positive control and line 19 negative control, 1kb: Marker of molecular weight.

RFLP analyses. The band pattern obtained for the 262 samples amplified with the three evaluated enzymes enabled us to confirm that the products belonged to the 16SrIII group (X-disease) **(Figure 2)**.

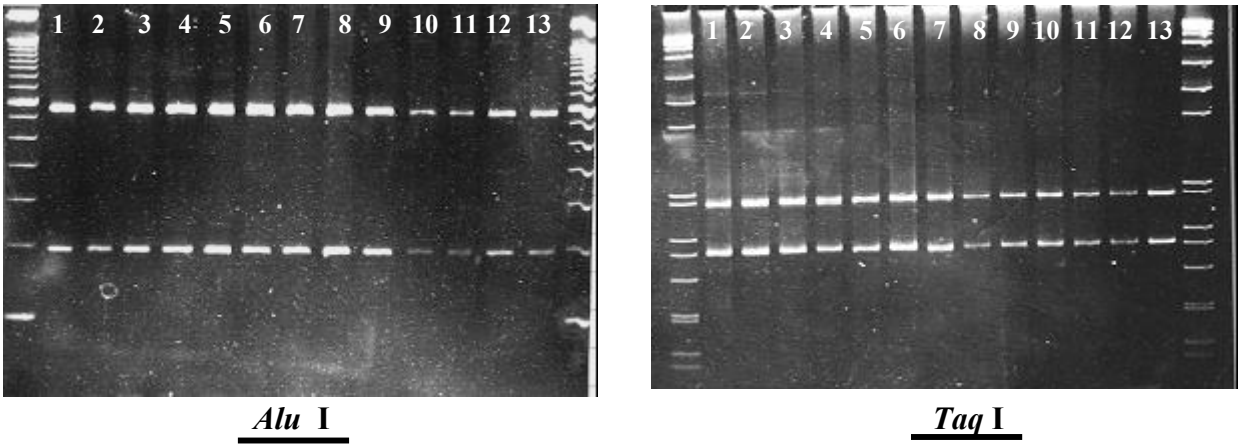

Figure 2. Cuts of the amplified product with primers R16(III)F2/R16(III)R1 by restriction enzymes *Alu*I and *Taq*I. Lines 1-6 (Glasshouse) and 7-13 (Screenhouse).

DNA sequencing. For the DNA amplifications, we took representative tissue samples from the roots and leaves (midrib and petiole) of infected cassava varieties in the field and in the greenhouse and screen house where the trials took place. The 40 fragments of plant DNA and 2 of insect DNA **(Table 1 and 2)** were then directly sequenced, purifying the PCR products.

The sequence analysis of the 42 fragments revealed that the cassava phytoplasma was similar to Cirsium white-leaf phytoplasma (GenBank accession no. AF373106, 16SrIII or X-disease group), with a sequence homology of 100% in fragments measuring 800 bp. These findings thus confirmed that the amplified products belong to a phytoplasma associated with FSD in cassava (CIAT 2003).

A homology of 90% was found among the sequenced fragments from insect tissue and from

tissues of the varieties Manzana and Catumare. Given these homology results, being based on the nested-PCR technique, new transmission trials are being evaluated by Cassava Entomology Section at CIAT. The Section will first evaluate plants regarded as healthy or diseased and then evaluate plants on which those homopterans insects identified as possible vectors have fed.

This study shows evidence of an association between FSD and phytoplasmas. By applying molecular tools, a phytoplasma was successfully detected in FSD-infected cassava roots and leaf midribs.

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Activity 9. Identifying phytoplasmas by sequencing PCR products.

Specific objective

To identify, through DNA sequencing, the phytoplasma associated with frogskin disease (FSD) of cassava.

Methodology

Plant tissues. Roots, petioles, and leaf midribs of both FSD-infected and healthy cassava plants, grown in the field and greenhouse, were processed. We evaluated 41 samples from cassava genotypes and varieties of three areas of Colombia—Atlantic Coast, Valle del Cauca, and Cauca—where FSD has high incidence. The goal was to confirm the presence of phytoplasmas in plants showing symptoms of FSD **(Table 1)**.

Table 1. List of DNA fragments obtained from samples of tissues of 41 cassava varieties infected with frogskin disease. The samples were amplified by nested and direct PCR, using universal primers and primers specific for phytoplasmas.

