Output 1: Pest and pathogen complexes in key crops described and analyzed.

Activity 1.1. Rapid identification of *Colletotrichum lindemuthianum*-specific microsatellite markers using 5'anchored PCR.

Contributors: G. Mahuku; M.A. Henriquez and M. Navia

Highlight:

- We identified and developed primers specific to 16 microsatellite loci in the bean anthracnose pathogen, *Colletotrichum lindemuthianum*, and showed the potential of these primers to distinguish between Andean and Mesoamerican groups and to provide information on the genetic structure of the pathogen.

Rationale

Knowledge of the genetic structure of plant pathogen populations is important because the amount of genetic variation that is maintained in a population reflects the capacity of a pathogen to evolve, information which is important in choosing the most effective control strategy (Burdon, 1993, Genetic Variation in pathogens populations and its implications for adaptation to host resistance, In: Durability of Disease resistance (Jacobs, T., Parleviet [eds]), Kluwer, Dordrecht, pp 41-56; McDonald & McDermott, 1993, Bioscience 43: 311-319). The distribution of genetic variation can be used to select the most effective resistance gene(s) that are better suited to manage pathogen populations in a given locality. Several tools have been used to characterize *C. lindemuthianum*, but the lack of a standardized molecular system makes it impossible to compare data coming from different laboratories and to have a concerted (unified) effort (strategy) for developing anthracnose management strategies. A standard set of differential cultivars is used. Although this has greatly facilitated comparison of data, the process is long and tedious, and subject to environmental conditions. Where conditions for inoculum preparation, inoculation and humidity are not controlled, the procedure is not precise. Several types of molecular markers have been used, but there are disadvantages with many of these. For example, although RAPD-PCR has high resolution, and is simple to carry out, it requires exquisite control to achieve robustness. AFLPs are also very informative but require careful optimization of conditions for restriction enzyme activity, ligation of adapters and PCR. Microsatellites are tandemly repeated copies of short nucleotide sequences that are useful for PCR-based DNA typing of fungi. Once developed, microsatellites behave like RAPD markers with the added advantage of robustness. We used the 5' anchored PCR method (Fisher *et al*., 1996, Nucleic Acid Research 24: 4369-4371), to identify microsatellite loci without the expenses of library screening. We report the isolation and characterization of microsatellite loci in the bean anthracnose pathogen *C. lindemuthianum*.

Materials and Methods

Fungal isolates and DNA extraction: Four *C. lindemuthianum* isolates previously characterized on a set of 12 anthracnose differential varieties and classified as Andean (two) and Mesoamerican (two), were used in this study. Isolates were recuperated from lyophilizaed samples. Mycelia production and DNA extraction was according to the method described by Mahuku *et al.* (2004, Plant Molecular Biology Reporter 22: 71-81). The DNA quality was assessed by electrophoresis in a 0.7% agarose gel and the quantity measured using the fluorometer (Hoefer® DyNA Quant 2000, Pharmacia Biotech, USA) and adjusted to 10 ng/µl in 0.1X TE buffer. A further 35 isolates

representative collected from Andean and Mesoamerican regions were used to standardize cycling protocol and test the suitability of developed microsatellites.

RAMs PCR, sequencing and microsatellite identification: A series of degenerate di – and tri - nucleotides microsatellite primers anchored at the 5' end (Table 1.1.1), were used to amplify genomic DNA from two Andean and two Misoamerican isolates. The 3'end of each primer was complemenatry to a common fungal repetitive sequence whilst the 5'end provided a degenarate anchor sequence to avoid slippage. The rationale behind this design was that if the primer annealed to two close and inverted simple sequences repeats, then the region between them, which may also contain repetitive sequences would be amplified. The conditions of the PCR amplification were according to Ganley and Bradshaw (Ganley and Bradshaw, 2001, Mycological Research 105: 1075-1078), in an MJ100 PTC model thermal cycler. PCR products were separatyed on 1.5% agarose gels. For each degenerate primer, descrite bands in the 600-1200 bp region were excised from the gels, gel purified with a QiaEX II gel Extraction Kit (QIAGEN) and cloned with a pGEM easy vector kit (INVITROGEN). Plasmid DNA was obtained and sequnced using the ABI PRISM™ 377 DNA automated sequencer (Perkin Elmer). DNA sequences were analysed using Seqman within DNAStar (DNAStar, Madison, WIS. USA) and PCR products containing repeated sequences were selected. Specific primers flanking the region of microsatellites were designed using Primer3 software (Center for Genome Research, Whitehead Institute, MA, USA - http://www-genome.wi.mit.edu/cgi-bin/primer/primer3), and commercially synthesized (Integrated DNA Technologies, Inc., Coralville, IA, USA). These primers were tested first on four isolates and on an additional set of 35 isolates representative of Andean and Mesoamerican groups.

Table 1.1.1. 5'anchored primer sequences (5'to 3').

ID	Sequence
PTC1	KKYHYHY$(GA)_6$
PTC3	KKYNSSH$(AAG)_5$
PTC4	KKVRVRV$(CT)_6$
PTC5	KKVRVRV$(TG)_6$
PTC6	KKBNVSS$(GATA)_4$

Results and Discussion

Amplification of four *C. lindemuthianum* isolates using the anchored primers gave distinct 'RAPD-type' DNA profiles; and example with the anchored PTC1, PTC4 and PTC5 primers are shown in Figure 1.1.1. No products were observed for PTC3 and PTC6 primers (Figure 1.1.1). Reproducible profiles were seen in replicate tests for all primers. Potentially informative microsatellite loci were identified by selecting anchored-PCR products from gels on the basis of their consistent amplification or their apparent uniqueness to an individual isolate. Thirty such PCR products were purified, cloned and sequenced.

Sixteen of the cloned microsatellite loci were chosen for further assessment on the basis of the size of the microsatellite repeat or the number of repetitive sequences present. For each of these a locus-specific primer was designed so that a PCR product of approximately 100-500 bp in length would be obtained when used in conjunction with the anchored primer or in pairs. Seventeen

primer pair combinations were obtained; eight of which gave single PCR products, one gave two, one gave four, and six consistently gave multiple PCR products (Figure and Table 1.1.2).

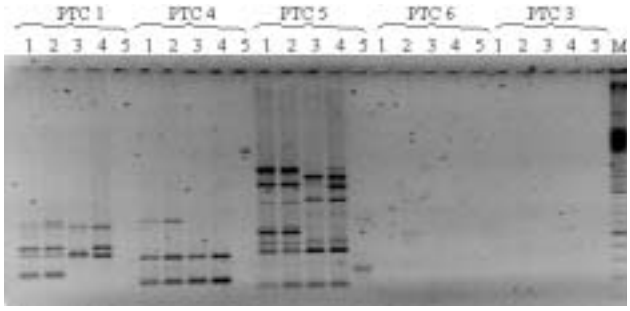


Figure 1.1.1. PCR amplification products from *Colletotrichum lindemuthianum* isolates obtained using the anchored PTC1, PTC3, PTC4, PTC5, and PTC6 primers. Lane 1 and 2 are *C. lindemuthianum* Andean isolates while lanes 3 and 4 a Mesoamerican isolates. Lane 5 is negative control (no fungal DNA was added) and lane M is the 100 bp molecular size marker. NO products were obtained with PTC3 and PTC6.

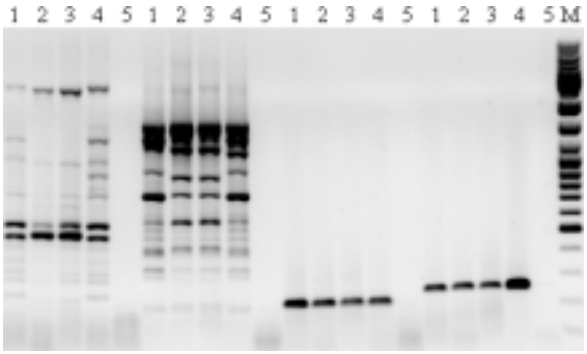


Figure 1.1.2. Microsatellite length variation between Andean and Mesoamerican isolates of *Colletotrichum lindemuthianum* isolates. Lanes 1 and 2 are Andean isolates; lanes 3 and 4 are Mesoamerican isolates, lane 5 is negative control (no DNA added), lane M is 100 bp molecular size marker.

We are in the process of optimizing the annealing temperature so that single fragments are consistently amplified, after which we will test promising microsatellite primers on 35 Andean and Mesoamerican isolates from different regions where anthracnose of common bean is a serious problem. Preliminary evaluation of nine *C. lindemuthianum* isolates collected from Andean and Mesoamerican beans and regions using primers specific to the Anth.CL_006 loci revealed four alleles (Figure 1.1.3), while amplification of 35 isolates from a world-wide collection using Anth.CL001 primer pair revealed two alleles, one that was consistently associated with isolates belonging to the Andean group, and another of isolates belonging to the Mesoamerican group (Figure 1.1.4). These preliminary results show the potential of these markers to distinguish between Andean and Mesoamerican groups and for population genetic studies.

Table 1.1.2 Polymorphic microsatellite loci for the bean anthracnose pathogen, *Colletotrichum lindemuthianum*.

Locus	Primer sequence 5' to 3' Forward	Reverse	# of alleles	Repeat type	size range
Anth.CL001	TGTCACAGGGGGGGG	GGCAGAACCAGGATG	multiple	PTC1	271
Anth.CL002	TGGGCTCTCTCTCTCTTCC	GGTGGGAGAA	2	PTC4	4
Anth.CL003	GGCTCTCTCTCTCT	CAGGAACAAACAGGG	multiple	PTC4	234
Anth.CL004	TCATGCTACTCTGACTCCTGG	GAGCGCTCTGC	multiple	PTC4	400
Anth.CL005	CCCTCAACCTCTTTGTGG G	GAGCGCTCTGC	PTC4	234	
Anth.CL006	TGGGATCCTGATGATG	GAGTGGTATAATCTG	2	PTC 5	270
Anth.CL007	GGTGTCTCGTACCTA	ACAAATGGAAGCG	2	PTC5	239
Anth.CL008	TGTGTATCAACGCCT ACCCCGTATCATACG		4	PTC5	234, 1200
Anth.CL009	TCTCTAACAGGG GAAG	GGGGGGGGGG	multiple	PTC5	274
Anth.CL010	TAGGACAGCGC GCATCGTCTGCACAT		2	PTC5	289
Anth.CL011	GTGGGGGGTG ATGTTCGTTGGTCTC		multiple	PTC5	641
Anth.CL012	TACCTATCTGCGAG GGGGGTGGAG		2	PTC5	335
Anth.CL013	GGTGGGGGG GAGTCCGTCACATCAA		4	PTC5	500
Anth.CL014	TCAGGGGG TGGG GCCATC TTTGATGCTG		multiple	PTC5	184
Anth.CL015	GATACAAAGTG CATG ACCCCTAGCGTGT		2	PTC5	340
Anth.CL016	GATAAAGCCTG CAAA	GCAGCACAGCACTTCTCG	2	PTC5	248

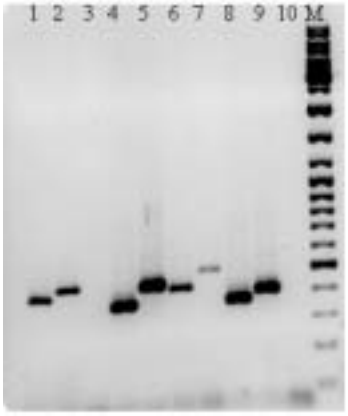


Figure 1.1.3. PCR amplification of nine *Colletotrichum lindemuthianum* isolates of a diverse geographical origin using primers specific to the Anth.CL006 primers. Lanes 1, 3, 4, 7, and 8 are *C. lindemuthianum* isolates collected from Mesoamerican genotypes while lanes 2, 5, 6, and 9 represent isolates collected from Andean genotypes. Lane 10 is negative control while lane M is the 100 bp molecular size marker.

Figure 1.1.4. Amplification of the SSR markers corresponding to specific Anth.CL001 locus from genomic DNA of 35 Colleto*trichum lindemuthianum* isolates collected from Andean and Mesoamerican bean genotypes and regions shown on a silver-stained polyacrylamide gel.

Conclusions: Primers specific to 16 microsatellite loci in *C. lindemuthianum* were developed. Six of these primers consistently gave multiple fragments. Preliminary evaluation of nine and 35 isolates using primers specific to two microsatellite loci revealed the potential of these SSR markers to distinguish between different *C. lindemuthianum* isolates and to generate information that can be used to infer the genetic structure of this fungus. We are currently optimizing the cycling conditions for these primers, and using them to characterize a set of 120 *C. lindemuthianum* isolates that have been collected from different regions of Colombia.

Activity 1.2. Develop multiplex PCR assay for simultaneous detection of 6 *Pythium* species in common bean soils.

Contributors: G. Mahuku and R. Buruchara

Highlight:

- Developed a multiplex PCR assay for simultaneous detection of six pathogenic *Pythium* species and a potential biocontrol agent.

Rationale

Root rots are an increase problem to bean production in East Africa that has resulted in 100 % yield losses, and in some cases, (e.g. western Kenya) farmers were forced out of bean production. Previous characterization work identified *Pythium* as the most important causative agent and six species (*P. ultimum,P. spinosum,P. deliense,P. salpingophorum,P. torulosum* and *P. nodosum*) were shown to be important in inciting root rots of bean. Of these species, *P. ultimum* var *ultimum* is the most prevalent and devastating. However, identifying and distinguishing between these species, using morphological or pathogenic characteristics is difficult and slow. In the soil, these species may be mixed with a range of other host pathogens and saprophytes (e.g. Mortilela spp. that is morphologically indistinguishable from Pythium). Detection and identification of the causative species is crucial for identifying resistance or introducing and targeting other disease management practices. This study was initiated to develop a multiplex PCR assay for the simultaneous detection and identification of the major *Pythium* species associated with common bean root rots. Multiplex PCR is a method for simultaneous amplification of several fragments in a single PCR and is one of the best molecular tools for species identification, as it enables the identification of several species by a single PCR followed by a single electrophoresis.

Materials and Methods

Primer design: The internal transcribed spacer region 1 (ITS 1) of the ribosomal DNA was targeted for the development of *Pythium* species-specific primers. DNA sequences from our own work (CIAT 2003, Bean Project Annual Report), and from the data bank of Dr Andre Levesque (AAFC Canada) were compared for the 6 target *Pythium* species, and based on sequence differences in the ITS 1 region, species-specific primers were designed. We also took note of the primers that had been developed and reported by other groups (Table 1.2.1), and where these matched what we had designed, the same primer sequences were synthesized. Where there were differences from our optimized sequences, the two primers, our own and the one reported were synthesized and tested for specificity by amplifying test strains.

Primer optimization : The specificity of the primers to the target strain was tested by amplifying DNA of several *Pythium* species that we have in our collection. Once found to be specific, the primers were optimized, to select suitable primer combination that avoid formation of heterodimers, before optimizing for magnesium concentrations, DNA concentrations, enzyme concentrations, annealing temperature and cycling and electrophoresis conditions so as to enable simultaneous detection of the target species.

Suitability for detecting different Pythium species: Two approaches were followed to test the suitability of the multiplex PCR assay for simultaneous detection of different *Pythium* spp: (1) primers were mixed, added one at a time and used to amplify DNA from individual species, until all primers were part of the mix; (2) DNA from different species were mixed and amplified, first with individual primers, then with different primer combinations.

Results and Discussion

Multiplex PCR assay for Pythium species: PCR primers specific to six *Pythium* species, including *P. oligandrum*, a known biocontrol agent designed based on sequences differences in the internal transcribed spacer region 1 of the ribosomal DNA were used in conjunction with an oomycete specific primer that was previously developed by Dr. Andre Levesque, AAFC, Canada. The Oomycete specific primer is based on conserved sequences in the 5.8S ribosomal genes. The designed primers, their sequences and the target fragment size are shown in Table 1.2.1.

Suitability for multiplexing: When DNA from different *Pythium* species was mixed at a concentration of 10 ng each, and used as template in PCR reactions with specific primers, only the target species was amplified in all cases, (Figure 1.2.1). Mixing primers of all species to a final concentration of 0.4 µM, and adding DNA from individual *Pythium* species resulted in the amplification of the desired fragment (Figure 1.2.2). Two fragments the desired 150 bp and a ~400 bp fragments were amplified *P. oligandrum* (Figure 1.2.2). Increasing the PCR buffer concentration to 1.5 X and annealing temperature to 65°C resulted in the disappearance of the larger fragment. The annealing temperatures for the different primers ranged from 56 – 70, and as such, the multiplexing should take this into consideration.

Table 1.2.1. Amplification results from the designed primers using genomic DNA from different *Pythium* species.

Species	Primer ID	Size (bp)	Sequence 5' to 3'	Source
P. spinosum	Pspi (old)	200	TGT GTG TTG TGA TCG TGC CT	Wang *et al*., 2003, Lett. Appl. Microb.37: 127-132
P. spinosum	Psp1 (new)	325	TGT TGT GTG TCT GCG CCG TTG TTG G	This project
P. ultimum	PuK1	192	ACG AAG GTT GGT CTG TTG	Kageyama *et al*., 1997, Plant Dis. 81:1155-1160
P. deliense	Pdel1	182	GCT GAA CGA AGG TGG GCT GCT	This project
P. salpingophorum	Psal1	217	TTA TGT TCT GTG CCT TCT CTC G	This project
P. oligandrum	Po1	150	TGC GTC TAT TTT GGA TGC GG	Wang et al., 2003
P. nodosum	Pnod1	232	ATC TGC TCT CTG TGC CTT TCG	This project
P. torulosum	Pto 1	177	AGG TAG AGC TGC ATG TAA AAG T	Wang et al., 2003
OOM-lo 5.8S47B			CGC ATT ACG TAT CGC AGT TCG CAG	Schurko *et al*., 2003, Myco. Res 107: 537-544

*All primers were paired with the oomycte specific primer OOM-Lo5.8S 47 B

Reaction and cycling conditions: The PCR reaction contained: 0.2 mM dNTP, 0.4 µM each primer, 2 mM Mg^{2+}, 1U *Taq* DNA polymerase (Promega) and 10-30 ng genomic DNA in a 12.5 µL PCR reaction volume. The optimum cycling conditions, taking into consideration the differences in annealing temperatures were: 94°C 2 min; 35 cycles of 94°C for 30 sec, annealing at 63°C for 30 sec; extension at 72°C for 40 sec; followed by a final extension at 72°C for 10 minutes. However, these should be optimized for each laboratory, taking into account differences in thermocyclers, enzyme used and source of different reagents.

Electrophoresis conditions: Optimum electrophoresis condition to be able to distinguish all *Pythium* species was in 1.5% agarose gel. The small fragment size might warrant higher concentrations of agarose, and these have to be optimized for local conditions before large-scale application.

Conclusion: A multiplex PCR assay was developed to simultaneously detect and identify 6 *Pythium* species that are pathogenic to beans, and *P. oligandrum*, the biocontrol agent. We are

currently testing different methods for extraction DNA from plant tissues to assess the effect of DNA purity on PCR amplification efficiency. In addition, we are adopting different methods for extracting DNA from different soils with different clay content, so as to establish the suitability of the developed PCR assay for direct detection of these *Pythium* species in soil.

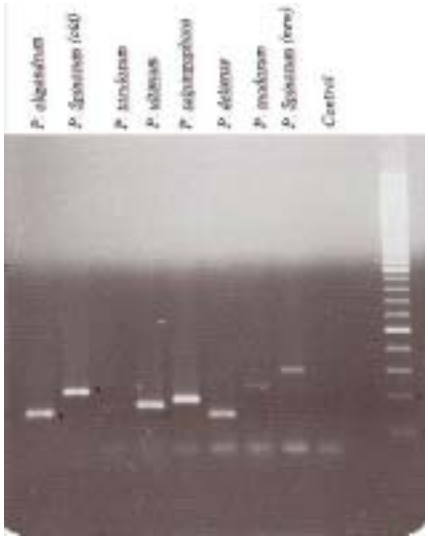


Figure 1.2.1. Detection of 6 *Pythium* species using a multiplex PCR assay. DNA from different *Pythium* species was mixed and amplified using species-specific primers that target the ITS 1 region of ribosomal DNA.

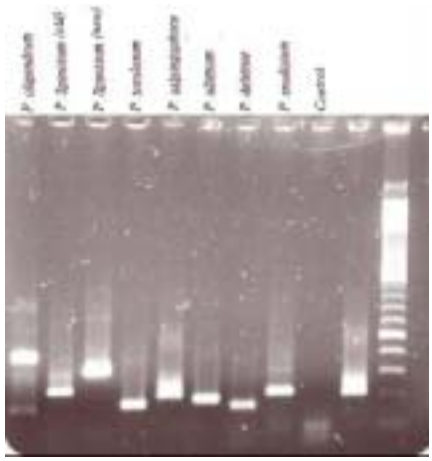


Figure 1.2.2. Suitability of multiplex PCR assay to detect different *Pythium* species. Primers specific to seven *Pythium* species were mixed, and DNA from individual *Pythium* species was added. In all cases, the desired fragment was amplified. Only with *P. oligandrum* were two fragments amplified, the desired 150 bp and a second larger fragment of approximately 400 bp.

Activity 1.2.1. Development of a molecular-based quantitative assay for *Pythium* species

Contributors: G. Mahuku and R. Buruchara

Highlight:

- We developed a competitive PCR assay for *Pythium ultimum* var *ultimum* and showed its utility to determine DNA concentration from unknown samples**.**

Rationale

The incidence and severity of root rots caused by soil borne pathogens is directly related to levels of pathogen propagules in the soil. Several management strategies are directed at reducing soil inoculum to below economic threshold levels. Although the effects of management practices can be assessed or measured on the basis of disease severity, the latter are not always the best indicators of soil pathogen population. There is therefore need to develop tools and procedures that are simple, fast and accurate for the quantification of pathogen populations directly in soils. This would facilitate development of prediction models but more importantly will facilitate determining the effects of various root rot management options on the pathogen population. It will also facilitate the understanding of factors or practices, which influence the increase of the pathogen population in the soil subsequently leading to root rots. *P. ultimum* var *ultimum* has been identified as the most important cause of bean root rots in East Africa. The objective of this project is to develop a molecular based quantitative assay, based on the competitive PCR technique that could be used to monitor propagules of *P. ultimum* var *ultimum* in the soil and to assess the effect of different management options on inoculum levels of this pathogen in the soil. It is hoped that this assay will yield information that can lead to recommendations on best practices to include in an integrated root rot management strategy.

Materials and Methods

PCR amplifcations: An isolate of *P. ultimum* obtained form diseased bean roots was used in this study. Amplification of the target *P. ultimum* DNA was done using the primers Puk1 and OOM-lo 5.8S47B previously developed by Kageyama et al., 1997, Plant Disease 81: 1155-1160; Schurko et al., 2003, Mycological Research 107: 537-544, respectively, using the following conditions: 0.5 µM of each primer, 0.2 mM dNTP mixture, 1x PCR buffer, 1.5 mM Mg^{2+}, 1U Taq DNA polymerase and 20 ng genomic DNA in a 12.5 µL PCR reaction volume. The cycling conditions were as follows: 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for I min, annealing at 55°C for 1 min and extension at 72°C for 2 min, with a final extension at 72°C for 10 min.

Creation ofa heterologous internal Standard: An internal standard was prepared by amplifying genomic DNA extracted from the soil pathogen *Macrophomina phaseolina* with primers Puk1 and OOM-lo5.8S47B (Schurko *et al*., 2003, Mycological Research 107: 537-544) at low stringency. These two primers amplify a fragment of approximately 200 bp in *P. ultimum*. Two fragments of approximately 250 and 190 bp were excised from the gel, purified and amplified at high stringency (55°C) using the same primers. After confirming the size, the fragment was cleaned and cloned into pGEM T-easy vector and maintained in transformed *Ecoli* . DNA for further studies was extracted and quantified using a scpectrophotometer and used as control DNA in developing a *P. ultimum*-specific competitive PCR assay. To determine the utility of this fragment as control DNA, varying concentrations of control DNA, ranging from 100 ng to 10 fg

were amplified either alone or in the presence of a fixed amoung of *P. ultimum* DNA, using the cycling conditions previously described.

Results and Discussion

Generation of competitor DNA fragments: The amplification of *M. phaseolina* genomic DNA under low stringency annealing conditions resulted in the production of several PCR fragments. Two fragments of 250 and 190 bp in size were excised from the gels, purified and cloned into pGEM T-easy vector. These fragments were engineered to contain the priming sites of the two *P. ultimum*-specific primers, through low stringency amplification, followed by size selection before the fragment was cloned in *Ecoli* (Figure 1.2.1.1) In competitive PCR, it is extremely important to have a competitor with a greater degree of similarity (size fragment) to the target, so as to allow for more even amplification efficiencies. In addition, the two fragments should be easily distinguished upon electrophoresis. The usefulness of the designed heterologous probe was tested by amplifying different concentrations of competitor DNA in the presence of a fixed amount of target DNA (Figure 1.2.1.2). As the concentration of competitor DNA decreases, the intensity of the amplified target DNA increases. By comparing the relative band intensities of the two fragments, a ratio is reached where the amount of target and competitor DNA are in a 1:1 ratio. The sensitivity of detection of was measured by amplifying known concentrations of the control DNA. The assay could detect down to 100 fg of competitor DNA (Figure 1.2.1.3).

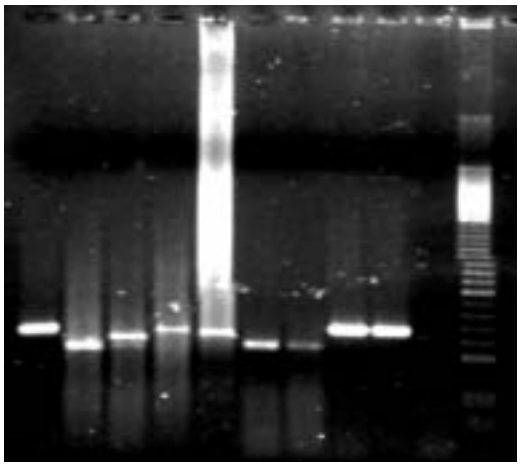


Figure 1.2.1.1. Ethidium bromide stained gel of products of candidate internal fragments for competitive PCR. Lanes 1, 8 and 9 represent *Pythium ultimum* DNA, while lanes 2 to 7 are different individual bands obtained from amplifying *Macrophomina phaseolina* DNA under low stringency conditions using *P. ultimum* specific primers Puk1 and Puk3. Fragments from lanes 2 and 3 were eventually cloned and subsequently tested for their suitability as internal controls in competitive PCR assays.

Conclusion: We successfully developed control fragment that can be used in the competitive PCR assay to estimate *P. ultimum* DNA concentration, and hence estimate pathogen inoculum levels in the soil. We are in the process of developing standard curves by amplifying DNA isolated from known concentrations of zoospores and oospores obtained from *P. ultimum* var *ultimum*. In addition, we are in the process of testing the suitability of the designed competitive PCR assay for direct estimation of *P. ultimum* propagules directly in the soil. Several procedures

for direct extraction of *P. ultimum* DNA from soils are currently being tested, by extracting known concentrations of DNA from soils differing in structure, clay and humus content. This is important, to optimize the conditions under which this assay could be applied.

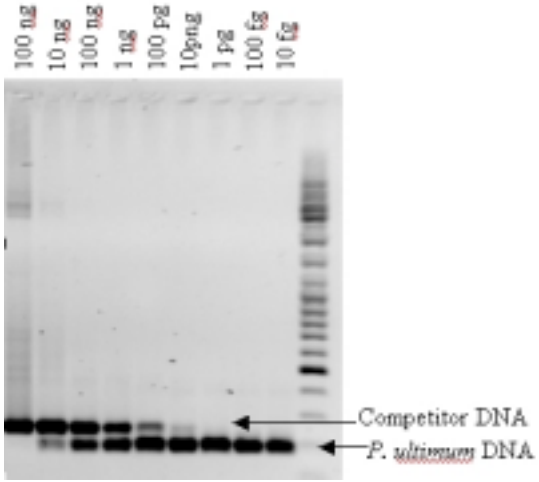


Figure 1.2.1.2. Competitive PCR products of *Pythium ultimum*. A constant unknown concentration of *P. ultimum* DNA was co amplified in the presence of competitor DNA ranging from 100 ng to 10 fg. At 1 ng concentration of competitor DNA shows an almost 1:1 ratio of control: *P. ultimum* DNA concentration.

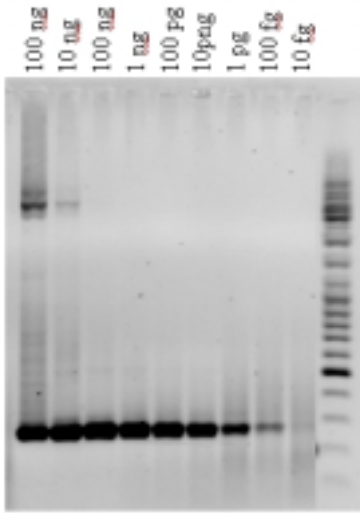


Figure 1.2.1.3 Sensitivity of the competitive PCR assay to detect target DNA

Activity 1.3. Development of molecular markers linked to the *Pythium* resistance genes in common bean genotype RWR 719

Contributors: G. Mahuku, and M. Navia, R. Buruchara (PABRA) and R. Otsyula (KARI)

Highlights:

- Three markers linked in coupling to the resistance gene in RWR 719 were identified, two were turned into SCAR markers and the potential use of these markers in MAS was demonstrated.
- This is the first report of tagging and developing a SCAR marker for a Pythium gene in common bean.

Rationale

Previous studies have shown that Pythium root rot resistance in RWR719, AND 1062 and MLB 48-89A is controlled by single dominant gene(s). Allelism tests among the resistant germplasm showed no segregation, revealing the possibility of a common locus controlling resistance to *Pythium* spp. in common bean. Field and screen house evaluations of RWR719, AND 1062 and MLB 48-89A using seven *Pythium* species confirmed the potential of this resistance locus as a source of resistance to this pathogen. This study was initiated to develop molecular markers that are linked to the resistance gene(s) so as to use marker assisted selection (MAS) to facilitate the introgression of resistance into varieties preferred by small-scale farmers in east and central Africa.

Materials and Methods

Plant material: Crosses were made between GLP2 and each of the DNA resistant parents (RWR719, AND 1062 and MLB 48-89A) to create F_2 populations. All F_2 populations were evaluated using *Pythium ultimum*, previously established as the most important and widely distributed species causing bean root rots in East and Central Africa (Mukalazi *et al*., 2001, African Crop Sci. Conference, Lagos, Nigeria). Inoculum production, and inoculation were done as described previously (Mukalazi *et al.*, 2001). Seeds of parental materials and F_2 individuals were planted in inoculated soil in wooden trays. Germinated seedlings were watered 2 times a day for three weeks to provide a favorable environment for fungal infection, establishment and development. Individual seedlings were uprooted, washed in tap water and roots scored using the CIAT 1-9 scale (Schoonhoven and Pastor-Corrales, 1987, Standard system for the evaluation of bean germplasm, CIAT). Plants with no or limited symptoms (score 1-3) were rated as resistant, and the rest of the plants as susceptible.

DNA extraction and Marker identification: Young trifoliate leaves were collected from the resistant and susceptible parents and resistant and susceptible F_2 progenies, and DNA was extracted using the procedure described by Mahuku (Mahuku *et al*., 2004, Plant Molecular Biology Reporter 22: 71-81). Five resistant and 5 susceptible plants, including the parents were used to evaluate 300 RAPD and 50 RAMS primers as previously described (CIAT, Bean Project Annual Report 2004). Candidate markers showing evidence of correlation to disease resistance or susceptibility were further evaluated on an additional 10 resistant and susceptible F_2 plants. Where polymorphism was maintained, the potential markers were evaluated on the entire F_2 population (Table 1.3.1). The marker scoring data in the F_2 were merged with the disease scoring data for linkage analysis using the computer program MAPMAKER (Lander *et al.,* 1987, Genomics 1: 174-181).

Table 1.3.1. Response of common bean genotypes to inoculation with three-root rot causing *Pythium* species.

Bean genotype	Reaction to 6 *Pythium* species [a]	Candidate markers		
		Scar-PAA19	Scar-BA08	(GT)n
A 240	1	+[b]	+	+
MLB-49-89A	1	+	+	+
MLB-40-89A	1	+	+	-
RWR 719	1	+	+	+
Scam80-cm/15	1	+	+	+
AND 1064	1	+	+	+
AND 1062	1	+	+	+
RWR 1091	1	+	+	+
GLP 585	9	-	-	-
Scam- KWD	9	-	-	-
GLP2	9	-	-	-
URUGEZI	9	-	-	-
CAL 96	9	-	-	-

[a]The genotypes were evaluated with 6 *Pythium* species; *P. ultimum*, *P. salpingophorum,P. spinosum,P. tolorosum,P. chamaehyphom* and *P. pachycaule*. The reactions are based on the CIAT 1-9 scale and different scores for the same genotype represence different scores from different Pythium species.
[b] + represent presence of a marker and – absence of a marker.

Establishing the suitability ofcandidate marker for MAS: Candidate markers were evaluated on nine bean genotypes (RWR 719, MLB 49-89A, AND 1062, A 240, SCAM 80-CM/15, MEX 54, CAL 96, Urugezi and GLP 2) that are either resistant or susceptible to Pythium root rots under greenhouse and field conditions (Table 1.3.1)

Development ofSCAR markers: Candidate fragments were excised from agarose gels, cloned and sequenced as described by Mahuku *et al.* (2004, Crop Science 44: 1817-1824). Primers were designed using the Primer3 software (Center for Genome Research, Whitehead Institute, MA, USA - http://www-genome.wi.mit.edu/cgi-bin/primer/primer3). Developed primers were used to amplify DNA from parental materials, and two resistant and susceptible F_2 individuals. If polymorphism was maintained, the designed SCAR primers were tested in ten resistant and ten susceptible individuals. In the case of an identical sequence length, the fragment from the susceptible individuals was cloned and sequenced. The sequences derived from resistant and susceptible individuals were then aligned using the program MEGALIGN within DNAStar, and where possible the primer pairs were re-designed to exploit differences between the resistant and susceptible sequences.

Results and Discussion

Marker identifcation: Of the 300 RAPD and 50 RAMS primers evaluated, four RAPD primers, (OPAA19, OPY20, OPG3 and OPBA08) and one RAMS marker $(VHVGT)_5G$) segregated in coupling phase with the resistance gene in RWR 719 (Figure 1.3.1 A, B, and C), three RAPD primers (OPY20, OPF3 and OPG3) segregated in coupling phase with the resistance gene in MLB 49-89A. No marker was observed for the gene in AND1062. Further testing of RWR 719 potential markers on 10 resistant and susceptible individuals confirmed the potential of OPAA19, OPBA08 and (GT) n as molecular markers linked to the RWR 719 resistance gene. Since RWR 719 is currently being used in programs to introgress resistance to pythium root rot into commercial varieties in Eastern Africa, we focused our marker development efforts on this

genotype. Linkage analysis after testing the marker on 150 F_2 individual plants showed that the OPAA19 marker was located at 1.5 cM, the OPBA08 at 4 .0 cM and (GT)n marker at 6.3 cM from the resistance gene.

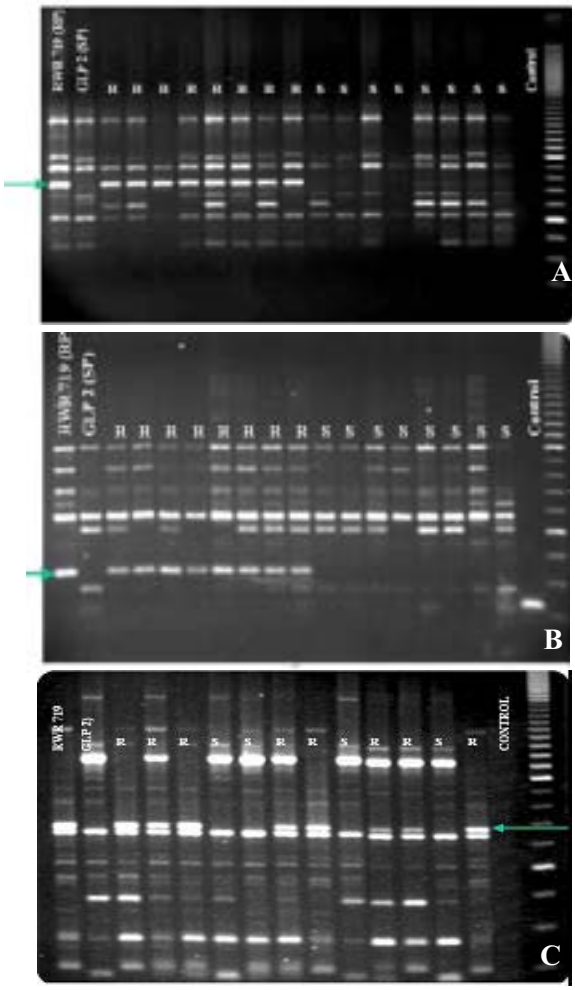


Figure 1.3.1. RAPD (A) OPAA19, (B) OPBA08, and RAMS GT $(VHVGT)_5G$) linked in coupling to the pythium resistant gene in the common bean genotype, RWR 719.

Validation of markers for MAS: Amplification of parental materials and selected susceptible and resistant bean genotypes revealed the potential of all three markers for marker assisted selection (MAS) breeding. The fragments associated with resistance were present in all resistant and absent from susceptible genotypes (Figure 1.3.2A and B), raising the possibility that the resistant genotypes tested in this study might carry the same resistance gene locus, with the same or different alleles for conditioning pythium root rot resistance. These results concur with the allelism test, where no segregation was observed in resistant x resistant crosses involving RWR 719, AND1062, SCAM 80/15 and MLB49-89A. Furthermore, all cultivars tested (RWR 719, AND1062, SCAM 80/15 and MLB49-89A) have A240 in their pedigree. Greenhouse evaluations of A240 showed that it was resistant to *P. ultimum* var. *ultimum*, and the fragment associated with resistance for all potential markers was present in A240. It is likely that this genotype is the origin

of resistance to pythium root rots. We are currently evaluating other genotypes that have A240 as one of the parents to test this hypothesis.

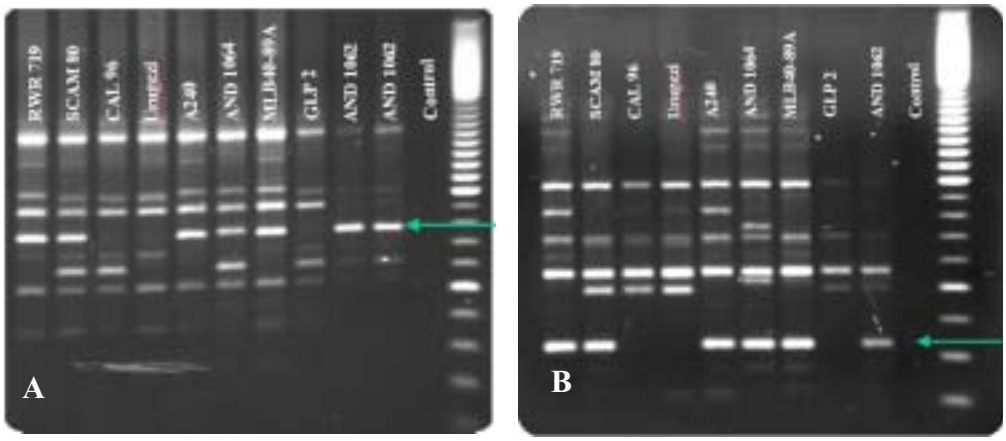


Figure 1.3.2. Validation of molecular markers outside the mapping population: (A) OPAA19 and (B) OPBA08. The fragments associated with resistance are present in all resistant and absent from susceptible genotypes.

SCAR Marker: Three sequence characterized amplified region (SCAR) markers were developed for Of the three SCAR markers developed OPAA19, OPBA08 and (GT) n markers. The SCAR marker derived from OPAA19 was polymorphic and co-dominant (Figure 1.3.3). The SCAR marker derived from BA08 was polymorphic and dominant, while the $(GT)_n$ derived marker amplified a similar sized fragment from susceptible and resistant plants. PCR products from resistant and susceptible parents amplified using these primers were cloned and sequenced. Alignment of resistant and susceptible plant sequences revealed polymporphisms and new primers targeting these differences were designed and are currently being synthesized. In addition, RILs have been developed from the RWR 719 x GLP 2 cross and these will aid further identification and development of other markers, and increase the efficiency of marker use in MAS.

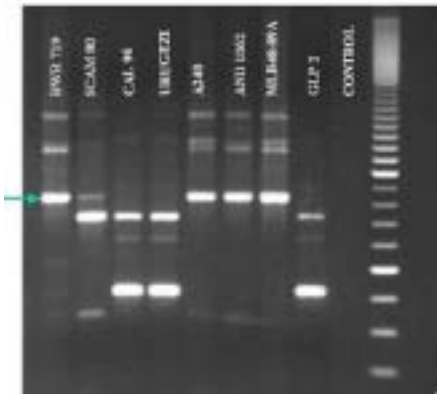


Figure 1.3.3. Amplification of resistant and susceptible bean genotypes using the SCAR primers derived from the OPAA19. The fragment associated with resistance was present in resistant and absent from susceptible genotypes.

Conclusions: Three markers linked in coupling to the resistance gene in RWR 719 were identified, and the potential of these markers in MAS was established. SCAR markers derived from the OPBA08and OPAA19 RAPD primers were developed and their potential utility for MAS demonstrated. We are in the process of validating these SCAR markers outside the mapping population. In addition, we are currently in the process of developing SCAR markers for the VHVGT)$_5$G markers, as well as evaluating RGAs and bean microsatellites so as to identify more markers and saturate the region. This is important as it will increase the utility of these markers in MAS.

Activity 1.4. Characterizing and monitoring pathogen and insect diversity

Contributors: R. Buruchara, V. Gichuru, (graduate student), S. Buah, C. Acam and S. Musoke (CIAT), F. Opio and M.A. Ugen (NARO)

Highlights:

- Three major intercrops of beans i.e. maize, sorghum and peas, in the bean-based system of southwestern Uganda were affected by root rots implying that they may be hosts of the pathogens.
- Management options effective for bean root rots are also beneficial to other crops such as sorghum and field peas in bean based cropping system. Formulating management strategies for root rots need to consider a systems approach rather than a crop's approach.
- Several Pythium spp were recovered from crops grown in association with beans. *P. ultimum* is the most frequent.
- Mortierella (MS10) has was shown been observed to have antagonist effects to pathogenic Pythium isolates with marked reduction in disease severity in screenhouse studies offering potential as a biocontrol agent against Pythium root rot.