	Variety	Tissue	Site[a]	PCR	Primers[b]
1	CM 6740-7	Leaf midrib	Agrovélez	+	A
	CM 6740-7	Root	Agrovélez	+	A
	CM 6740-7	Leaf midrib[c]	CIAT-greenhouse	+	A
	CM 6740-7	Root	CIAT-greenhouse	+	A
	CM 6740-7	Leaf midrib[c]	Santa Elena-field	+	C
	CM 6740-7	Root	Santa Elena-field	-	C
2	Parrita	Shoot	Agrovélez	+	B
	Parrita	Leaf midrib	Agrovélez	+	B
	Parrita	Stem	Agrovélez	+	B
	Parrita	Petiole	Agrovélez	+	B
	Parrita	Root	Agrovélez	+	B
	Parrita	Leaf midrib	CIAT-greenhouse	-	B
	Parrita	Petiole	CIAT-greenhouse	-	B
	Parrita	Stem	CIAT-greenhouse	-	B
	Parrita	Rootlet	CIAT-greenhouse	-	B
3	Catumare	Leaf midrib	Montenegro	-	B
	Catumare	Root	Montenegro	-	B
	Catumare	Leaf midrib[c]	Rozo-field	+	B
	Catumare	Root	Rozo-field	+	B
	Catumare	Leaf midrib	CIAT-screen house	+	C
4	Manzana	Leaf midrib	Montenegro	-	B
	Manzana	Root	Montenegro	-	B
	Manzana	Leaf midrib	Rozo	+	B
	Manzana	Root	Rozo	+	B
5	M Bra 383	Root	Quilichao	+	B
	M Bra 383	Root	Quilichao	+	B
	M Bra 383	Root	CIAT-field	+	B
	M Bra 383	Root	CIAT-field	+	B
6	CM 849-1	Leaf midrib	Agrovélez	+	B
	CM 849-1	Petiole	Agrovélez	+	B
	CM 849-1	Stem	Agrovélez	+	B
	CM 849-1	Rootlet	Agrovélez	+	B
7	SM 1219-9	Leaf midrib	CIAT-field	+	B

	Variety	Tissue	Site[a]	PCR	Primers[b]
	SM 1219-9	Root	CIAT-field	+	B
8	CM 2177-2	Leaf midrib	CIAT-field	+	B
	CM 2177-2	Root	CIAT-field	+	B
9	CM 4919-1	Leaf midrib	CIAT-field	+	B
	CM 4919-1	Root	CIAT-field	+	B
10	M Col 2063	Leaf midrib[c]	CIAT-greenhouse	+	B
	M Col 2063	Root	CIAT-greenhouse	+	B
	M Col 2063	Leaf midrib	CIAT-screen house	+	B
	M Col 2063	Root	CIAT-screen house	+	B
11	M Bra 383	Leaf midrib[c]	CIAT-greenhouse	+	B
	M Bra 383	Rootlet	CIAT-greenhouse	+	B
12	Venezolana	Root	Sincelejo-field	+	A-B
	Venezolana	Root	Sincelejo-field	+	A-B
13	CM 3306-9	Leaf midrib[c]	CIAT-greenhouse	+	B
	CM 3306-9	Petiole	CIAT-greenhouse	+	B
14	CM 3306-19	Leaf midrib[c]	CIAT-greenhouse	+	B
	CM 3306-19	Petiole	CIAT-greenhouse	+	B
15	M Bra 856-54	Leaf midrib[c]	CIAT-greenhouse	+	B
	M Bra 856-54	Petiole	CIAT-greenhouse	+	B
16	M Col 634	Leaf midrib	Quilichao-field	+	C
	M Col 634	Root	Quilichao-field	+	C
17	M Bra 829	Leaf midrib	Quilichao-field	+	C
	M Bra 829	Root	Quilichao-field	-	C
18	M Per 16	Leaf midrib	Quilichao-field	+	C
	M Per 16	Root	Quilichao-field	+	C
19	M Bra 856	Leaf midrib	Quilichao-field	+	C
	M Bra 856	Root	Quilichao-field	+	C
20	M Bra 856	Leaf midrib	Quilichao-field	+	C
	M Bra 856	Root	Quilichao-field	+	C
21	M Chn 2	Leaf midrib	Quilichao-field	-	C
	M Chn 2	Root	Quilichao-field	-	C
22	HMC 1	Leaf midrib	Quilichao-field	+	C
	HMC 1	Root	Quilichao-field	+	C
23	M Arg 2	Leaf midrib	Quilichao-field	-	C
	M Arg 2	Root	Quilichao-field	-	C
24	M Bra 325	Leaf midrib	Quilichao-field	+	C
	M Bra 325	Root	Quilichao-field	+	C
25	M Bra 839	Leaf midrib	Quilichao-field	+	C
	M Bra 839	Root	Quilichao-field	+	C
26	M Col 1178	Leaf midrib	Quilichao-field	+	C
	M Col 1178	Root	Quilichao-field	+	C
27	M Col 1468	Leaf midrib	Quilichao-field	+	C
	M Col 1468	Root	Quilichao-field	+	C
28	M Cub 74	Leaf midrib	Quilichao-field	-	C
	M Cub 74	Root	Quilichao-field	+	C
29	M Bra 886	Leaf midrib[c]	Quilichao-field	+	C
	M Bra 886	Root	Quilichao-field	+	C
30	M Bra 882	Leaf midrib	Quilichao-field	-	C
	M Bra 882	Root	Quilichao-field	-	C
31	CM 5460-10	Leaf midrib[c]	CIAT-screen house	+	C
	CM 5460-10	Petiole	CIAT-screen house	+	C
32	SM 909-25	Leaf midrib[c]	CIAT-screen house	+	C
	SM 909-25	Petiole	CIAT-screen house	+	C
33	CG 6119-5	Leaf midrib[c]	Santa Elena-field	+	C
	CG 6119-5	Root	Santa Elena-field	+	C