Activity 1.4.1. Characterization and distribution of *Pythium spp*. associated with other crops in a bean based cropping system in southwestern Uganda

Rationale

Mukalazi (2002, PhD dissertation, Makerere University, Kampala, Uganda, pp 59-60) identified eleven *Pythium* species from Kabale district associated with bean root rot in Uganda, five of which were pathogenic on beans under field and screenhouse conditions. He also observed wide diversity of *Pythium spp* based on morphological, cultural and molecular characteristics. *Pythium* is an oomycete with a wide host range, which renders it a highly likely source of increased epidemics. There is very limited information on the role of various cropping systems on *Pythium* diversity and their role in root rot epidemics of beans in East Africa. Advances in plant and molecular pathology in particular provide a host of tools, which could be used to elucidate the diversity of *Pythium* spp under different cropping systems. We carried out studies to investigate species of *Pythium* associated with the crops grown as intercrops or in rotation in the bean based systems in southwestern Uganda.

Materials and Methods

Eighty *Pythium* isolates were obtained from intercrops of beans in Kabale district. DNA was extracted from harvested mycelia according to the procedure described by Mahuku (2004, Plant Molecular Biology Reporter 22:71-81). PCR analysis was performed using Oomycete ITS region primers to differentiate *Pythium* from other closely related fungi. The amplified products were purified and sequenced. The resultant sequences were edited for accurate peak scorings and later subjected to a nucleotide-nucleotide blast search at National Center for Biotechnology Information (NCBI) website. The sequences were compared to 10 most relevant matches in the GenBank and the best match was taken as the species of the isolate.

Results and Discussion

Out of the 80 isolates characterized, 19 species were identified (Table 1.4.1.1). Five of these have been reported in our previous pathogen characterization studies in Uganda but fourteen were new additions. Thirty percent of the isolates were *Pythium ultimum* followed by *P. acanthicum* (12.5%), *P. spinosum* (11.25%) and *P. torulosum* (7.5%). *P. arrhenomanes, P. macrosporum*, *P. mamilatum, P. orthogonon*, *P. conidiophorum, P. erinaceum, P. periplocum* and *P. vexans* were the least prevalent, each of which occurred only once. Species distribution map is shown (Figure 1.4.1.1). These results are consistent with past observations that overall *P. ultimum* is the most frequent species in the region in the case of beans and their intercrops. These results show that other crops in the bean system play a role as likely hosts to bean pathogenic *Pythium spp*. It also indicates that other crops are likely hosts to *Pythium spp*, which had not been previously isolated from, beans. Further studies will determine the pathogenicity of *Pythium spp*. from other crops on beans to establish if other crops in the bean system play a role as likely hosts to bean pathogenic *Pythium spp*. The knowledge generated from this study is crucial to formulating an effective management strategy for bean root rot in similar cropping systems.

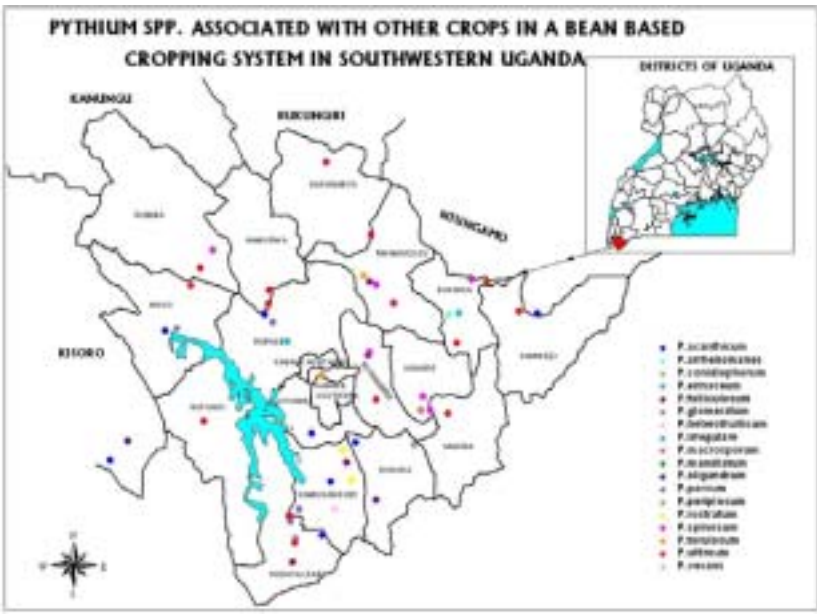


Figure 1.4.1.1. A distribution map of *Pythium* species identified and found to be associated with other crops in a bean based cropping system in southwestern Uganda

Table 1.4.1.1. Identification by sequencing of *Pythium* isolates from other crops in a bean based cropping system in southwestern Uganda

	Major Crops sampled												
***Pythium* spp.**	**Irish**	**Sorghum**	**Maize**	**Bean**	**Sweet potato**	**Cabbage**	**Peas**	**Tomatoes**	**Millet**	**Wheat**	**Bananas**	**Weed**	**Total**
P. ultimum	8	6	4	-	1	2	1	-	-	1	1	-	24
P. acanthicum	5	3	-	1	-	-	-	-	-	-	-	1	10
P. spinosum	3	4	-	-	-	-	1	1	-	-	-	-	9
P. torulosum	2	-	2	1	-	-	1	-	-	-	-	-	6
P. folliculosum	1	1	1	2	-	-	-	-	-	-	-	-	5
P. oligandrum	1	2	2	-	-	-	-	-	-	-	-	-	5
P. parvum	-	1	-	-	-	2	-	-	1	-	-	-	4
P. irregulare	2	-	-	-	-	-	-	1	-	-	-	-	3
P. glomeratum	2	-	-	-	-	-	-	-	-	-	-	-	2
P. heterothallicum	-	-	-	-	2	-	-	-	-	-	-	-	2
P. rostratum	1	-	-	-	1	-	-	-	-	-	-	-	2
P. arrhenomanes	-	1	-	-	-	-	-	-	-	-	-	-	1
P. macrosporum	1	-	-	-	-	-	-	-	-	-	-	-	1
P. mamilatum	1	-	-	-	-	-	-	-	-	-	-	-	1
P. orthogonon	-	-	-	1	-	-	-	-	-	-	-	-	1
P. conodiophorum	-	-	-	1	-	-	-	-	-	-	-	-	1
P. erinaceum	-	1	-	-	-	-	-	-	-	-	-	-	1
P. periplocum	-	-	-	-	1	-	-	-	-	-	-	-	1
P. vexans	-	1	-	-	-	-	-	-	-	-	-	-	1
Total	**27**	**20**	**9**	**6**	**5**	**4**	**3**	**2**	**1**	**1**	**1**	**1**	**80**

Activity 1.4.2. Pathogenicity of *Pythium* spp on beans, Kawanda, screen house

Rationale

The genus *Pythium* consists of more than 100 species with unique ecological preferences and includes important plant pathogens, parasites of other soil or water organisms, and saprophytes that survive on dead organic matter. Close to 30 different *Pythium* species have been encountered in the East African region. However, not all of the identified species have been checked for pathogenicity on important crops of the region. Therefore, the objective of this study was to test the pathogenicity of some isolates associated with beans. The knowledge obtained will help in designing management strategies for the disease in the region.

Materials and methods

Pythium isolates stored on PDA slants at 4°C were reactivated and sub-cultured at 24 C. Inoculum of each isolate was raised independently on millet grains for 12 days and incorporated in sterilized soil at a ratio of 1:8 v/v inoculum to soil. Three susceptible bean varieties (CAL 96, Kanyebwa, and K20) and one resistant variety (RWR 719) were grown on infected soil Disease evaluation was done 3 weeks after planting using the 1-9 CIAT scale. Isolates with mean disease score of 5.0 on CAL 96 were considered to be pathogenic to beans.

Results and Discussion

Of the 6 isolates tested two were found to be non-pathogenic (*P. diclinum*), whereas four were pathogenic (Table 1.4.2.1).

Table 1.4.2.1. Pathogenicity of different *Pythium* species on bean cultivars, Kawanda.

		Bean cultivars			
Isolate	***Pythium* spp.**	**CAL 96**	**Kanyebwa**	**RWR 719**	**K20**
GS1	*P. diclinum*	3.15	2.20	1.24	2.11
GS4	*P. diclinum*	3.26	3.22	1.65	3.65
KB15A	*P. rostratum*	6.41	6.29	2.38	6.34
KIS8B	*P. irregulare*	5.07	4.23	1.23	3.80
MC2C	*P. torulosum*	5.97	4.57	1.66	3.55
MR11a	*P. vexans*	7.46	7.57	1.58	6.96
MS61 (+ve)	*P. ultimum*	8.53	8.57	1.90	8.49
-ve control	No *Pyth*_spp	1.00	1.03	1.00	1.00

Note: Disease scale (1-9) 1 = no root symptoms; 3 = 10% of the hypocotyl and root tissues have lesions; 5= 25% of the hypocotyl and root tissues lesions 7= 50% the hypocotyl and root tissues have lesions and the root system suffers a considerable decay; 9= 75% or more of the hypocotyl and root tissues have lesions and the root system suffers advanced stages of decay and considerable reduction (Abawi and Pastor Corrales, 1990, CIAT, Cali, Colombia, 114 pp).

These results from four plantings clearly showed that *P. ultimum* was the most pathogenic species followed by *P. vexans*, *P. rostratum*, *P. torulosum*, and *P. irregulare* in that order. These results are consistent with pathogenicity tests done by Mukalazi (2000, PhD thesis, Makerere University, Uganda) in which he recorded disease scores 6-9 for *P. ultimum* and *P. torulosum* for several isolates. There is no previous pathogenicity data on *P. diclinum*, *P. rostratum*, *P. irregulare*, and *P. vexans* from this region.

Activity 1.4.3. Characterization of bean leaf crumple virus

Contributor: F. Morales

Bean leaf crumple virus continues to affect snap beans in the Cauca Valley of Colombia (Figure 1.4.3.1). All of the available snap bean lines evaluated to date (over 120 lines) and some backcrosses made to red-seeded genotypes from Honduras, have been severely affected by this whitefly-transmitted virus. It is necessary to undertake a serious breeding for resistance project for the predominant snap bean variety grown in the Cauca Valley ('Blue Lake'), using the black-seeded Mesoamerican sources of resistance identified by Bean Virology. This virus has been occasionally observed to attack bush beans under experimental field conditions. Recently, a line identified as SUG 131, showed a 100% disease incidence due to this virus under field conditions at CIAT.

Figure 1.4.3.1. Broad pathogenicity range of a new whitefly-borne virus in common bean genotypes under field conditions in the Cauca Valle, Colombia.

Activity 1.4.4. Monitoring Peanut stripe virus in common bean

Peanut stripe virus (PStV) is currently considered a strain of BCMV, but this virus has a distinct biological (pathological) behavior (CIAT, 2002, Bean Project Annual Report). In 2005, reports of a new viral disease affecting common bean in Mexico, was brought to our attention. In serological assays, the causal virus was shown to be a member of the *Potyvirus* genus. The virus was amplified using potyvirus-specific primers and then cloned for partial sequencing. The sequence obtained corresponded to Peanut stripe virus, indicating that this virus is being disseminated in Latin America by aphids and probably through sexual seed of common bean and other legumes, including peanut. The Peanut stripe strain of BCMV from CIAT (Figure 1.4.4.1**)** and Mexico show capsid protein aminoacid homologies > 95% (Figure 1.4.4.2). Peanut stripe has a pathogenicity range in common bean similar to the New York 15 strain of BCMV, but differs in certain groups of differential cultivars. For instance, NY 15 infects Michelite 62 and Red Mexican 34, whereas PStV does not; but PStV infects Great Northern 123, not attacked by NY 15. PStV does not induce systemic necrosis in the BCMV differential genotypes, and there are several sources of resistance to this virus that could be used, should the need arise.

Figure 1.4.4.1. Symptoms induced by *Peanut stripe mosaic virus* (currently *Bean common mosaic virus*) in a susceptible common bean cultivar.

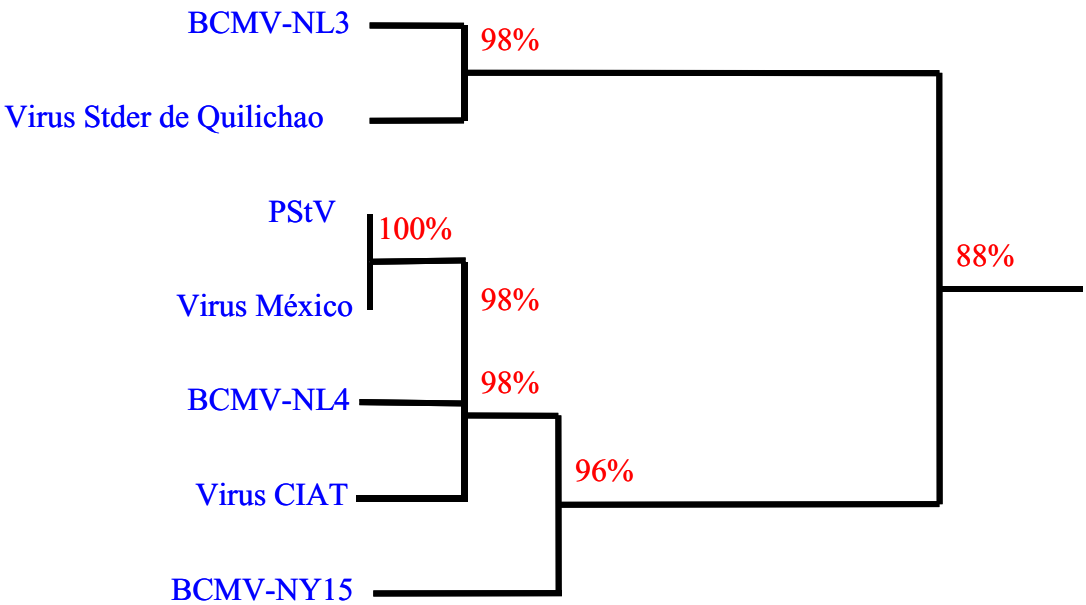


Figure 1.4.4.2. Phylogenetic Tree. Multiple alignment of the aminoacid sequence of the 3' of the Coat protein gene of BCMNV NL3, and BCMV NL4 and NY15; *Peanut stripe virus* (PStV) and bean viruses detected in Santander de Quilichao, CIAT and México (DNAMAN versión 4.13).

Activity 1.4.5. Characterization of a virus inducing systemic necrosis in common beans planted in Santander de Quilichao, Cauca, Colombia.

The potyvirus isolated from common bean in Santander de Quilichao, Cauca, in 2004, has been shown to be similar in the genomic composition of their capsid protein genes, but quite distinct from the necrosis-inducing BCMNV-NL3 strain in their host range. The pathogenicity spectrum of this legume potyvirus isrestricted to cultivars Widusa and Black Turtle Soup (Group 8), whereas BCMNV-NL3 is known to cause systemic necrosis in *I* gene groups 8, 9a and 9b.The virus also differs from BCMNV-NL3 in its pathogenicity to strain-specific recessive genes. The SQ potyvirus is particularly virulent in Groups 1 and 4, and does not induce systemic infection in various cultivars shown to be susceptible to BCMNV NL3. The phylogenetic position of this new virus is shown in Figure 1.4.4.2.

Activity 1.4.6. Detection and characterization of viruses affecting common bean in northwestern Argentina

Argentina is the main exporter of black-seeded beans in the world thanks to the improved cultivars developed by CIAT. This technical assistance has been provided in view of the high yields and low production costs that characterize bean production in northwestern Argentina (NWA), which makes black-seeded beans available to developing countries at highly reduced prices. In 2005, a virus survey was conducted in NWA to monitor the phytosanitary situation of the crop. The results of this survey showed that *Bean golden mosaic virus* is still the predominant virus in the region, and that this virus has not changed significantly in the past decade. The 'achaparramiento' (bean dwarf mosaic) disease of common bean that greatly affected common bean production in NWA in the late 1970s and early 1980s, is still present but only affects common bean genotypes that have not been improved for their resistance to these viruses (Figure 1.4.6.1). Whereas genetic recombination between the causal agents of bean dwarf mosaic and bean golden mosaic has been detected in some samples, the genetic resistance deployed in NWA is effective against both viruses.

Figure 1.4.6.1. Bean dwarf mosaic-affected field in N. W. Argentina, showing that this virus is still present in this region. All commercial cultivars deployed by CIAT in this region since 1980 are resistant to this virus.

Because these virus problems are associated with the presence of their whitefly vector, *Bemisia tabaci* , a study on the distribution of biotypes in NWA was carried out. Interestingly, the original A biotype was

still present in all the samples assayed, but there are reports on the presence of the new B biotype in NWA.

The high populations of *Btabaci* in NWA, however, pose a constant threat to common bean production, not only as vectors of begomoviruses, such as bean golden mosaic and bean dwarf mosaic viruses, but as vectors of other genera of viruses as well. In the survey conducted, a carlavirus (*Cowpea mild mottle virus*), was detected in some samples. Fortunately, this virus transmitted by *Btabaci*, does not affect bean production significantly. In Brazil, where this virus probably comes from, it only moderately affects 'Jalo' and 'Manteiga' common bean genotypes. In NWA, virus symptoms are so mild that they are usually overlooked (Figure 1.4.6.2).

Another begomovirus found to affect common bean in NWA in 1995 (CIAT, Bean Project Annual Report 1995), and later on reported as a pathogen of tomato in Brazil, appears to be Potato deforming mosaic virus, originally found in 1981 affecting potato in the province of Buenos Aires, Argentina. This virus seems to affect common bean as well in Brazil and Argentina.

Figure 1.4.6.2. Symptoms induced by *Cowpea mild mottle virus* in common bean, a filamentous virus transmitted by the whitefly *Bemisia tabaci*.

Activity 1.5. Identification of a virus associated with cassava plants affected by frogskin disease

Contributors: L.A. Calvert, M. Cuervo, I. Lozano, N. Villareal and J. Arroyave.

Rationale

The causal agent of cassava frogskin disease (CFSD) has remained unknown since the disorder was first identified in 1971. Although a viral agent has been suspected, it has been difficult to determine the etiology of CFSD. Virus-like particles of 70 and 45 nm in diameter were found in affected cassava plants and partially purified preparations, respectively. Nine species of dsRNA were associated with this disease. Eight cDNA clones were synthesized from the dsRNAs extractions. The putative proteins predicted from the sequence of the cassava virus cDNA clones have similarity with the P1, P3, P4, P5, P10 proteins of rice ragged stunt virus. Hybridization analyses of the dsRNA extractions identified the S1, S3, S4, S5 and S10 genomic segments in the CFSD affected plants, but not in healthy controls. Blind testing of diverse isolates identified most but not all the positive samples. No false positives were detected.

Introduction

Cassava frogskin disease (CFSD) is a disorder of unknown etiology that affects cassava and was first reported in 1971 from southern Colombia (Kimura *et al.*, 2001, Biosci. Biotechnol. Biochem. 65: 125-1283), and is endemic in the Amazon regions of Colombia, Peru, and Brazil. In the Amazon region, one common name for CFSD is "Jacaré" (caiman). The disorder is also present in Venezuela and Costa Rica. While the principal means of transmission are infected stem cuttings that are used to propagate cassava, there is circumstantial evidence suggesting that an aerial vector spreads disorder. The disease is transmitted through grafting (Upadhyaya *et al*., 1998, Arch. Virol. 143: 1815-1822).

In a typical CFSD affected cassava plant, there are no leaf symptoms, but the base of the stem can be swollen and the root periderm and corky layers enlarge to form raised lip shaped fissures (Fukushi *et al*., 1962, Virology 18: 192-198). Roots that are severely affected do not fill with starch. In areas where the disorder is prevalent, the yield losses can be 100%. In a limited number of cassava landraces, CFSD affected plants are stunted and the leaves develop mosaic symptoms. The roots of these varieties are either stunted or have root fissures typically associated with CFSD. When the temperatures are cooler, the symptoms tend to be more severe. When the temperature is kept constant at 30^{o}C, there is no symptom development in those landraces that normally express foliar mosaic symptoms (Fukushi *et al*., 1962, Virology 18: 192-198). CFSD can be eliminated from an affected plant by heat treatment and meristem tip culture (Milne & Lovisolo, 1977, Advances in Virus Research 21: 267-341). *In vitro* shipment of disease free certified plants is recommended for the safe movement of cassava germplasm (Calvert, 1994, International Crop Network Series Report 10: 163-165).

This report includes evidence on dsRNA genomic segments and cDNA clones that were associated with CFSD affected plants. To date about 5000 bases or 20% of the genome of CFSV has been sequenced.

Materials and Methods

Source of host plants and isolates: The CFSD affected plant materials were collected in the Andean and Amazonian regions of Colombia and maintained in greenhouses by vegetative propagation. The CFSD isolates used in this study were obtained from the cassava clones designed as Secundina 5, Secundina 80, Valluna 29, CM-5460-10, SM 909-25, Regional Tolima, CMC40, Amazonas 16, and Catumare Jamundi.

The healthy control plants were obtained from CFSD-free materials that were subjected to heat therapy and cultured *in vitro*. The *in vitro* plants were hardened and subsequently maintained in a greenhouse. When Secundina (CIAT accession CM 6014) is affected with CFSD, it develops foliar mosaic symptoms. Control test plants were periodically grafted to Secundina to assure that they had not become affected with CFSD.

Double-stranded RNA extraction and análisis: A modification of the method of Morris and Dodds (1979, Phytopathology 69: 854-858) was used to extract double-stranded RNA (dsRNA) from CMD or FSD affected plants. Young leaves or petioles were frozen with liquid nitrogen and homogenized with two volumes of extraction buffer (2X STE, 10% SDS, 1% bentonite, and 0.5% 2-mercaptoethanol) and 0.5 volumes of chloroform:pentanol (24:1). The extracts were centrifuged for 10 min at 8,000 *g*, and the aqueous phase was collected. Ethanol was added to a final volume of 16.5%, and for each gram of tissue, 0.1 g CF-11 cellulose was added to the extracts. The slurries were stirred for 30-60 min and poured into columns and drained. The columns were rinsed with 100 ml of 1X STE containing 16.5% ethanol. The columns were rinsed with 0.1 ml of 1X STE, and the ds-RNAs were eluted using three 0.1 ml aliquots of 1X STE. The nucleic acids were precipitated with 2.5 volumes of absolute ethanol followed by centrifugation. The pellets were dried and resuspended in 1X TAE buffer (0.04M Tris acetate, 1 mM

EDTA adjusted to pH 7.8 with acetic acid) containing 5% glycerol, bromo-phenol blue and xylene cyanol. The preparations were run on a 5% polyacrylamide or 1% agarose gels using a continuous buffer system of 1X TAE. On most gels, the 1-kilobase ladder from BRL (Bethesda, MD) was used as the marker. To more accurately estimate the M_r of the dsRNA species, a series of polyacrylamide gels were run using Bozarth's dsRNA standard markers (Bozarth & Harley, 1976, Biochim. Biophys. Acta 432: 329-335). The gels were stained with ethidium bromide (1 εg/ml) and examined using a UV-transilluminator.

In order to test the type of nucleic acid, the samples were subjected to digestion using a concentration of 0.1 U/µl RQ-1 Dnase (Promega), 1.2 U/µl S1 nuclease, or 0.9 εg/ml Rnase A in the presence of either high (2X) or low (0.1 X) SSC (0.15M NaCl, 0.015 M sodium citrate, pH 7.0). The samples were analyzed using TAE polyacrylamide electrophoresis.

In some cases, the dsRNAs were purified from 2% low melting point agarose gels. After staining with ethidium bromide, selected gel pieces were melted a 70 ºC, 2X STE, 2% SDS and 0.1% bentonite were added, and the extraction procedure describe for the plant material was followed.

The synthesis of cDNA from the dsRNA: The syntheses of cDNAs were started by denaturing 5 σg dsRNA and 500 ng of random (hexameros) (Gibco BRL) or 10 bases oligonucleótidos (Operon) primers using 40 Mm methyl-mercuric hydroxide at a final volume of 13 σl. The mixtures were incubated for 10 min at room temperature and frozen using liquid nitrogen. The samples were the allowed to thaw out and immediately processed. The first strand was synthesized in a final volume of 40 σl containing 50Mm Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dATP, dCTP, dGTP, dTTP, 40 U of Rnase in (Promega) and 400 U of SuperScript II RT (Gibco, BRL). The mixture was incubated for 60 min a 37∀C. Then an additional 200 U of SuperScript II RT was added to the mixture and the reaction was allowed to continue for 30 min. The reactions were subjected to 70∀C for 1 min and placed in ice water for 2 min. To the 40σl of the first strand reaction 25 mM Tris-HCl (pH 7.5), 100 mM KCl, 5 mM $MgCl_2$, 10 mM $(NH_4)_2SO_4$, 0.15 mM η-NAD^+, 0.25 mM each dATP, dCTP, dGTP, dTTP, 1.2 mM DTT, 25 U *Ecoli* Ligase, 40 U *Ecoli* Polymerase, 4 U *Ecoli* RNase H were added and the final volume was 150σl. After the mixture was incubated for 3 h at 16∀C, 30 U of T4 DNA Polymerase was added and the reaction was continued at 16∀C for 10 min. The reaction was stopped by the addition of 10 σl of 0.5 M EDTA, pH 8.0 and treated with phenol:chloroform:isoamyl alcohol (25:24:1). The cDNAs were precipitated with 1/10 volume of 7 M ammonium acetate and 2.5 volumes of 95% ethanol and resuspended in sterile DEPC treated water. The cDNAs were modified by adding a 3'A-overhangs and ligated in to the pCR 2.1 vector (TA cloning Kit, Invitrogen).

Elution and amplification of selected cDNA: The polymorphic cDNA amplified products were eluted from pieces of the agarose or polyacrylamide gels. The selected pieces of the polyacrylamide gels were soaked in water for 10 min at room temperature then an additional 15 min at 65∀C. The aqueous portion containing the cDNA was subject to an ethanol precipitation and the pellet was resuspended in sterile water. Five σl of each sample was amplified using 10 mM dNTPS, 1X PCR buffer, 2 mM $MgCl_2$, 0.2 σl de 10 U/σl Taq polymerase final volume of 20 σl. The PCR profiles were the same used in the original amplification, and the products were visualized used agarose gel electrophoresis.

Cloning and sequencing of PCR products: The PCR products were cloned into the TA plasmid (Invitrogen). Plasmid DNA was purified using Wizard™ plasmid purification columns (Promega). Nucleotide sequences were determined using an ABI Prism 377 sequencer (Perkin-Elmer) by the dideoxynucleotide chain termination procedure (Shikata *et al*., 1979, Ann. Phytopathol. Soc. of Jpn. 458: 436-443) using the ABI dye terminator reaction ready kit. The sequence data were analyzed using SEQUENCHER version 4.1.2 for Macintosh, NCBI BLASTX, and DNAMAN Version 4.13 (Lynnon Biosoft, Vaudreuil, Quebec).

Results and Discussion

Double-stranded RNA extraction and analysis: Nine species of dsRNA were visualized in plants affected with CFSD, but not in the healthy controls. The nine species of nucleic acids present in the PAGE gels were determined to be dsRNA by digestion experiments using RQ1 DNase, RNase A, and S1 nuclease. These nine species of nucleic acids were not digested by RQ1 DNase or S1 nuclease; were resistant to digestion by RNase A in high salt, but were digested in low salt. It was determined that these were ds-RNAs and their size was estimated using ds-RNA markers. The sizes of the nine species were estimated to be between 4000 to 1000 bases (Table 1.5.1).

Complementary DNA cloning of the dsRNA associated with CFSD: The cDNA clone designed CFSV-S1 and was 228 nucleotides in length and contained an open reading frame of 62 amino acids. The putative protein had 39% identity and 55% similarity with the homologous region of the RRSV P1 protein (Figure 1.5.1). The clone CFSV-S1 was used to localize the S1 genomic segment in agarose gels. The dsRNA genomic segments in this region were eluted from the agarose gels and cDNA clones were obtained. One cDNA clone was 867 nucleotides in length and contained an open reading frame of 288 amino acids. When it was determined that this cDNA clone had similarity with the RNA dependent RNA polymerase protein encoded by the genomic segment 4 of RRSV, and it was designated as CFSV-S4-867. A second cDNA clone was 580 nucleotides in length and contained an open reading frame of 159 amino acids. It had 367 nucleotides in common with CFSV-S4-867 and the contig of these clones were designated as CFSV-S4-1 and were submitted as one accession. The putative protein based on the contig of the cDNA clones was 355 amino acids and had 37% identity and 55% similarity with RRSV P4 (Figure 1.5.2). A third cDNA clone with similarity to RRSV RNA 4 was identified (Figure 1.5.1) and designated CFSV-S4-2. A comparison of the protein encoded by RRSV RNA 4 (Upadhyaya *et al*., 1997, Arch Virol. 142:1719-26) and the putative protein derived from these three CFSV cDNA clones is shown in Figure 1.5.3. Two additional cDNA clones were identified to that have similarity with RRSV RNA 3 (Figure 1.5.1) and one was designed as CFSV-S3-1 (GenBank accession DQ139868) and the other as CFSV-S3-2. Another cDNA clone was identified as having similarity with the RRSV RNA 10 (Vos *et al*., 1995, Nucleic Acids Res. 23: 4407-4414) and was designated at CFSV-S10 (Figure 1.5.1).

Association of cDNA clones with CFSD: Double-stranded RNA was extracted from individual healthy and CFSD affected plants and transferred to membranes. The CFSV-S5-351 clone hybridized to a species of dsRNA of approximately 3000 nucleotides that was extracted from CFSD affected plants. This genomic segment was detected in all nine isolates of CFSD that were tested, and was absent in the healthy controls. Both the CFSV-S1 and the CFSV-S4 clones hybridized to species of dsRNA that were approximately 4000 nucleotides that were extracted from CFSD affected plants. The genomic segment 4 was detected in all nine isolates of CFSD that were tested, but the S1 hybridized readily with Valluna 29 and Amazon 16, but less so with the other seven isolates (data not shown). A genomic segment of approximately 3000 nucleotides of this cassava virus were identified using the cDNA clone CFSV S5 as a hybridization probe (Figure 1.5.2B). The dsRNA for these hybridization experiments were run in agarose gels using DNA markers. The estimated size of the S5 segment was 3000 nucleotides as compared with the estimate of 2700 nucleotides when the size estimate was made in polyacrylamide gels using dsRNA markers.

Using primers designed from the cDNA S4 clones, the PCR products were amplified from either leaves or root tissues in nine isolates of CFSD but were not amplified in healthy controls (Figure 1.5.2C). The PCR products were transferred to membranes and visualized using the largest CFSV-S4 clone as the hybridization probe. It detected specific products of the expected size of 1000 bp from the leaf tissues in the CFSD affected plant but did not detect any products in the healthy controls (Figure 1.5.2D). This experiment was repeated using extracts from root tissues and the genomic S4 segment and the rt-PCR products were detected in all of the isolates tested.

Table 1.5.1. A comparison of the known size of the genomic segments of rice ragged stunt virus (RRSV) with the estimated size of the cassava frogskin virus genomic segments.

Genomic Segment No.	RRSV Size (bp)	CFSV Size (M_r) expressed as bp
1	3849	4000
2	3810	
3	3669	3700
4	3823	3900
5	2682	2600
6	2157	1800
7	1938	1750
8	1814	1700
9	1132	1100
10	1162	1000

The 70 nm virus-like particles and inclusion bodies found in CFSD affected cassava plants are similar to the virions and the inclusion bodies that are characteristic of plant viruses in the family *Reoviridae* (Nuss & Dall, 1990, Advances in Virus Research 38: 249-306). While most of the plant reoviruses are restricted

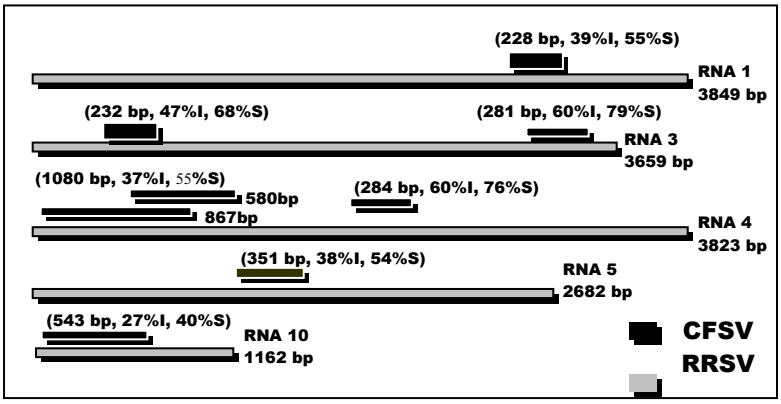


Figure 1.5.1. A comparison of the putative amino acid sequences deduced from cDNA clones of a virus found in cassava. The putative protein of CFSV-S4 is compared with the protein encoded by the segment 4 of rice ragged stunt virus.

to phloem or neoplastically developed phloem-derived tissues (Frison & Feliu, 1991, Technical guidelines for the safe movement of cassava germplasm. FAO/IBPGR), rice dwarf phytoreovirus (RDV) is found in both phloem and parenchyma cells. In most cassava landraces, the virus-like particles associated with CFSD were found only in phloem or associated cells of the affected plants. In the cassava landraces that developed leaf symptoms, virus-like particles and inclusion bodies could be found in many cell types, including the parenchyma cells of the leaves. CFSD is characterized by the hyperplasia of the root cortex. The fissures in the cortex of the cassava roots are similar to the symptom of longitudinal splitting in roots of maize infected with maize rough dwarf fijivirus (Milne & Lovisolo, 1977, Advances in Virus Research 21: 267-341).

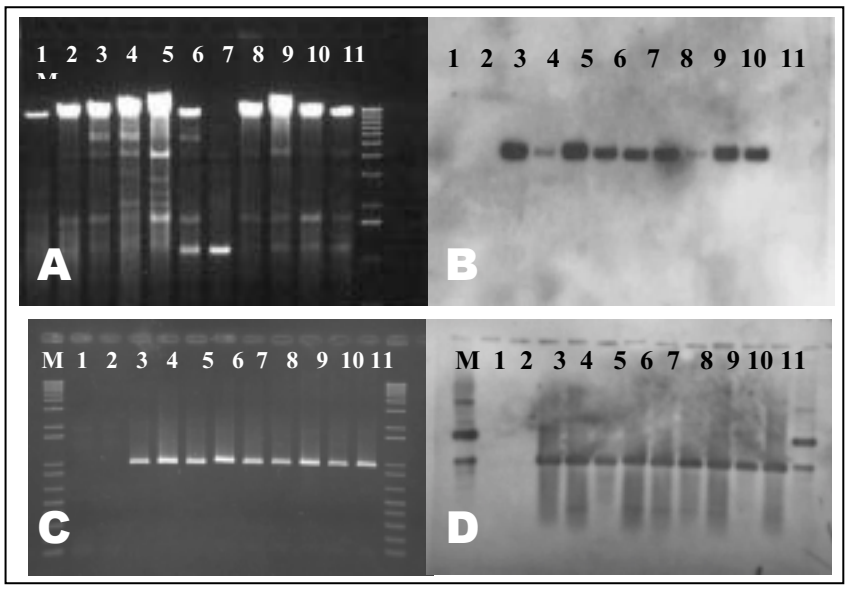


Figure 1.5.2. A series of dsRNA extractions and rt-PRC amplifications with their hybridizations using a cDNA probe derived from the segment S5: A) dsRNA Profiles isolated from healthy and affeted varieties in 1% agarose gel stained with ethidium bromide, B) hybridization using a cDNA probe derived from the segment S4, C) rt-PCR products using CFSV-S4 primers from healthy and CFSD affected varieties in 1% agarose gel stained with ethidium bromide, D) hybridization with the S4 probe. Healthy varieties: 1: Secundina, 2: CMC40. CFSD affected varieties: 3: Secundina 5, 4: Secundina 80, 5: Valluna 29, 6: CM-5460-10, 7: SM 909-25, 8: Regional Tolima, 9: CMC40, 10: Amazonas 16, 11: Catumare Jamundi, M: 1Kb + DNA molecular marker.

The virus-like particles found in the partially purified preparations were 45-50 nm in diameter and are similar to the core particles found during purification of other reoviruses (Pineda *et al*., 1983, ASIAVA 4: 10-12; Upadhyaya *et al*.,1998, Arch. Virol. 143:1815-1822). These particles were labile and were disrupted if subjected to additional steps of centrifugation. The size and fragility of these virus-like particles are similar to the plant reoviruses sub-viral particle that consists of double stranded nucleic acids and the inner shell or core (Bozarth & Harley, 1976, Biochim. Biophys. Acta 432: 329-335).

The relative molecular weights (M_r) of the dsRNAs found in CFSD affected plants are similar to those of RRSV (Collinge & Boller, 2001, Plant Mol. Biol. 46: 521-529), but only nine species of ads-RNA could be visualized using polyacrylamide gel electrophoresis. It was first reported that there were only eight genomic segments of RRSV, but the molecular characterization confirmed that genome of the RRSV consists of 10 species of dsRNA. It is expected that the existence of the 10 species of this cassava virus will be resolved through the molecular analysis of the genomic segments.

The use of RAPD and AFLP analyses to detect genetic differences is well documented and rt-AFLP is used as a means to identify differentially expressed genes (Kimura *et al*., 2001, Biosci. Biotechnol. Biochem. 65: 125-1283). Cytokinin-induced gene expression in cultured green cells of *Nicotiana tobacum* identified by fluorescent differential display). The successful cloning of a portion of this plant virus genome demonstrates that rt-RAPD and rt-AFLP can be useful techniques to characterize elusive causal agents.

Based on the amino acid similarity of putative proteins of portions of the segment 1, 3, 4, 5 and 10, the virus affecting cassava is most similar to the RRSV in the genus *Oryzavirus* of the family *Reoviridae* (Lozano & Nolt, 1989, Plant Protection and Quarantine: Selected Pests and Pathogens of Quarantine Significance 2:174-175; Upadhyaya *et al*., 1997, Arch Virol. 142:1719-26; Vos *et al*.,1995, Nucleic Acids Res. 23: 4407-4414). The two members of the Genus *Oryzavirus* are RRSV and ERSV, but there is sequence information only for RRSV, and it has little similarity with the reoviruses in the other genera. Given the geographical separation of the range of RRSV and its distant relation with other reoviruses, it is surprising that this cassava virus is related to it. The nucleotide or amino acid sequence analysis, of the eight cDNA clones of this cassava virus, revealed only significant similarity for RRSV.

The genomic segments of this cassava virus were identified in hybridization studies using cDNA clones. Also, the cDNA clones can detect these genomic segments in all nine isolates of CFSD tested to date, but they cannot be detected in CFSD free cassava. There are consistent differences in the intensity of the hybridization with the different isolates and this suggests that they may be significant variation between the isolates of this virus. Nevertheless, the virus can only be detected in CFSD affected plants, which suggests that this virus is associated with cassava frogskin disease.

Activity 1.6. Transmission of cassava frog skin disease: evaluation of homopteran species as vectors.

Contributors*:* Ma.P. Hernandez and A.C. Bellotti

Highlight:

∉ Several Delphacidae species, possible vectors of CFSD, collected from cassava fields infested with CFSD.

Rationale

Cassava frog skin disease (CFSD) is native to the Neotropics, probably originating the Amazon region of South America. It causes reductions in cassava root yields and hinders the movement of germplasm within and between countries. Detection of the disease is made difficult due to the lack of foliar symptoms on most cassava varieties. The disease has been described as a virus of the Family Reoviridae and/or a phytoplasm. The epidemiology of CFSD is still being studied. CFSD dissemination by infected planting material (stem cuttings) is well documented but the roll of an insect vector has not been adequately determined. Although previous research indicated the involvement of a whitefly (*Bemisia tuberculata*) vector, this is being questioned and re-evaluated (Calvert and Thresh, 2001, *In*: Hillocks *et al*., (eds), Cassava: Biology, Production and Utilization, CABI Publishing, pp 237 - 260). The beginnings in 2002 field surveys have been conducted in several regions of Colombia to collect possible vectors of CFSD. Emphasis is being given to homopteran species, especially those of the family Delphacidae, which are known vectors of Reoviruses. The objective of these studies is to determine the actual vector of CFSD (See CIAT PE-1 Annual Reports, 2003, 2004).

Materials and Methods

During the last trimester of 2004 and the early months of 2005, homopteran specimens from the families Delphacidae and Cixiidae were collected from cassava fields known to contain CFS diseased plants. Specimens were collected from six localities in the Valle del Cauca, Cauca, Quindio and Risaralda departments of Colombia. Additional samples were collected from vegetation in fields adjacent to cassava fields.

Collected insect specimens were brought into the laboratory and prepared for taxonomic identification. Preliminary identification can be done at CIAT while specimens are also sent to Dr. Stephen Wilson of the University of Missouri, a specialist in the families, for further verification.

Results and Discussion

Delphacidae species collected at CIAT, Palmira, were identified in the CIAT laboratory as *Peregrinus maidis* (Ashmead), *Sogatella kolophon* kirkaldy and *P. saccharicida* (identifications were corroborated by Dr. Stephen Wilson) [Table 1.6.1]

P. saccharicida was also collected from fields in the Rozo municipality. Adults of *P. maidis* were collected from several experimental fields at CIAT where CFSD was present. It should be noted that several cassava fields were weedy, especially with *Rottboellia exalta* (caminadora) and all nymphal instars of *P. maidis* were captured from this species. When weeds were removed, additional collections were made from these fields and high populations of *P. maidis* were again collected. This indicates that *P. maidis* can successfully feed on cassava in the absence of its preferred host.

The presence of *P. saccharicida* in cassava can be explained by the considerable cultivation of sugarcane on the CIAT farm. The ability of these species to successfully feed and reproduce on cassava needs to be determined.

Table 1.6.1. Homopteran species associated with the cassava.

Dept.	Municipality	Locality	Host	Family	Crop species
Valle del Cauca	Palmira	CIAT	*M. esculenta* *Rotboellia exaltata*	Delphacidae	*Peregrinus maidis* (Ashmead)
			M. esculenta *Digitaria sp*	Delphacidae	*Sogatella kolophon* Kirkaldy
			M. esculenta		*Perkinsiella saccharicida*
			M. esculenta	Cixiidae	*Oliarus* sp.
	Rozo		*M. esculenta*		*P. saccharicida* *Oliarus* sp.
Cauca	Santander de Quilichao	Granja CIAT	*M. esculenta* *Digitaria sp*		*S. kolophon*
			M. esculenta		*Oliarus* sp.
Quindío	Armenia	Marmato	*M. esculenta*		*S. kolophon* *S. molina*
Risaralda	Morelia	Sta . Rita	*M. esculenta*		*Oliarus* sp
	Cerritos		*M. esculenta*		*Oliarus* sp.