	Variety	Tissue	Site[a]	PCR	Primers[b]
34	M Per 335	Root	Santa Elena-field	+	C
35	ICA Nataima	Leaf midrib	Santa Elena-field	-	C
	ICA Nataima	Root	Santa Elena-field	+	C
36	SM 1201-5	Leaf midrib	Santa Elena-field	-	C
37	GM 228-14	Leaf midrib	Santa Elena-field	-	C
38	CM 9582-64	Leaf midrib	Rozo-field	+	A-B-C
	CM 9582-64	Root	Rozo-field	+	A-B-C
39	CM 9582-65	Leaf midrib	Rozo-field	+	A-B-C
	CM 9582-65	Root	Rozo-field	+	A-B-C
40	CM 9582-24	Leaf midrib	Rozo-field	+	A-B-C
	CM 9582-24	Root	Rozo-field	+	A-B-C
41	M CR 81	Leaf midrib	Rozo-field	+	A-B-C
	M CR 81	Root	Rozo-field	+	A-B-C

a. Agrovélez S.A., CIAT, Rozo, and Santa Elena are found in the Department of Valle del Cauca; Quilichao in Cauca; Sincelejo in Atlántico; and Montenegro in Quindío.

b. Primers used for amplification were **(A)** P1/P7–R16F2N/R16R2, **(B)** R16mF2/R16mR1–R16F2N/R16R2, and **(C)** R16F2/R16R2–R16(III)F2/R16(III)R1.

c. Also showing foliar symptoms of mosaic and deformation of leaf blade.

Sequencing the 16S rRNA region. DNA obtained from plants with symptoms of FSD was used to amplify fragments of the 16S region of ribosomal DNA, using polymerase chain reaction (PCR) and two pairs of universal primers P1/P7 and R16mF2/R16mR1. The products were re-amplified, using nested PCR and primers R16F2N/R16FR2, to detect and confirm that the phytoplasma is associated with the disease. The products of the nested PCR (1.2 kb) were digested with the enzymes *Alu*I, *Mse*I, *Rsa*I, and *Taq*I. The band patterns obtained with the restriction fragment length polymorphism (RFLP) technique made it possible to locate the group to which the phytoplasma belongs.

These results were confirmed by re-amplifying the products R16F2/R16R2 with primers R16(III)F2/R16(III)R1 (0.8 kb) specific to the 16SrIII group. The fragments of 1.2 kb and 0.8 kb were cloned and sequenced. Purified PCR products were ligated in pGEM®-T Easy Vector, which was introduced into the *Escherichia coli* strain DH5-α by electroporation at 2.4 kV/cm^2.

Transformants were selected on a blue-white screen by plating on an LB/ampicillin/IPTG/Xgal medium. Positive inserts were observed with plasmid restriction with *Eco*RI and electrophoresis in 1.5% agarose gel. Different-sized fragments were selected and sequenced by automated dideoxy sequencing (ABI PRISM® 377-96 DNA Sequencer, Applied Biosystems).

Results

A phytoplasma was successfully detected, using nested PCR, in all FSD-infected tissues. Of the methods used in this study, PCR was the most sensitive for detecting, identifying, and classifying phytoplasmas.

A sequence from a cloned fragment, obtained from an infected cassava plant, showed a 99% homology with the Chinaberry yellows phytoplasma and 100% with that of Cirsium white leaf.

These results allow us to infer that a phytoplasma plays a role in this disease.

As criteria, we took the number of correctly read bases of the amplified fragment, amplifications of the characteristic symptoms of the disease, and differences of genotype, and obtained two complete sequences, measuring 1260 and 1298 bp of 16Sr DNA gene region of two different cassava varieties, M Col 2063 (Y17) (leaf midrib and petiole) and SM 1219-9 (Y29) (external phloem from roots), which were classified and reported in GenBank with the accession numbers AY737646 (1260 bp) and AY737647 (1298 bp).

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Continued