Identifications verified by:Dr. Stephen Wilson (Central Missouri State University).

S. kolophon was collected from several of the sites surveyed, while *S. molina* was only collected from fields in Armenia, Quindio. *Cixiidae* species are considered as possible vectors of phytoplasmas, but few species have been studied. *Oliarus* sp was collected from most of the sites surveyed; it would be

interesting to evaluate its relationship to cassava and CFSD since nymphs are found feeding on cassava roots.

Activity 1.7. Rearing of Delphacidae species, possible CFSD vectors on natural hosts.

Contributors: Ma.P Hernández and A. C. Bellotti

Highlight:

- Two Delphacidae species, *Peregrinus maidis* and *Sogatella kolophon,* possible vectors of CFSD successfully reared on gramineous hosts in the greenhouse.

Rationale

The search of an insect vector of CFSD has, in recent years led to the identification of numerous homopteran species found in association with the cassava crop. With the exception of whiteflies, mealybugs and scale insects, none of these are considered economic pests of cassava, causing yield losses. Most species collected belong to the homopteran families of Cicadellidae, Delphacidae and Cixiidae (See CIAT PE1 Annual Reports 2002, 2003 and 2004). However, species within these three families are known vectors of virus or phytoplasm diseases. Cassava is often grown in association with other crops and cassava fields will often contain several weed species that could be the preferred hosts of these homopterans. It is therefore necessary to determine if these insect species can successfully infest cassava and hence be a possible vector of CFSD. The objective of this research is to develop rearing methods for two Delphacidae species collected from cassava, first on their native hosts and secondly evaluate their ability to colonize cassava.

Materials and Methods

Nymphs and adults of *P. maidis* and *S. kolophon* (Fam. Delphacidae) were collected from field plantings of cassava at CIAT, Palmira and Santander de Quilichao, Cauca, during the first trimester of 2005. Specimens were also collected from weeds adjacent to the cassava plots. Field captures were done with the aid of a sweep-net or a buccal aspirator. Specimens were than placed in plastic boxes containing cassava leaves or grasses for live transportation to the laboratory.

Rearing is initiated in nylon meshed cages in the greenhouse. *P. maidis* is placed on six plants of its natural host, *Rottboellia exalta* and *S. kolophon* on its host *Digitaria sp*. (Figures 1.7.1 a, b). At the same time *P. maidis* nymphs and adults were placed in a nylon meshed cage containing both cassava and *R. exalta* (50% - 50%) in order to facilitate adaptation of *P. maidis* to cassava. Greenhouse conditions were; temp. 21 to 29∀C; 63-100% RH and 12:12 hrs photoperiod. Lifecycles studies for both species on their grass hosts were carried out in the greenhouse.

Results and Discussion

P. maidis and *S. kolophon* have been successfully reared on their natural hosts (*R. exalta* and *Digitaria sp,* respectively) in the greenhouse. Experiments to adapt these homopteran species to cassava have been discouraging. Both species prefer to feed on their natural gramineous hosts rather than on cassava. Maximum survival on cassava is only four days once the grass hosts are removed. Although *P. maidis* is oligophagous, the adaptation to a "new" host appears to be difficult. However, these studies will continue.

Biological characteristics of P. maidis: Hosts: this species is frequently found associated with maize, sorghum, millet, gramineous weeds and some shrubs and horticultural plants (Denno and Roderick, 1990, Annual Review Entomol. 35: 489-520).

Figures 1.7.1 a, b. *P. maidis* colonizing its native host *Rottboellia exalta* in the greenhouse.

Natural Enemies: Strepsitera parasites, possibly *Elenchus sp* were reared from field collected *P. maidis* individuals.

Geographic distribution: Widely distributed, especially in the tropical and sub-tropical regions.

Lifecycles studies: Under greenhouse conditions the egg to adult stages were 29-30 days. The egg stage is 10 to 12 days and nymphal development is 15 to 18 days. Females oviposit 17 $\pm$ 2.0 eggs per day and sex relation is 1.6 ♀ ; 1 ♂ .

Eggs are translucent, becoming more whitish as they develop. They are elongated, slightly curved near the cephalic region (Figure 1.7.2). Eggs are inserted in the central leaf vein.

1.7.2 **1.7.3** **1.7.4**

Figures 1.7.2-4. Eggs, nymph (N4) and adult *Peregrinus maidis* feeding on *Rottboellia exalta* leaves.

There are five nymphal instars; coloration during the first 3 instars is yellowish and dark spots appear on the thorax and abdomen during the latter instars (Figure 1.7.3). During the N4 nymphal stage the genus is distinguishable. It is common to find branchipterous individuals within the population, owing to conditions of confinement.

Adults can be characterized by their general coffee-yellow coloration with darkend areas around the distal zones of the first two antennal segments, on the terminal parts of the costal nerves and more diffuse on the dorsal-apical area and on the tergos, pleuras and exterior of the abdomen (Figure 1.7.4). Legs are yellowish with the coxas and femurs a dark brown.

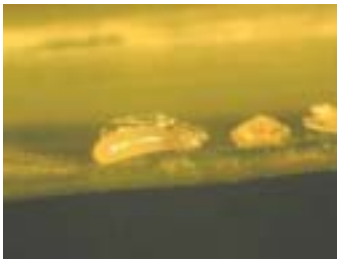
P. maidis is the recognized vector of five virus diseases, maize stripe, Iranian maize mosaic, maize mosaic, maize sterile stunt and finger millet mosaic, that affect maize and sorghum (S. Wilson, personell communication).

Biological characteristics of Sogatella kolophon: Hosts: Found on grasses, often at low densities. Sporadically recovered from maize and oats.

Natural Enemies: Similar to *P. maidis*, Strepsiptera parasites, possibly *Elenchus sp* recovered from field collected specimens.

Geographic distribution: Amply distributed, including the eastern Pacific, the Ethiopian region and in the new world it is reported from the USA, Mexico, Bermuda, Guyana and Argentina. This is the first established report from Colombia.

Lifecycle studies: The egg to adult stage under greenhouse conditions was 26 to 39 days. Egg eclosion is in 10 days and the average nymphal period is 15 to 17 days. Eggs are inserted in the central leaf vein and stem by using the ovipositor as a sword. Eggs are cream colored, turning more yellowish as they advance in development. Although eggs are similar to *P. maidis,* they are smaller (Figure 1.7.5).



1.7.5

1.7.6

1.7.7

Figure 1.7.5-7. Eggs, nymph (N4) and adult of *Sogatella kolophon* feeding on *Digitaria* sp. in the greenhouse.

Nymphs have five instars, are clear yellow in color, becoming reddish as development advances (Figure 1.7.6). Adults of this species are easily distinguishable by the presence of a whitened longitudinal dorsal band on the vertex and thorax of the pronotum. The tegman is translucid with a dark spot on the apical-dorsal area.

Sexual dimorphism occurs in color and size; females are a clear yellow in color (Figure 1.7.7) while males are dark brown. *S. kolophon* is considered to be the vector of Brasilian wheat spike disease, Digitaria striate virus and maize sterile stunt virus.

Activity 1.8. Evaluation of *Peregrinus maidis* and *Sogatella kolophon* as possible vectors of CFSD.

Contributors: Ma.P Hernández and A. C. Bellotti

Highlights:

- Planthoppers species are the primary reovirus vectors.
- A two field collected species suspected of being reovirus vectors in cassava crops: *S. kolophon* and *P. maidis.*
- Studies of transmission were carried out with healthy insect raised in the laboratory; different acquisition, latency and inoculation periods were test.
- Virus detection in inoculated plants have so far been unsuccessful

Rationale

The causal agent of cassava frog skin disease has been described as a virus from the family Reoviridae. Reoviruses are known to be transmitted or vectored by homopterans of the family Delphacidae. There is a hypothesis that reoviruses of plants are originally from insects (Noda and Nakashima, 1995, Seminars in Virology 6: 109-116). According to results reported by the CIAT Virology group (PE1 Annual Report 2004) the virus found in CFSD infected materials shows a 72% homology with Rice ragged oryzaviruses (RRSV). The Delphacidae is an economically importantly family within the Superfamily Fulgoridae; most species feed on monocodyledonous plants and are known vectors of viruses in maize, sugarcane, rice and other crops (Denno and Roderick, 1990, Annual Review Entomol. 35: 489-520). There are nearly 50 genera and about 255 known species in South America, compared with 290 species reported from Mexico.

The objective of this research is to evaluate these Delphacidae species as possible vectors of CFSD in healthy cassava, var. Secundina.

Materials and Methods

Virus acquisitions were conducted by first selecting 50 N3 and N4 nymphs from established colonies of P. *maidis* and *S. kolophon* in the greenhouse. These were allowed to feed on two-week cassava plants that showed marked symptoms of CFSD. These plants originated from stem cuttings of plants naturally infected with CFSD in the field that displayed typical root symptoms. This should guarantee the recovery of native strains of the CFSD virus.

The virology unit verified the presence of the CFSD pathogen in the plant, when the stem cuttings were selected. Samples were taken from the youngest leaves of the selected plants. The insects feed on cassava plants for 48 hours to acquire the virus. After this period the Virology Unit examined five individuals using PCR.

Individuals feeding on CFSD infected plants were separated into two groups: half were placed on *Digitaria sp.* plants (*S. kolophon*) and *Rottbollia (P. maidis)* for an initial period of 5 days. The second group were placed on healthy Secundina plants for 48 hours or until insect death. It is not known if these species can transmit the virus immediately after feeding on infested plants. To insure feeding the methodology used employed small cages that confined the insect to a specific site on the cassava plant. Nine cassava plants were used in the transmission trial and nine plants were employed as controls and held under the same conditions.

Inoculated plants were placed in the Virology Unit greenhouse under controlled conditions to await expression of CFSD symptoms. Plants were observed daily during a one month period or until the appearance of symptoms (Figure 1.8.1).

Results and Discussion

At present CFSD virus transmission has not been achieved as Secundina plants do not show leaf symptoms. During the course of these evaluations, changes in methodologies have occurred, but it is recognized that longer observational periods may be required as well as more detailed studies with these organisms.

Molecular studies with those individuals that have fed on plants with CFSD symptoms have not provided conclusive results. It is considered that the PCR methodology that was designed for plants will need to be further adjusted for virus detection in these insects.

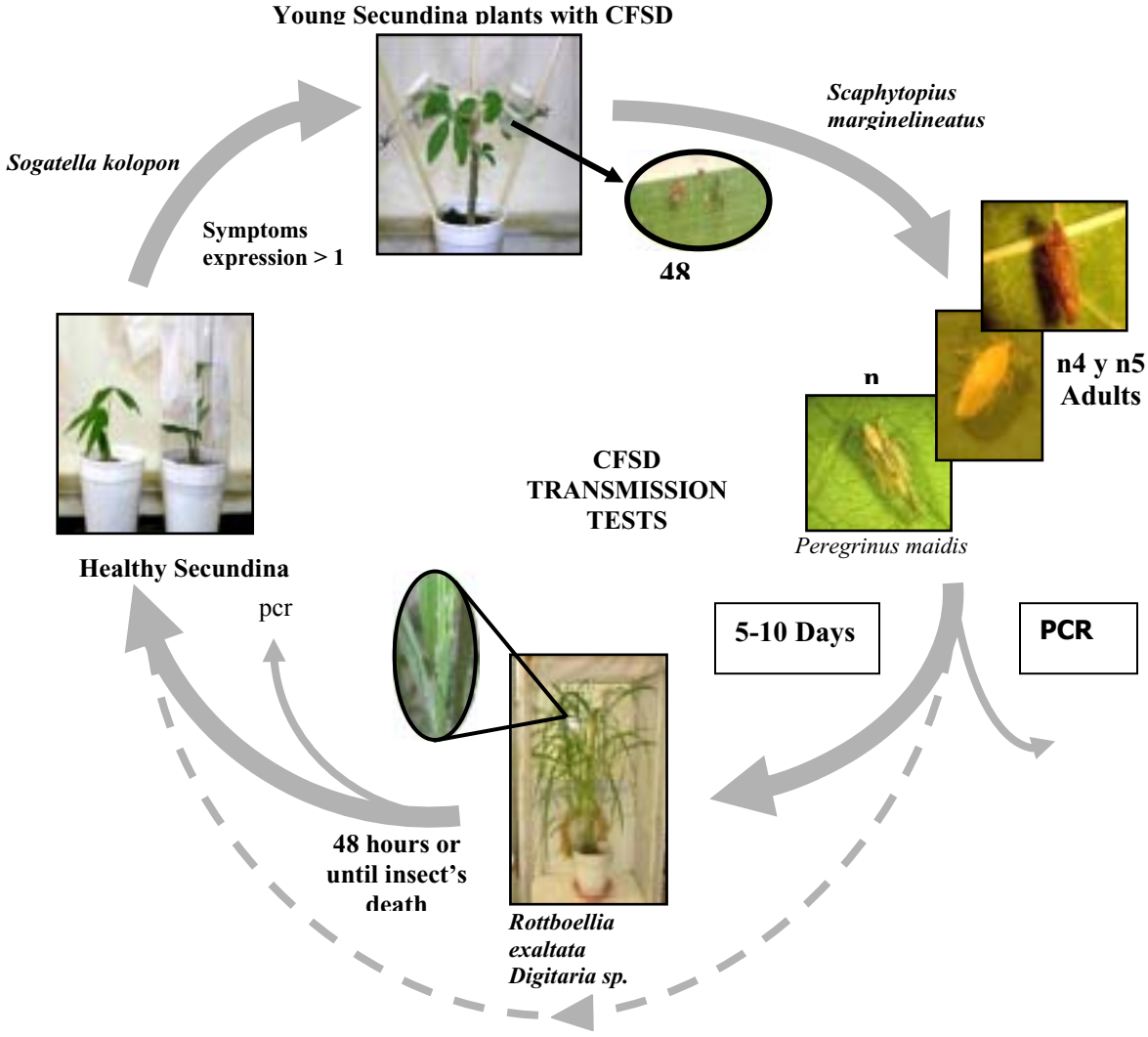


Figure 1.8.1 Cassava Frog Skin disease transmission test scheme

Activity 1.9. Evaluating the influence of soil as a source of cassava frog skin disease vectors

Contributors: E. Álvarez, J. F. Mejía, and J. Loke

Rationale

Phytoplasmas arze a group of bacteria that lack cell walls. They are obligate pathogens of a wide range of different plants. Phytoplasmas can spread through infected plant material or insect vectors. Because phytoplasmas do not grow on artificial media, studying them is extremely difficult, and many questions about their biology, spread, pathology, and control remain unanswered.

Phytoplasmas are spread mainly by cicadas and planthoppers, which have sucking-piercing mouth parts (order: Hemiptera, families: Jassidae, Cixiidae, and Psyllidae). Both adults and nymphs can spread phytoplasmas. Host specificity is variable between vector species. Some vectors are polyphagous and feed on a variety of different host plants, for example, Hyalesthes obsoletus on grapevine (giving rise to "bois noir"). In general, three types of host plants can be distinguished: those on which vectors feed throughout their whole life cycle (both imagos and larvae); those on which imagos (but not larvae) feed regularly; and those on which imagos feed only arbitrarily.

The vectors cannot transmit the phytoplasmas immediately after feeding on an infected plant, but only after incubation, the length of which depends on temperature, among other parameters. During incubation, the phytoplasmas multiply and spread within the insect. No vertical infection of insects is known, meaning no phytoplasmas are transmitted from one generation to the next one via infected eggs. Phytoplasmas also have been found in some insect groups that most likely do not transmit them. In these insects, phytoplasmas either do not multiply or transmission to plants is inefficient because the mouth parts are different.

Phytoplasmas can survive in the soil; they are also found in certain soilborne insect stages and in living roots, but are unlikely to survive in pure (mineral) soils and dead plant material. Our study aimed to evaluate the soil as a possible source of microorganism vectors of cassava frogskin disease (CFSD), and whether the presence of aerial vectors is related to the dissemination of this important disease.

Materials and Methods

In Santander of Quilichao (Cauca, Colombia), a field trial was established with cassava variety HMC-1, using stakes from infected plants (Jamundí, Valle del Cauca, Colombia) and healthy plants (Montenegro, Quindío, Colombia). To cut the stakes, a machete was disinfected in a solution of 1% sodium hypochlorite. The plants were planted inside and outside a screen house.

The outside plants were separated into two blocks, one receiving a weekly application of insecticides to control aerial vectors and the other no applications. Plots of 12 plants were established in a randomized complete block design with 3 replications, where the main plots were with insecticides and without insecticides. The plants inside the screen house received weekly applications of insecticides, rotating two products: either Sistemin® (dimethoate, 3 cc/L of commercial product) or Malathion® (malathion, 1 cc/L of commercial product). The subplots, both inside and outside the screen house, were plants from healthy seed and plants from seed infected with CFSD.

On day 30 after planting, stake germination was evaluated and records periodically taken on the presence of pests and diseases. When the plants were 6 months old, the presence or absence of symptoms of CFSD was recorded for roots in a sample of 3 plants per plot. In addition, samples of roots and leaves were collected for detecting phytoplasmas through nested PCR, using the methodology described in Activity 2.

When the plants were 10 months old, cuttings from 3 plants in each plot were taken to root in water and observe the expression of symptoms in leaves under laboratory conditions (23–30 °C). At 11 months, all the plants were harvested, symptoms in roots recorded, and DNA extracted for detecting phytoplasmas through PCR.

Results and Discussion

Plants from infected stakes presented symptoms of CFSD, whereas plants from healthy seed did not show symptoms.

Variety HMC-1 expressed leaf symptoms (chlorosis and mild curling) (Figure 1.9.1) in the cuttings that rooted under laboratory conditions. The plants with symptoms in the laboratory were from plants that expressed CFSD in the field, at a correlation of 0.82.

Figure 1.9.1. Leaves from cassava plants grown from cuttings rooted in the laboratory. The cuttings were taken from both healthy and diseased plants growing in a trial established in the field with variety HMC-1. The leaves show different degrees of severity of cassava frogskin disease: (A) healthy plant; (B) initial symptoms presenting minor chlorosis and curling in some leaf lobes; and (C) severe symptoms of chlorosis and curling.

Table 1.9.1 illustrates the different degrees of severity of disease per plant observed during evaluations at harvest and the symptoms expressed by the laboratory cuttings. The evaluation by nested PCR for phytoplasma was carried out for leaves and roots obtained from plants established in the field. A correlation of 0.37 was obtained between the degree of severity and detection by PCR. This correlation is high if we take into account that many of the plants from healthy seed and evaluated as healthy at harvest may possibly have acquired the pathogen and only in the following cycle would the symptoms be observed, thus demonstrating the technique's potential to carry out early detection of the pathogen in plants that as yet do not express symptoms.

To correlate presence of the disease and detection by PCR for phytoplasmas, the frequencies of amplification were analyzed between the number of plants amplified by plot according to treatment (i.e., outside screen house and not fumigated; outside and fumigated; inside and fumigated weekly with insecticide—*see* Table 1.9.1).

Table 1.9.1. Evaluation of different treatments in the field in terms of severity of disease, symptoms in leaves, and nested PCR for cassava frogskin disease.

Plot	Treatment[a]	Rep.	Source of seed[b]	Germin -ation	Population (%)	Severity per plant[c]	Symptoms in cuttings/plant[d]	PCR[e] (+/-)
1	Outside, not fumigated	1	D	100	100	4, 4, 4	nd, Cl, nd	3/3
2	Outside, not fumigated	1	H	91.7	83.3	1, 1, 1	nd, nd, nd	1/3
3	Outside, not fumigated	2	D	100	100	3, 4, 3	Cl, nd, nd	2/3
4	Outside, not fumigated	2	H	100	83.3	1. 1, 1	Ns, nd, nd	1/3
5	Outside, not fumigated	3	H	100	100	1, 1, 1	nd, nd, Ns	3/3
6	Outside, not fumigated	3	D	100	100	4, 4, 4	nd, nd, nd	2/3
7	Inside, fumigated	1	H	91.7	91.7	1, 1, 1	Ns, Ns, Ns	0/3
8	Inside, fumigated	2	H	91.7	83.3	1, 1, 1	Ns, Ns, Ns	1/3
9	Inside, fumigated	3	H	100	91.7	1. 1, 1	nd, nd, nd	0/3
10	Inside, fumigated	1	D	58.3	41.7	2, 2, 4	Ns, nd, nd	1/3
11	Inside, fumigated	2	D	83.3	58.3	2, 2, 2	Cl, Mc, Cl	2/3
12	Inside, fumigated	3	D	58.3	50	4, 4, 2	Ns, nd, nd	1/3
13	Outside, fumigated	1	D	100	91.7	4, 2, 4	nd, Mc, nd	2/3
14	Outside, fumigated	1	H	100	91.7	1. 1, 1	Ns, Ns, Ns	1/3
15	Outside, fumigated	2	D	100	100	4, 4, 2	Cl, Cl, Mc	2/3
16	Outside, fumigated	2	H	100	91.7	1, 1, 1	Ns, Ns, Ns	1/3
17	Outside, fumigated	3	D	100	100	2, 3, 4	Ns, Cl, Cl	3/3
18	Outside, fumigated	3	H	100	100	1, 1, 1	Ns, Ns, Ns	0/3

a. Outside = cassava plants were planted in the field; Inside = cassava plants were planted inside a screen house.
b. Seed evaluated at harvest according to symptoms in root: D = diseased seed (Jamundí, Valle del Cauca); H = healthy seed (Montenegro, Quindío).
c. Three plants per plot evaluated for diseased roots at harvest (September 2005) according to a disease severity scale, where 1 = no symptoms; 2 = mild; 3 = moderate; 4 = moderate to high; 5 = severe symptoms.
d. Evaluation of symptoms in cuttings, where Ns = no symptoms; Cl = chlorosis; Mc = minor chlorosis; nd = not determined.
e. Primers used for the evaluation were P1/P7–FSD-F/R and R16F2/R2–R16(III)F2/R1. Two evaluations were made, one in May and another in September at harvest. Values refer to number of replications out of 3 where detection was successful.

The results of the study to detect phytoplasmas through PCR were as follows:

- *In the plots with plants from healthy seed* (plots 2, 4, and 5; outside and not fumigated), the frequency of positive samples was 55% (i.e., 5 positive plants out of a total of 9 in the 3 plots), 11% (plots 7, 8, and 9; inside), and 22% (plots 14, 16, and 18, outside and fumigated).

- *In the plots with plants from diseased seed*, the frequency of positive samples was 77% (plots 1, 3, and 6; outside and not fumigated), 44% (plots 10, 11, and 12; inside), and 77% (plots 13, 15, and 17; outside and fumigated) (Table 1.9.1).

The absence of phytoplasmas through amplification of DNA obtained from plants in the plots with healthy seed and the application of insecticides indicates that insect vectors of phytoplasmas may exist.

The number of phytoplasmas detected in plants in the plots inside the screen house, both in healthy and diseased seed, was low, compared with the plots outside the screen house. The few infected plants from healthy seed inside the screen house may have been infected through homopterous species of insects of the Cixiidae family. These planthoppers, in their subterranean nymphal stage, feed on grass roots, and have been reported as vectors of phytoplasmas. An example is *Myndus crudus* Van Duzee on coconut palms; nymphs develop at or just under the soil surface on grasses or sedges, while adults feed on palms. At least 37 species of grasses (Gramineae) and 4 species of sedges (Cyperaceae) have been reported as hosts to nymphs of *M. crudus* (Howard and Ropeza, 1998, Florida Entomol 81:92–97).

The rate of detection of phytoplasmas in the plots with plants infected by CFSD and healthy plants is 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, low concentrations of the microorganism, or environmental effects, as occurred with cv. Manzano with apple proliferation phytoplasma, where proliferation is often disseminated in scion wood. Although the causal agent does not appear systemic, trees may yield a high proportion of apparently healthy but infected buds.

Distribution of phytoplasmas in the tree is not constant over the year. In winter, the content of phytoplasmas declines in the tree as a result of sieve tube degeneration. They also concentrate more in the roots but, during April to May, they reinvade the stem from the roots and reach a peak in late summer or early autumn. The distribution pattern of the phytoplasmas in the tree is also dependent on temperature. In France, phytoplasmas could be found throughout the trees at temperatures of 21–25 °C, causing symptoms. At 29–32 °C, symptoms were inhibited and phytoplasmas were found only in the roots, but reinvaded the stems when plantlets were stored at lower temperatures (EPPO/CABI 1996).

When a tree is inoculated with an infected bud, the first symptoms appear the following year, mostly on the inoculated branches. When carried in the rootstock, the causal agent produces symptoms on the first growth of the scion. It appears to be localized mainly in suckers and terminal shoots, where it can be found in the phloem of leaf petioles, midribs, and stipules (EPPO/CABI 1996).

From this same trial, we carried out a replication with cuttings to reduce the number of dead plants and be able to complete the data as yet not determined. We are also evaluating cuttings from 30 varieties with and without symptoms in the field under laboratory conditions to confirm the results obtained.

Activity 1.10. Studying the transmission of a phytoplasma belonging to the 16SrIII group (cassava frogskin disease phytoplasma; Cfdp) associated with cassava frogskin disease (CFSD)

Contributors: E. Álvarez, J. F. Mejía, and J. Loke

Highlight:

- Characteristic symptoms of foliar frogskin disease (FSD) were developed in the parasitic dodder plant (*Cuscuta* sp.) after infection by a pathogen transmitted from infected *in vitro* plants of cassava variety Secundina. This result provided evidence that FSD is caused by a phytoplasma.

Rationale

Definitive proof that a microorganism causes a disease is obtained by fulfilling Koch's postulates. However, because phytoplasmas are not cultivable, the fulfillment of Koch's postulates is very difficult. In general, phytoplasmas are believed to cause a certain plant disease if the plant shows typical "phytoplasma-related" symptoms, and phytoplasmas can be detected regularly in the diseased plants. Our study aimed to examine the transmission of a phytoplasma of the 16SrIII group (Cfdp), associated with cassava frogskin disease (CFSD), thereby fulfilling Koch's postulates. This may confirm that a phytoplasma is the causal agent of the disease.

Materials and Methods

The transmission of the pathogen was carried out on two cassava varieties infected with CFSD and evaluated with PCR as positive to Cfdp, using the ectoparasite *Cuscuta* sp. as a bridge transmitter and grafts (clefts and splices) into periwinkle (*Catharanthus roseus* (L.) G. Don) and healthy cassava.

The cassava variety used was SM 909-25, selected for severe symptoms in roots and the presence of symptoms—chlorosis and curling—in leaves under greenhouse conditions (23 °C and 80% RH) (Figure 1.10.1a). A clone (CW 94-21) from the CW family in CIAT's cassava genetic improvement program was also used. It had been found in the field with the characteristic symptoms of the disease in roots.

For transmission with *Cuscuta*, sexual seed was planted in soil, together with the cassava plants corresponding to each treatment. Once germinated, the seeds were connected with each other through the long extensions issued by the parasite (Figure 1.10.1b). Periwinkle seedlings were obtained from sexual seed and the plants ascertained as free from phytoplasmas through nested PCR . Transmission time was estimated from the establishment of *Cuscuta* in the plants corresponding to each treatment. The transmission period was about 2 months under greenhouse conditions (20–25 °C and 50%–90% RH). For transmission, the following treatments were carried out: (1) from infected cassava plants to healthy periwinkle plants; (2) infected periwinkle plants to healthy periwinkle plants; (3) infected cassava plants to healthy cassava plants; (4) healthy periwinkle plants to healthy periwinkle plants; and (5) healthy cassava plants to healthy periwinkle plants.

Transmissions by graft were carried out with cleft and splice grafts, using the leaf central nervure and terminal shoots of infected cassava plants. As stock, we used 2-month-old cassava plants and 5-to-6-week-old periwinkle plants. The treatments for this trial were (1) infected cassava plants to healthy periwinkle plants; (2) healthy periwinkle plants to healthy periwinkle plants; and (3) healthy cassava plants to healthy periwinkle plants. Each treatment was carried out with 6 replications. Once the *Cuscuta* plants were established and the grafts had developed well, monthly evaluations by PCR and by characteristic symptomatology reported in periwinkle were carried out.

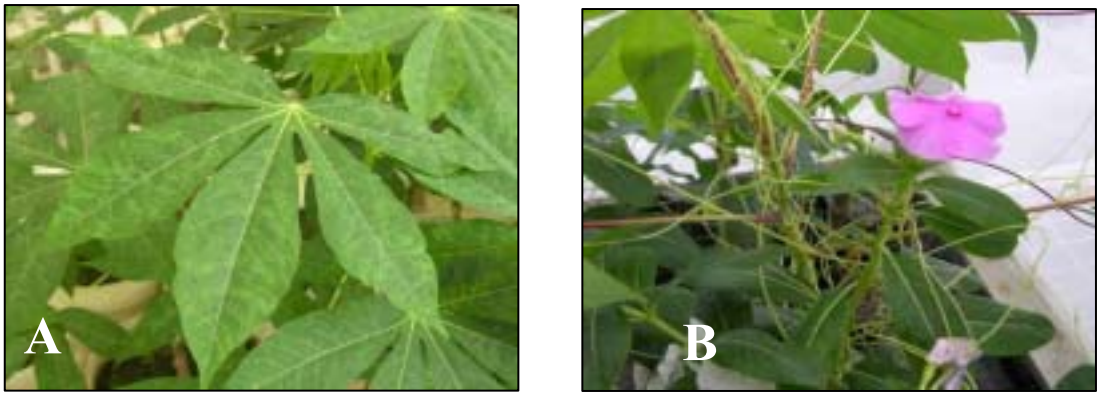


Figure 1.10.1. (A) Cassava variety SM 909-25, which is highly susceptible to cassava frogskin disease, showing leaf symptoms of chlorosis and curling under greenhouse conditions. (B) *Cuscuta sp*. parasitizing cassava and periwinkle, and acting as a bridge transmitter of phytoplasmas.

Results and Discussion

After 4 months of exposure, the pathogen's transmission was achieved through *Cuscuta* from infected cassava plants to healthy periwinkle plants and from infected to healthy periwinkle plants (treatments 1 and 2). Detection was achieved with nested PCR in 2 of the 6 replications for each treatment with variety SM 909-25. For the other three treatments and the other variety, positive amplifications were not obtained (Table 1.10.1).

Transmission by graft was generally high for treatment 1—of the 6 replications, 66% were detected as positive by nested PCR in the two infected varieties (SM 909-25 and CW 94-21). For the other two treatments, no positive samples were detected. Phytoplasmas were detected, using grafts, 3 to 4 months after exposure (Table 1.10.1).

So far, we have not observed the characteristic symptoms caused by phytoplasmas in periwinkle, even though we detected the presence of phytoplasmas with the nested-PCR technique in infected cassava plants and later in periwinkle plants infected through *Cuscuta* or grafting. A major cause is the rather unfavorable environmental and greenhouse conditions for symptom expression.

Valencia *et al*. (1993, Fitopatol Colomb 17:39–45) in studies on the transmission of the causal agent of witches' broom in cassava, caused by a phytoplasma, achieved 100% transmission between cassava and periwinkle, using *Cuscuta* sp. and grafts, after 3 months of exposure. This demonstrated that transmission of phytoplasmas between these two different species is possible. These studies reported the expression of symptoms under growth chamber conditions with temperatures at 18–20 °C and RH at 44%–84%. The symptoms were not very severe, but vegetative depressions were observed in less than 6 months since transmission began.

The periods of incubation and optimal greenhouse conditions are fundamental for the expression of symptoms characteristic of phytoplasmas in periwinkle plants. The plants evaluated as positive by PCR will be exposed to different periods and greenhouse conditions to seek the optimal for reproducing symptoms.

Table 1.10.1. Results obtained with *Cuscuta* sp. and grafts as transmitters of phytoplasmas from cassava infected with Cfdp to periwinkle.

Treatment		PCR (+)[a]	
		SM	CW
No.	Description	909-25	94-21
Cuscuta sp.			
1	Infected cassava to healthy periwinkle	2/6	0/6
2	Infected periwinkle to healthy periwinkle	2/6	0/6
3	Infected cassava to healthy cassava	0/6	0/6
4	Healthy periwinkle to healthy periwinkle	0/6	0/6
5	Healthy cassava to healthy periwinkle	0/6	0/6
Grafts (clefts and splices)			
1	Infected cassava to healthy periwinkle	5/6	3/6
2	Healthy periwinkle to healthy periwinkle	0/6	0/6
3	Healthy cassava to healthy periwinkle	0/6	0/6

a. Values refer to number of replications out of 6, where detection was successful.

Activity 1.11. Evaluating specific primers for high-specificity detection of phytoplasmas associated with cassava frogskin disease (CFSD)

Contributors: E. Álvarez, J. F. Mejía, G. Llano, and J. Loke

Highlight:

∉ FSD-specific primers proved highly specific and sensitive for detecting the phytoplasma. The PCR assay designed provided an effective alternative to conventional tests.

Rationale

Recent research on phytoplasma diseases has focused on developing techniques for detecting the pathogens and diagnosing their associated diseases. Such techniques include assays with antibodies, genomic DNA probes, and, more recently, PCR-based DNA markers. Other focal areas of phytoplasma research have been genetic variation among phytoplasmas and phylogenetic relationships among phytoplasma groups and other Mollicutes. Using heterogeneous DNA probes and differential screening, the 16S ribosomal RNA (rRNA) gene and ribosomal protein genes have been cloned from a phytoplasma. Universal primers were then designed and used to amplify 16S rRNA gene segments from each known phytoplasma group. Overall classification of phytoplasmas and general phylogenetic relationships were derived from extensive studies of those conserved genes. The objectives of the present study are to achieve high specificity in detection, using nested PCR, in order to improve the detection of phytoplasmas in cassava plants presenting symptoms of CFSD, weeds, and potential insect vectors. A protocol will be standardized for detecting phytoplasmas of the 16SrIII group associated with CFSD in cassava by using specific primers.

Materials and Methods

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. 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.

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.

The results of the phylogenetic and homology analyses showed that the CFSD 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 CFSD phytoplasma and the 16SrIII-group phytoplasmas in the 16S rRNA gene, generating sufficient information to design primers (CIAT 2004). The primers designed were FSD-F (5'-TTT GAA GGT ATG CTT AAG GAG-3') and FSD-R (5'-GGA GTC CCG TCA ATT CCT T-3').

Standardizing primers: The principal objective for which the primers were designed was to avoid the need for nested PCR, using the primers directly and seeking to make detection more efficient, thus saving time and increasing the number of samples processed per day. Primers FSD-F/R were carried out in a total volume of 25 µL, containing 1X PCR buffer (supplied by the manufacturer of the polymerase), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.1 U of *Taq* polymerase (Promega, Madison, WI), and 0.5 pmoles of each primer. Reactions were heated at 94 °C for 2 min and then cycled through 35 cycles at 94 °C for 1 min, passing through 48, 50, 52, and 55 °C for 2 min each, and 72 °C for 3 min, followed by a final extension of 10 min at 72 °C. As positive controls, DNA of different cassava varieties infected with CFSD was evaluated as positive for phytoplasma with the universal primers and sequencing (Table 1.11.1) [CIAT, PE-1 Annual Report 2004].

Table 1.11.1. DNA evaluated with direct PCR, using specific primers FSD-F/R.

Sample code number	Variety	Tissue	PCR universal primers[a]
1	M Col 2063	Leaf	+
2	CM 6740-7	Leaf	+
3	SM 909-25	Leaf	+
4	SM 909-25	Root	+
5	SM 1219-9	Leaf	+
6	SM 1219-9	Root	+
7	M Col 2063 (Invitro)-1	Leaf petiole (healthy)	-
8	M Col 2063 (Invitro)-1	Stem (healthy)	-
9	M Col 2063 (Invitro)-2	Leaf petiole (healthy)	-
10	M Col 2063 (Invitro)-2	Stem (healthy)	-
11	Control (Sqmp)[b]	Leaf petiole	+
12	Control (Ccp)[c]	Petiole/stem	+

a. Samples positive to phytoplasmas through PCR with universal primers and sequencing.
b. Sqmp = *Solanum quitoense* machorreo phytoplasma (AY731819).
c. Ccp = coffee crispiness phytoplasma (AY525125).

DNA sequencing: The product amplified from direct and nested PCR was purified, following the protocol described for the QIAquick PCR Purification Kit, and sequenced. The fragments were sequenced, 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 version 4.13. Homology was sought in the NCBI's GenBank (www.ncbi.nlm.nih.gov), using the tool BLAST®n.

Results and Discussion

Before evaluating the primers, their sequences were aligned with phytoplasma accessions of the 16SrIII group in GenBank (including those of cassava, AY737646 and AY737647) (Figure 1.11.1) to confirm that the primers' sequences were correct. On evaluating the different annealing temperatures (48, 50, 52, and 55 °C), two fragments of about 0.6 and 0.7 kbp were obtained for all DNA evaluated, except the positive controls Sqmp and Ccp of the 16SrIII group (Figure 1.11.2; Table 1.11.1), showing in-specificity of the primers.

Origin 1
ccctaagac gaggataacg attggaaaca gttgctaaga ctggatagga aaagtaaagg
61 catctttact tttttaaaag accttt tttg aaggtatgct taaggag ggg cttgcgacac
121 attagttagt tggcagggta aaggcctacc aagactatga tgtgtagctg gactgagagg
181 ttgaacagcc acattgggac tgagacacgg cccaaactcc tacgggaggc agcagtaggg
241 aatttt cggc aatggaggaa actctgaccg agcaacgccg cgtgaacgat gaagtacctc
301 ggtatgtaaa gttcttttat taaggaagaa aa aagagtgg aaaaactccc ttgacggtac
361 ttaatgaata agccccggct aattatgtgc cagcagccgc ggtaatacat aaggggcgag
421 cgttatccgg aattattggg cgtaaagggt gcgtaggcgg tttaataagt ctatagttta
481 atttcagtgc ttaacgctgt tgtgctatag aaactgtttt actagagtga gttagaggca
541 agcggaattc catgtgtagc ggtaaaatgc gtaaatatat ggaggaacac cagaggcgta
601 ggcggcttgc tgggacttta ctgacgctga ggcacgaaag cgtggggagc aaacaggatt
661 agataccctg gtagtccaca ccgtaaacga tgagtactaa gtgtcgggta aaaccggtac
721 tgaagttaac acattaagta ctccgcctga gtagtacgta cgcaagtatg aaactta aag
781 gaattgacgg gactcc gcac aagcggtgga tcatgttgtt taattcgaag atacacgaaa
841 aaccttacca ggtcttgaca ttttcttgcg aagttataga aatataatgg aggttatcag
901 gaaaacaggt ggtgcatggt tgtcgtcagc tcgtgtcgtg agatgttagg ttaagtccta
961 aaacgagcgc aacccttgtc gttaattgcc agcatgtaat gatggggact ttaacgagac
1021 tgccaatgaa aaattggagg aaggtgggga ttacgtcaaa tcatcatgcc ccttatgatc
1081 tgggctacaa acgtgataca atggttgata caaagagtag ctgaaacgcg agttcttagc
1141 caatctcaca aaa tcaatct cagttcgga t tgaagtctgc aactcgactt catgaagttg
1201 gaatcgctag taatcgcgaa tcagcatgtc gcggtgaata cgttctcggg gtttgtacac
1261 accgcccgtc aaaccacgaa agttggcaat accccaaa

Figure 1.11.1. Cassava frogskin disease phytoplasma strain FSDY29 16S ribosomal RNA gene, partial sequence, accession number AY737647. Yellow = primers designed for cassava frogskin disease phytoplasm (Cfdp); green = primers designed for 16S rRNA gene (X-disease phytoplasma group).

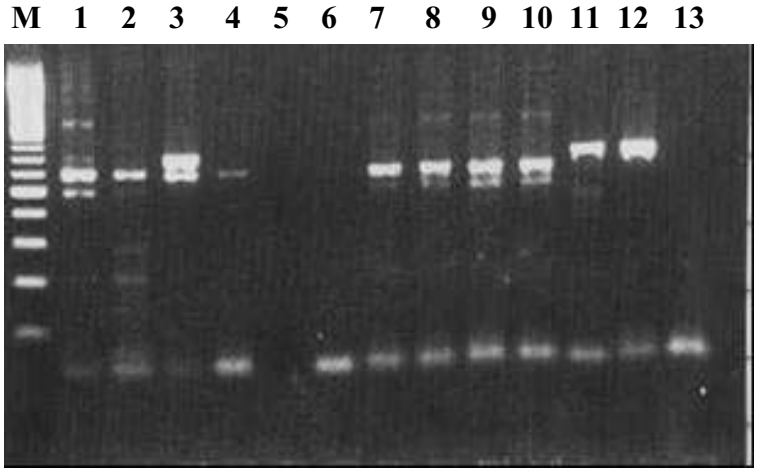


Figure 1.11.2. Two fragments, about 0.6 and 0.7 kbp long, were obtained through direct PCR with primers FSD-F/R. Lanes 1–10 = samples 1–10 (Table 1.11.1); lanes 11 and 12, respectively = positive controls Sqmp (*Solanum quitoense* machorreo phytoplasma) and Ccp (coffee crispiness phytoplasma); lane 13 = negative control; M = molecular weight marker of 1 kbp.

To confirm this result, the number of cassava samples was broadened, taking 32 samples of DNA from healthy and diseased plants of the varieties Manzana and ICA Catumare (8 healthy and 8 diseased of each variety), obtaining bands of the two sizes (0.6 and 0.7 kbp) in both healthy and diseased tissues, confirming what was evaluated with the samples described in Table 1.11.1 and Figure 1.11.2, very possibly because of in-specific amplifications with the plant's DNA (Figure 1.11.3). The 0.6 and 0.7-kbp bands were sequenced to confirm that the fragments that amplified to a molecular weight equal to that of the controls belonged to a phytoplasma and not to the plant's nuclear or chloroplastic DNA. For the 0.6-kbp fragments, we found homologies with 80% of accessions of *Lycopersicon esculentum* Mill. (CT476825) and with chromosome 5 of *Solanum demissum* Lindley (AC149301). In contrast, the 0.7-kbp fragments showed a 99% homology with cassava frogskin disease phytoplasma strain FSDY29 (AY737647).

Harrison *et al*, (2002, Plant Dis 86: 676–681) had similar problems of amplification, improved with the use of universal primers (P1/P7) that amplified genes 16S rRNA, 23S rRNA, and the 16S/23S spacer region from genomic DNA. These primers were used in the first cycle of a nested PCR, to be later nested with more specific primers or with primers designed for the phytoplasma identified. PCR amplification of the 16S rRNA gene and the 16S/23S spacer region from genomic DNA yielded one fragment of about 0.78 kbp, from cassava frogskin disease phytoplasm (Cfdp) isolates (Figure 1.11.4). Amplification fragments of the same size were obtained from the Sqmp and Ccp phytoplasmas. From 10 cassava samples, 6 showed positive amplification for the 16S rRNA gene. These gene segments were not amplified during PCR from healthy control plants. Similar results were obtained when the 32 DNA samples from varieties ICA Catumare and Manzana were evaluated (Figure 1.11.5).

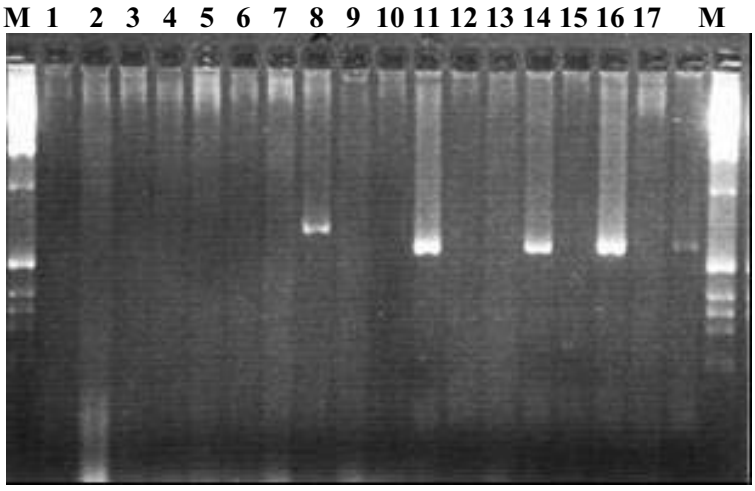


Figure 1.11.3. Amplified fragments of 0.6 and 0.7 kbp obtained with primers FSD-F/R from healthy and diseased plants of cassava varieties Manzana and ICA Catumare. Lanes 1–4 = amplified DNA from healthy tissue of variety Manzana; lanes 5–8 = infected tissue from variety Manzana; lanes 9–12 = amplified DNA from healthy tissue of variety ICA Catumare; lanes 13–16 = infected tissue from variety ICA Catumare; lane 17 = negative control; M = molecular weight marker of 1 kbp.

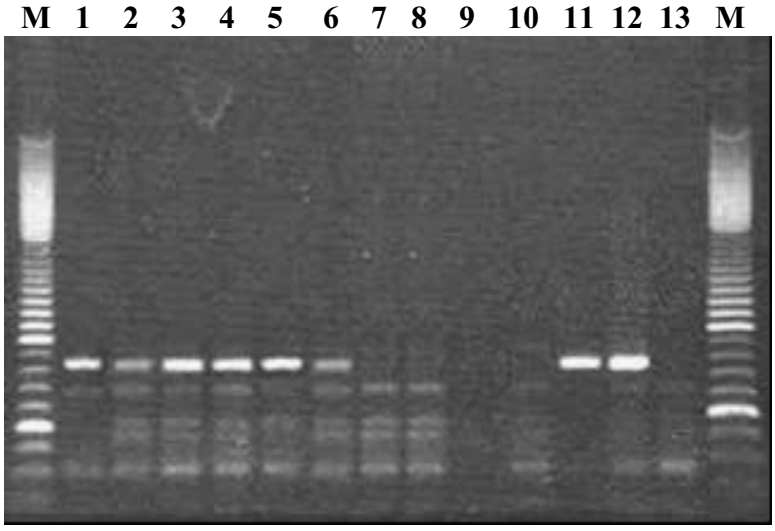


Figure 1.11.4. PCR amplification of one fragment of about 0.78 kbp with nested PCR, using primers P1/P7 and FSD-F/R. Lanes 1–10 = cassava frogskin disease phytoplasm (Cfdp) isolates (see Table 1.11.1); lanes 11 and 12, respectively = positive controls of Sqmp (*Solanum quitoense* machorreo phytoplasma) and Ccp (coffee crispiness phytoplasma); lane 13 = negative control; M = molecular weight marker of 1 kbp.

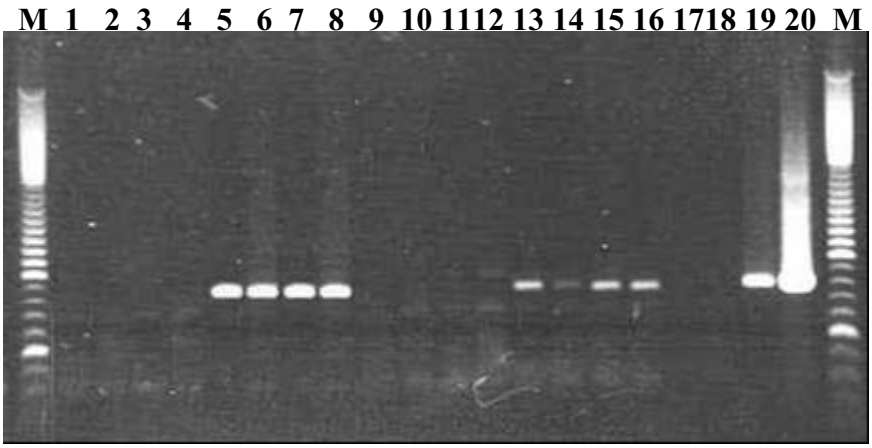


Figure 1.11. 5. Amplification of a fragment of 0.78 kbp with nested PCR, using primers P1/P7 and FSD-F/R, from healthy and diseased plants of varieties Manzana and ICA Catumare. Lanes 1–4 = healthy tissue of variety Manzana; lanes 5–8 = infected tissue; lanes 9–12 = healthy tissue from variety ICA Catumare; lanes 13–16 = infected tissue; lane 17 = negative control; M = molecular weight marker of 1 kbp.

Activity 1.12. Detecting phytoplasmas in cassava infected by cassava frogskin disease (CFSD), using nested PCR

Contributors: E. Álvarez, J. F. Mejía, G. Llano, and J. Loke

Highlight:

∉ FSD-specific primers proved highly specific and sensitive for detecting the phytoplasma. The PCR assay designed provided an effective alternative to conventional tests.

Rationale

Cassava frogskin disease (CFSD) attacks cassava roots, causing increasing numbers of deep lesions that eventually deform the roots. Despite its economic significance, the disease's causal agent has remained unknown for many years. Recently, CFSD has been reported with increasing frequency in Colombia, Brazil, and Venezuela. In Colombia, for example, incidence of up to 70% has been recorded in commercial fields in the production areas of Valle del Cauca, Cauca, Meta, and the North Coast. To develop appropriate management strategies for controlling the disease, the pathogen must be identified. Preliminary studies evidenced the existence of an association between CFSD and phytoplasmas. To confirm these results, molecular tools will be applied to detect phytoplasmas in CFSD-infected roots, leaf midribs, petioles, and peduncles in the different varieties and genotypes of cassava found in various regions of Colombia.

Materials and Methods

Phytoplasma sources: We took samples of leaf, stem, and root tissues from 39 genotypes of cassava (*Manihot esculenta* Crantz) grown in the field and greenhouse and naturally infected by CFSD. The plants were collected from 2002 to 2004 in three areas of Colombia—North Coast, Valle del Cauca, and Cauca—where the incidence of CFSD is high.

We included as negative checks cassava plants obtained *in vitro* through meristem culture. Positive checks were samples of periwinkle (*Catharanthus roseus* (L.) G. Don) that clearly showed typical symptoms induced by phytoplasmas—internode stunting or a clumping form of stunting in terminal buds.

Other checks included DNA from coffee crispiness phytoplasma (Ccp; GenBank Accession Number AY525125), facilitated by the National Coffee Research Center (CENICAFE, Colombia) and used as reference for the 16SrIII group (X-disease group); remolacha phytoplasma (Rp; 16SrIII group) and pimenton clover proliferation phytoplasma (Pcpp; 16SrVI group), both facilitated by the National Institute for Agricultural and Food Research, Madrid, Spain; and lethal wilt oil palm phytoplasma (Lwop) (GenBank Accession Number AY739024)

DNA extraction: DNA was extracted, following the protocol of Gilberston et al.(Gilbertson *et al.*, 1991, J Gen Virol 72:2843–2848.), from root, stem, and leaf petiole and nervure tissues sampled from plants in each geographical area.

PCR amplification: Three pairs of universal primers—**P1/P7** (Smart *et al*., 1996, Appl Envir Microbiol 62: 2988-2993.), **R16mF2/R1** (Gundersen and Lee, 1996, Phytopath Medit 35:114–151); Schneider *et al.*, 1993, J Gen Microbiol 139: 519–527.), and **R16F2n/R2** (Gundersen and Lee, 1996, Phytopath. Med 35:114-151)—were used in nested PCR to amplify the region of the genes 16S rRNA and 23S rRNA. The amplified products of P1/P7 and R16mF2/R1 were diluted at 1:50 with sterilized and distilled water for use as DNA mold in 1-µL quantities with primers R16F2n/R2.

Each reaction was put in 0.2-mL tubes carrying a volume of 25 µL, using final concentrations of 100 ng of DNA, 1X buffer, 3 mM $MgCl_2$, 1 U *Taq* polymerase (Promega, Madison, WI), 0.8 mM dNTPs (Invitrogen Life Technologies, Carlsbad, CA), and 0.1 µM of each primer (Operon Technologies, Inc., Alameda, CA).

For primers P1/P7, 35 cycles were carried out in a PTC-100 thermal cycler with a hot cover unit (MJ Research, Inc., Waltham, MA), following these conditions: 30 s (90 s for the first cycle) of denaturation at 94 °C, annealing for 50 s at 55 °C, and extension of the primer for 80 s (10 min in the final cycle) at 72 °C. Primers R16mF2/R16mR1 were amplified with 28 cycles, using the same conditions. The primer pair R16F2n/R16R2 was evaluated in a similar manner, but with an annealing temperature of 50 °C.

The PCR products were visualized in a 1.5% agarose gel, stained with 0.75 µg/mL ethidium bromide, and analyzed in a Stratagene Eagle Eye® II video system (La Jolla, CA).

PCR amplification with specific group primers: The DNA amplified with primers R16F2/R2 (16) was diluted at 1:50 and used as sample for re-amplification in nested PCR, using primers R16(III)F2/R16(III)R1 (16), designed specifically for the 16SrIII group of phytoplasmas (X-disease group), using the same PCR conditions as mentioned above, carrying out 35 cycles of 1 min (2 min for the first cycle) of denaturation at 94 °C, annealing for 2 min at 50 °C, and extension of the primer for 3 min (10 min in the final cycle) at 72 °C.

Results and Discussion

Detecting phytoplasmas associated with CFSD: Infected cassava and periwinkle plants exhibited symptoms typical of witches' broom in phytoplasmal infections. Nested PCRs primed by phytoplasma universal primer pair R16F2n/R2 resulted in the amplification of 1.2-kb DNA fragments of the 16S ribosomal DNA, indicating that the symptomatic cassava and periwinkle plants were infected by phytoplasmas. Phytoplasmas were detected in 35 of the 39 cassava varieties tested, exhibiting symptoms of CFSD, representing 89% of amplification (Table 1.12.1). The specific group primer products indicated

that the diseased cassava plants were infected by strains of a phytoplasma belonging to the 16SrIII group (X-disease group). The presence of a 16SrIII-group phytoplasma in all DNA samples was verified by nested PCR assays primed by primer pair R16(III)F2/R16(III)R1, which yielded an amplified product of about 0.8 kb. No amplified products were observed for Pcpp, Lwop, and periwinkle, which were used as controls.

Table 1.12.1. List of DNA fragments obtained from tissue samples from 39 varieties infected with cassava frogskin disease. The samples were amplified by direct and nested PCR, using universal primers and primers specific for phytoplasmas.

Sample code number	Variety number	Genotype	Site[a]	Tissue[b]	PCR[c]	Primers[d]
Y1	1	CM 6740-7	VC	LmP/R[e]	+/+	A–C
Y2	2	CIAT Parrita	VC	LmP/S/R	+/+/+	B
Y3	3	ICA Catumare	VC	LmP/R[e]	+/+	B–C
Y4	4	Manzana	VC	LmP/R[e]	+/+	B–C
Y5	5	M Bra 383	VC	LmP/R	+/+	B–C
Y6	6	CM 849-1	VC	LmP/R	+/+	B–C
Y7	7	CM 5460-10	VC	LmP	+/+	C
Y8	8	CM 2177-2	VC	LmP/R	+/+	B–C
Y9	9	CM 4919-1	VC	LmP/R	+/+	B–C
Y10	10	CM 3306-9	VC	LmP[e]	+	B–C
Y11	11	CM 3306-19	VC	LmP[e]	+	B–C
Y12	12	M Bra 856-54	VC	LmP[e]	+	B–C
Y13	13	M Per 335	VC	R	+	C
Y14	14	M Bra 856	C	LmP/R	+/+	C
Y15	15	SM 909-25	VC	LmP/R[e]	+/+	C
Y16	16	CG 6119-5	VC	LmP/R	+/+	C
Y17	17	M Col 2063	VC	LmP[e]	+	A–B–C
Y18	18	ICA Nataima	VC	LmP/R	-/+	C
Y19	19	SM 1201-5	VC	LmP	-	C
Y20	20	GM 228-14	VC	LmP	-	C
Y21	21	CM 9582-64	VC	LmP/R	+/+	A–B–C
Y22	22	CM 9582-65	VC	LmP/R	+/+	A–B–C
Y23	23	CM 9582-24	VC	LmP/R	+/+	A–B–C
Y24	24	M CR 81	VC	LmP/R	+/+	A–B–C
Y25	25	Venezolana	S	R	+	A–B–C
Y26	26	M Per 16	C	LmP/R	+/+	C
Y27	27	M Col 634	C	LmP/R	+/+	C
Y28	28	M Bra 829	C	LmP/R	+/+	C
Y29	29	SM 1219-9	VC	LmP/R[e]	+/+	A–B–C
Y30	30	M Chn 2	C	LmP/R	-/-	C
Y31	31	HMC-1	C	LmP/R	+/+	C
Y32	32	M Arg 2	C	LmP/R	-/-	C
Y33	33	M Bra 325	C	LmP/R	+/+	C
Y34	34	M Bra 839	C	LmP/R	+/+	C
Y35	35	M Col 1178	C	LmP/R	+/+	C
Y36	36	M Col 1468	C	LmP/R	+/+	C
Y37	37	M Cub 74	C	LmP/R	-/+	C
Y38	38	M Bra 886	C	LmP/R	+/+	C
Y39	39	M Bra 882	C	LmP/R	+/+	C
Y40	5	M Bra 383	C	LmP/R	+/+	B
Y41	3	ICA Catumare	Q	LmP/R	-/-	B–C
Y42	4	Manzana	Q	LmP/R	-/-	B–C

a. VC = Department of Valle del Cauca; C = Cauca; S = Sucre; Q = Quindío.
b. LmP = leaf midrib and petioles; R = roots; S = stems.
c. + = amplification positive for phytoplasma; - = amplification negative for phytoplasma.
d. Primers used for amplification were A = P1/P7–R16F2N/R16R2; B = R16mF2/R16mR1–R16F2N/R16R2; C = R16F2/R16R2–R16(III)F2/R16(III)R1.
e. Also showing foliar symptoms of chlorosis and deformed leaf blades.

Activity 1.13. Characterization of avirulence and resistance genes in the rice blast pathosystem

Contributors: D. Pulgarin and F. Correa

Highlight:

- The blast resistance genes present in 211 commercially grown Latin American rice cultivars were identified and nine groups of potential sources of complementary resistance genes were defined for their use in a breeding program aiming at developing commercial rice cultivars combining desired agronomic traits and blast resistance

Rationale

Rice blast is the most destructive disease of the crop in the world. Blast resistance in commercially released cultivars has not been durable, due to the lack of knowledge on the genetic constitution for blast resistance in the cultivars and the great variability of the blast pathogen. The blast resistance genes present in commercial rice varieties from Latin America are not known. One of the reasons for this situation includes the lack for many years of a differential system for the identification of the resistance genes. The main objective of this study was to identify blast resistance genes in 211 commercial rice cultivars from different Latin American countries following a differential system based on the gene-for-gene relationship between rice resistance genes and avirulence genes in the blast pathogen. The resistance genes were inferred based on the reaction patterns of monogenic differentials against 28 blast isolates from Colombia. The varieties were classified in nine complementary resistance groups, which can be used in genetic crosses aiming at combining complementary blast resistance genes for developing rice cultivars with desired agronomic traits and durable blast resistance. The information produced in this study is very important in relation to rice breeding programs that need to use resistant donors with desired agronomic traits and blast resistance.

Materials and Methods

A group of 40 international blast differentials (Table 1.13.1) with known blast resistance genes was used for the inoculation of blast isolates collected from infected samples and for the identification of avirulence genes present in the isolates. A total of 211 commercial rice cultivars from Latin America were inoculated and evaluated with blast isolates under greenhouse conditions for the identification of their blast resistance genes. Cultivars were selected from: Argentina (3), Bolivia (7), Brazil (30), Chile (2), Colombia (29), Costa Rica (12), Cuba (19), Ecuador (6), Guatemala (10), Guyana (1), Honduras (2), Mexico (17), Nicaragua (3), Panama (10), Paraguay (3), Peru (21), Dominican Republic (9), Salvador (5), Surinam (2), Uruguay (9), USA (2), and Venezuela (9).

One hundred and twenty blast isolates were obtained from infected samples collected from the different Latin American rice cultivars and breeding lines planted at the Santa Rosa, Meta experiment station in 2004. Monosporic isoloates were obtained in the pathology laboratory of the rice project and stored at –20C after growing them on filter paper.

Blast differentials were grown in 14 inches pots and inoculated with a spore suspension of 5×10^5 of each blast isolate. Plants were inoculated 21 days after sowing or at the third leaf stage in two replications with ten plants each. After inoculation, plants were incubated in plastic chambers with high relative humidity for 15 days allowing reinfection of the plants after the first cycle of reproduction of the pathogen. Plants were evaluated for the lesion type and leaf area affected exhibited after 15 days of incubation. Lesion types were scored from 1 to 4 were 1-2 were resistant types, 3 an intermediate type and lesion 4 as susceptible. Disease leaf area was scored from 1 to 100%. Commercial cultivars from Latin America were

inoculated with 28 blast isolates carrying between 1 to 14 avirulence genes determined on the inoculations on the differential set. Inoculation, incubation and evaluations were performed as for the differential set of cultivars.

Results and Discussion

Inoculation of blast isolates on the differential set of rice cultivars with known blast resistance genes allowed the selection of 28 blast isolates carrying between 1 to 14 avirulecne genes (Table 1.13.2). A resistant reaction observed after the inoculation of a rice differential carrying a known resistance gene indicates the presence of the corresponding avirulence gene in the blast isolate. These 28 blast isolates were used to inoculate the 211 commercial rice cultivars to infer the potential resistance genes present in their genetic constitution. A resistant reaction observed after the inoculation of a rice cultivar with an isolate carrying a known avirulence gene indicates the presence of the corresponding resistant gene in the cultivar.

As an example, Table 1.13.3 shows the possible resistant genes present in the cultivar ANAYANSI determined after the inoculation of the cultivar with the 28 blast isolates.
The compatible isolates A24, A19, A28, A20, A22, A5, and A17 (in red color) indicate that the resistant genes corresponding to the avirulence genes present in those 7 isolates can not be present in the cultivar ANAYANSI, otherwise the reaction would have been resistant. The resistant reaction observed for other isolates carrying similar avirulence genes (blue color) present in the 7 compatible isolates, cannot be due to corresponding resistance genes, but to other resistance genes. As a result, it can be concluded that the resistant reaction observed for those isolates exhibiting an incompatible reaction with the cultivar ANAYANSI should be the result on the interaction of the other avirulence genes present in the incompatible isolates as defined in Table 1.13.3. Based on this analysis, the resistance genes present in this cultivar were inferred as Pi-i, Pi-k, Pi-k^s, Pi-sh, Pi-t, Pi-ta^2, and Pi-z. Table 1.13.4 gives examples of 21 commercial rice cultivars from different Latin American countries indicating the diversity observed in terms of the potential number of blast resistant genes they carry, with 0 to all the 21 resistant genes present in the differentials. Further genetic studies including allelism tests will be developed to corroborate the presence of those genes in those cultivars.

Analysis of the frequency of the avirulence genes present among the 28 blast isolates used in this study were between 0.04 (Avr Pi-sh) and 0.96 (Avr Pi-9), indicating that most blast isolates have lost the avirulence gene for the resistance gene Pi-sh, while most still conserve the avirulence gene for the resistance gene Pi-9. This suggests that the resistant gene Pi-sh has not a valuable use as an individual gene and would have to be used in combination with other resistance genes. On the other hand, the high frequency observed for the avirulence gene Avr Pi-9, indicates that the Pi-9 gene could even be used as a single gene, although using it in combination with other blast resistance genes would ensure a longer life of the gene before the pathogen mutates to a compatible reaction. It should be noted however that given that one isolate (A27) exhibited a compatible reaction with the Pi-9 gene (Table 1.13.2), other blast resistant genes effective to this isolate should be combined with the Pi-9 gene (Table 1.13.2).

Analysis of the candidate blast resistant genes present in the 211 Latin American rice cultivars studied show in general a high negative correlation with the frequency of avirulence genes present in the pathogen population studied. A low frequency observed for an avirulence gene is associated with a high frequency of the corresponding resistance gene in the cultivars (Table 1.13.5 and Table 1.13.6). The frequency observed for Avr Pi-sh (0.04) is associated with a high frequency (0.84) of the resistant gene Pi-sh in the 211 commercial cultivars. On the other hand, the high frequency observed for the Avr Pi-9 (0.96) was associated with a low frequency (0.06) observed for the presence of the gene in the 211 cultivars (Table 1.13.5 and Table 1.13.6). For breeding purposes, those genes with a low frequency of use

(Table 1.13.6) would be of higher value than those genes that have been widely exposed to blast populations.

The 211 Latin American rice cultivars were classified into nine variety groups (Figure 1.13.1) based on the presence of 21 blast resistance genes analyzed in this study. These groups are considered complementary for different blast resistance genes and careful selection of different cultivars can provide useful combinations of resistance genes, which can confer durable blast resistance. As an example, Table 1.13.7 shows the frequency observed for three different blast resistant genes in three different variety groups an the complementarities among them to select rice cultivars for designing crosses aiming at combining the different resistance genes (Pi-1, Pi-2, Pi-33). While the three genes are in high frequency in group 3, the genes Pi-1 and Pi-33 are high in group 6 and gene Pi-2 is high in group 9. Crosses can be made between rice cultivars selected from groups 6 and 9 to combine the three genes, conferring a wider spectrum of resistance. Similar observations have been made for other genes and crosses have been designed with the purpose of pyramiding complementary blast resistance genes.

Commercially grown rice cultivars are characterized by having desired agronomic traits such as high yield, good grain quality, good adaptation, good plant type, and in many cases resistance to several pests and diseases. Most rice cultivars grown in Latin America have traits highly desired by rice farmers, however they have lost their resistance to blast, and therefore many of them are not grown anymore. A short-term breeding strategy aiming at developing rice cultivars with desired agronomic traits and combined blast resistance can be achieved if appropriate crosses between commercially blast susceptible rice cultivars are made. The information produced in this study is very important in relation to rice blast resistance breeding involving commercially grown blast susceptible cultivars from Latin America. This study demonstrates the utility of the differential system in elucidating the genetic constitution for blast resistance of 211 Latin American rice cultivars. Differential varieties are a very useful tool to identify blast resistance genes in rice and to characterize the pathogenicity of the blast pathogen. Latin American rice cultivars have been classified in different groups with complementary resistance genes, which can be used for designing genetic crosses aiming at combining resistance genes that can confer durable blast resistance. To confirm the different blast resistance genes identified in the rice cultivars from Latin America, genetic analysis using different progenies derived from crosses with susceptible varieties, allelism tests, and molecular markers associated with blast resistance genes will be conducted.

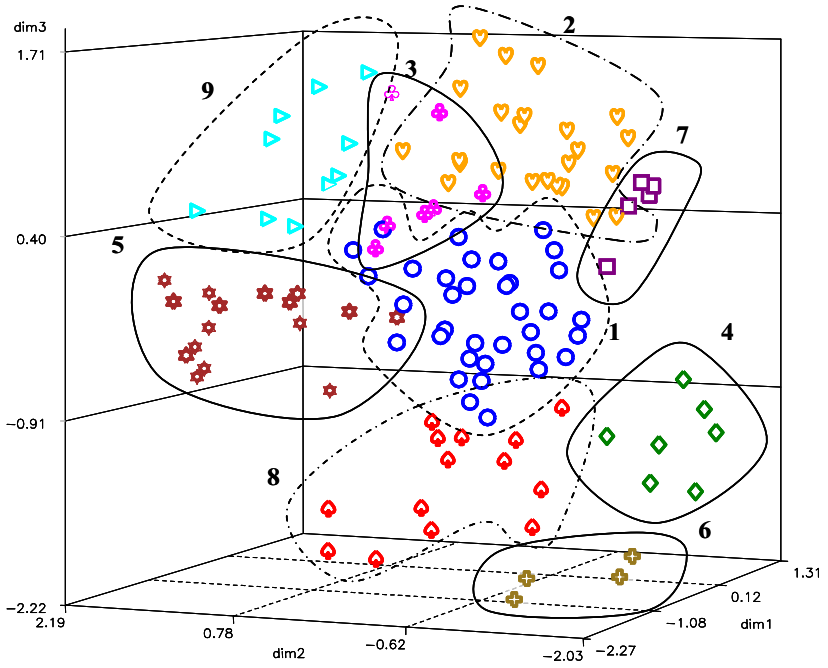


Figure 1.13.1 Spatial distribution of 211 Latin American rice cultivars in nine variety groups based on the presence of blast resistance genes

Table 1.13.1. Rice differentials with known blast resistance genes.

Number	Differential	Resistance Gene	Number	Differential	Resistance Gene
1	Fukunishiki	Pi-z, Pi-sh	21	Tsuyuake	Pi-k^{m}
2	Fujisaka 5	Pi-I, Pi-k^{s}	22	Nipponbare	Pi-sh
3	BL-1	Pi-b	23	Ou 244	Pi-z
4	BL-2	Pi-b	24	Ishikari Shiroke	Pi-I, Pi-k^{s}
5	Toride 1	Pi-z^{t}	25	Tetep	Pi-k^{h}
6	K3	Pi-k^{h}	26	IR 22	Pi-k, Pi-sh, Pi-ta^{2}
7	K 59	Pi-t	27	C 101 A 51	Pi-2
8	K60	Pi-k^{p}	28	C 101 LAC	Pi-1, Pi-33
9	C 104 LAC	Pi-1	29	C 101 PKT	Pi-4a
10	C 103 TTP	Pi-1	30	C 104 PKT	Pi-3
11	C 105 TTP-1	Pi-ta, Pi-4a	31	C 105 TTP-4	Pi-4b
12	F 128-1	Pi-ta^{2}	32	CT 13432-6	Pi-33
13	F 145-2	Pi-b	33	CT 13432 –68	Pi-1
14	Zenith	Pi-z, Pi-a	34	CT 13432-267	Pi-2
15	Pi No. 4	Pi-sh, Pi-ta^{2}	35	Dular	Pi-k^{a}
16	Rico 1	Pi-k^{s}	36	75-1-127	Pi-9
17	Norin 22	Pi-sh	37	K1(Francia)	Pi-ta
18	Nato	Pi-I	38	CT 13432-107	Pi-1, Pi-2, Pi-33
19	Shin 2	Pi-sh, Pi-k^{s}	39	Zenith (Brasil)	Pi-z, Pi-a
20	Kanto 51	Pi-k	40	Fanny	None

Table 1.13.2. Predicted avirulence genes present in 28 *Pyricularia grisea* isolates.

Isolates	Blast Resistance Genes																				Avr Gene No.
	Pib	**Pii**	**Pik**	**Pik^{a}**	**Pik^{h}**	**Pik^{m}**	**Pik^{p}**	**Pik^{s}**	**Pish**	**Pi-t**	**Pita**	**Pta^{2}**	**Pi-z**	**Piz^{t}**	**Pi1**	**Pi2**	**Pi3**	**Pi9**	**Pi33**	**Pi4a**	**Pi4b**	
A12. CT 13432-107 (14-1)																		1				1
A14. CT 13432-34 (25-1)																		1				1
A21. CL 00-2-1-38																		1				1
A 10. CT 13432-246 (13-1)										1								1				2
A 13. CT 13432-107 (25-1)										1								1				2
A08. O.Yacu 9-17-1													1					1	1			3
A01. Fanny 54													1	1				1	1			4
A24. CL 1-6-13														1		1	1	1				4
A25. CL 00-6-1-7				1													1	1	1			4
A02. O. Caribe 8-17-1			1	1	1	1												1				5
A19. CL 00-4-1-25					1	1									1			1	1			5
A23. CL 1-1-41				1	1	1									1			1				5
A11. Isolinea 6-7-1	1						1						1	1		1		1				6
A16. CT 13432-107 (12-1)			1	1	1							1			1			1				6
A28. CR 1821 (1-1)				1							1			1		1		1			1	6
A06. FL 00440-30P-2-2P-M			1	1	1	1				1					1			1				7
A09. FED 2000-5-1				1	1	1				1					1			1			1	7
A18. CL 00-4-1-24		1		1			1						1				1	1	1			7
A07. FED 50-24-1			1	1	1	1				1	1				1			1				8
A20. CL 3-6-25				1			1				1			1		1	1	1		1	1	9
A22. CL 00-1-1-36				1	1	1								1		1	1	1	1		1	9
A15. COL XXI		1		1	1	1	1						1		1		1	1	1			10
A26. Irga 409	1			1			1				1	1	1			1	1	1	1		1	11
A03. Metica 1-33-18			1	1	1	1							1		1	1	1	1	1	1	1	12
A05. Cica 9-15	1			1	1	1	1				1			1	1		1	1	1	1	1	13
A17. CL 00-3-1-29				1	1	1	1				1			1	1	1	1	1	1	1	1	13
A27. Cimarron 1-2	1			1	1		1	1	1	1			1	1	1	1	1		1			13
A04. Cica 9-37-1		1		1	1	1	1	1			1		1		1		1	1	1	1	1	14

1= incompatible reaction indicating the presence of the corresponding avirulence gene in the pathogen.

Table 1.13.3. Predicted resistance genes present in the commercial rice cultivar ANAYANSI (Pi-i, Pi-k, Pi-k^s, Pi-sh, Pi-t, Pi-ta^2, Pi-z) base susceptible reaction to isolates A24, A19, A28, A20, A22, A05, A17.

	Blast Resistance Genes																		
Isolates	**Pib**	**Pii**	**Pik**	**Pik^a**	**Pik^h**	**Pik^m**	**Pik^p**	**Pik^s**	**Pish**	**Pi-t**	**Pita**	**Pta^2**	**Pi-z**	**Piz^t**	**Pi1**	**Pi2**	**Pi3**	**Pi9**	**Pi33**	**Pi**
A12. CT 13432-107 (14-1)																		1		
A14. CT 13432-34 (25-1)																		1		
A21. CL 00-2-1-38																		1		
A 10. CT 13432-246 (13-1)										**1**								1		
A 13. CT 13432-107 (25-1)										**1**								1		
A08. O.Yacu 9-17-1													**1**					1	1	
A01. Fanny 54													**1**	1				1	1	
A24. CL 1-6-13														1		1	1	1		
A25. CL 00-6-1-7				1													1	1	1	
A02. O. Caribe 8-17-1			**1**	1	1	1												1		
A19. CL 00-4-1-25					1	1									1			1	1	
A23. CL 1-1-41				1	1	1									1			1		
A11. Isolinea 6-7-1	1						1						**1**	1		1		1		
A16. CT 13432-107 (12-1)			**1**	1	1							**1**			1			1		
A28. CR 1821 (1-1)				1							1			1		1		1		
A06. FL 00440-30P-2-2P-M			**1**	1	1	1				**1**					1			1		
A09. FED 2000-5-1				1	1	1				**1**					1			1		
A18. CL 00-4-1-24		**1**		1			1						**1**				1	1	1	
A07. FED 50-24-1			**1**	1	1	1				**1**	1				1			1		
A20. CL 3-6-25				1			1				1			1		1	1	1		
A22. CL 00-1-1-36				1	1	1								1		1	1	1	1	
A15. COL XXI		**1**		1	1	1	1						**1**		1		1	1	1	
A26. Irga 409	1			1			1				1	**1**	**1**			1	1	1	1	
A03. Metica 1-33-18			**1**	1	1	1							**1**		1	1	1	1	1	
A05. Cica 9-15	1			1	1	1	1				1			1	1		1	1	1	
A17. CL 00-3-1-29				1	1	1	1				1			1	1	1	1	1	1	
A27. Cimarron 1-2	1			1	1		1	**1**	**1**	**1**			**1**	1	1	1	1		1	
A04. Cica 9-37-1		**1**		1	1	1	1	**1**			1		**1**		1		1	1	1	

Red: compatible isolates predict susceptibility of resistance genes matching corresponding avirulence genes present in the islate; **blue**: inferred susceptibility of resistance gene; **black:** inferred resistance gene based on incompatible reactions with isolates carrying corresponding avirulence genes

Table 1.13.4. Predicted blast resistance genes present in 21 Latin American commercial rice cultivars based on greenhouse inoculations with rice blast isolates carrying corresponding avirulence genes

Commercial Cultivar	Country	Blast Resistance Genes																				
		Pi-b	Pi-i	Pi-k	Pi-k^a	Pi-k^h	Pi-k^m	Pi-k^p	Pi-k^s	Pi-sh	Pi-t	Pi-ta	Pi-ta^2	Pi-z	Pi-z^t	Pi-1	Pi-2	Pi-3	Pi-9	Pi-33	Pi-4a	Pi-4b	No.
P. industrial	**Chile**																						0
BR-Irga 412	**Brazil**			X																			1
CR 750	**Costa Rica**											X									X		2
Oryzica 1	**Colombia**	X								X			X										3
Juma 61	**R. Dominicana**			X						X			X				X						4
Linea 2	**Colombia**	X		X					X	X			X										5
Iniap 12	**Ecuador**	X	X						X	X				X						X			6
CR 1113	**Costa Rica**		X	X		X	X		X	X						X							7
Iacuba 16	**Cuba**		X	X		X	X		X	X			X			X							8
Palmar PA	**Argentina**	X	X					X	X	X							X	X			X	X	9
Damaris	**Panama**	X	X	X					X	X	X		X	X		X				X			10
Fedearroz V1	**Colombia**	X	X	X				X	X	X		X	X				X	X			X		11
Colombia 21	**Colombia**	X	X	X				X	X	X		X			X		X	X		X	X		12
Icta Virginia	**Guatemala**		X	X	X	X	X		X	X		X	X			X		X			X	X	13
Inia Zapata	**Uruguay**	X	X	X		X	X	X	X	X			X	X		X		X		X	X		14
Panacu	**Bolivia**	X	X	X		X	X	X	X	X	X		X	X		X		X		X	X		15
Icta	**Guatemala**	X	X	X	X	X	X	X	X	X		X	X			X	X	X			X	X	16
Colomgua																							
Altamira 7	**Nicaragua**	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	X			X	X	18
Fedearroz 50	**Colombia**	X	X	X	X	X	X	X	X	X		X	X	X	X	X	X	X		X	X	X	19
Cuyamel 3820	**Honduras**	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X		X	X	X	20
Triunfo	**Brazil**	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	21

Table 1.13.5. Frequency of avirulence genes in 28 blast isolates selected to screen commercial rice cultivars from Latin America.

Avirulence Gene	Isolate No.	Frequncy	Avirulence Gene	Isolate No.	Frequency
Avr Pi-sh	1	0.04	*Avr Pi-z*	9	0.32
Avr Pi-k^{s}	2	0.07	Avr Pi-z^{t}	9	0.32
Avr Pi-ta^{2}	2	0.07	Avr Pi-2	9	0.32
Avr Pi-i	3	0.11	Avr Pi-4b	9	0.32
Avr Pi-b	4	0.14	Avr Pi-k^{m}	12	0.43
Avr Pi-k	5	0.18	Avr Pi-1	12	0.43
Avr Pi-4^{a}	5	0.18	Avr Pi-3	12	0.43
Avr Pi-t	6	0.21	Avr Pi-33	13	0.48
Avr Pi-ta	7	0.25	Avr Pi-k^{h}	14	0.50
Avr Pi-k^{p}	9	0.32	Avr Pi-k^{a}	18	0.64

Table 1.13.6. Frequency of blast resistance genes in 211 commercial rice cultivars from Latin America.

Resistance Gene	Cultivars No.	Frequency	Resistance Gene	Cultivar No.	Frequency
Pi-9	12	0.06	***Pi-4b***	69	0.33
Pi-k^{a}	35	0.17	**Pi-3**	83	0.39
Pi-t	46	0.22	**Pi-ta**	94	0.45
Pi-z^{t}	48	0.23	**Pi-k**	99	0.47
Pi-k^{h}	55	0.26	**Pi-b**	112	0.53
Pi-k^{m}	55	0.26	**Pi-i**	117	0.55
Pi-z	62	0.29	**Pi-4a**	125	0.59
Pi-k^{p}	64	0.30	**Pi-ta^{2}**	137	0.65
Pi-1	64	0.30	**Pi-k^{s}**	158	0.75
Pi-33	64	0.30	**Pi-sh**	178	0.84
Pi-2	68	0.32			

Table 1.13.7. Example of complementary resistance genes between complementary groups of Latin American rice cultivars.

Complementary Group	Resistance Gene	Frequency
Group 3	Pi-1	1.00
	Pi-2	1.00
	Pi-33	0.82
Group 6	Pi-1	1.00
	Pi-2	0.00
	Pi-33	1.00
Group 9	Pi-1	0.10
	Pi-2	1.00
	Pi-33	0.10

Activity 1.14. Effects of endophytic bacteria on plant growth and development

Contributors: P. Fory, S. Kelemu, J. Ricaurte, R. Garcia and I. Rao

Highlights:

- Demonstrated that through tissue culture and spraying antibiotics (cefotaxime and vancomycin) we could eliminate endophytic bacteria in Brachiaria, which is necessary step to determine their fixing properties.
- Showed that through introduction of bacteria isolated from a Brachiaria hybrid (CIAT 36062) the Brachiaria hybrid cv. Mulato (CIAT 36061) exhibited improved growth (more litters and root development) relative to the control (indigenous bacteria only).
- Developed a specific primer useful to detect endophytic bacteria associated with Brachiaria using one step PCR instead of nested PCR.

Rationale

In both managed and natural ecosystems, plant-associated bacteria play key roles in host adaptation to changing environments. These interactions between plants and beneficial bacteria can have significant effect on general plant health and soil quality. Associative nitrogen-fixing bacteria may provide benefits to their hosts as nitrogen biofertilizers and plant growth promoters. Several endophytic bacteria have been reported to enhance growth and improve plant health in general (Sharma and Novak, 1998, Can. J. Microbiol. 44: 528-536; Stoltzfus *et al*., 1998, Plant Soil 194:25-36). Many plant-growth-promoting bacteria (PGPB) that include a diverse group of soil bacteria are thought to stimulate plant growth by various mechanisms such as plant protection against pathogens, providing plants with fixed nitrogen, plant hormones, or solubilized iron from the soil.

Brachiaria grasses of African savannahs have supported millions of African herbivores over thousands of years. Some of these *Brachiaria* species have many desirable agronomic traits. For example, they are persistent and can grow in a variety of habitats ranging from waterlogged areas to semi-desert. These grasses that are often grown under low-input conditions are likely to harbour unique populations of nitrogen-fixing or plant growth promoting bacteria. The aim of our study is to examine the effects of endophytic bacteria that were isolated from species of *Brachiaria* on plant development.

Materials and Methods

Bacterial inoculum preparation: Three endophytic bacterial isolates 01-36062-R2, 02-36062-H4, and 03-36062-V2 that were originally isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively, and that tested positive for sequences of the *nif*H gene (the gene that encodes nitrogenase reductase) are maintained at -80°C in 20% glycerol. Bacterial cells were removed from each of the stored samples, plated on nutrient agar medium (Difco, Detroit, MI) and incubated for 24 h at 28°C. The cells from each of the bacterial strains were collected from the plates, suspended in sterile distilled water and adjusted to a concentration of optical density (OD_{600}) = 1.0 with a spectrophotometer.

Plant inoculation: Twenty tillers of about a month old were prepared from a single mother plant of *Brachiaria* hybrid CIAT 36061 (cv. Mulato), their roots washed with sterile distilled water and made ready for inoculations. The roots of ten of these tillers were immersed in a beaker containing a mixture of equal volumes (50-ml each) of the three strains of endophytic bacterial suspension described above. The remaining ten plants were immersed in a beaker containing the same volume of sterile distilled water. All plants were kept immersed for 48 hours, after which they were removed and rinsed 3 times with sterile

distilled water. They were then each transplanted to pots containing sterile sand (95%) and soil (5%) and maintained in the greenhouse under natural day light and at temperatures between 19 and 30°C. No nutrients were applied.

Plant evaluations: Sixty-five days after inoculations the following measurements were taken in control and treated plants: 1) plant growth and development such as plant height, number of tillers, number of leaves, leaf area, 2) leaf chlorophyll content, 3) nitrogen content, and 4) soluble protein content in leaves.

Plant development and other measurements: Plant height was measured in centimeters from stem base to the highest part of the plant. Number of leaves per plant and the number of tillers were determined. Leaf area was determined in cm^2/plant and measured using a LI-300 leaf area meter (LI-COR, inc., Lincoln, NE). In addition, dry matter distribution among leaves, stems and roots was determined after drying each tissue separately in an oven at 70ºC for 48 hours. Leaf chlorophyll content was measured with a chlorophyll meter SPAD 502 (Minolta), taken across the third fully developed leaf as an average of 6 measurements. Soluble leaf protein was measured as described by Rao and Terry (Plant Physiol 90: 814-819). Nitrogen content in leaves and stems was determined using methods described by Salinas and García (1985, CIAT, Working document 83 p).

Bacterial population in the roots: Approximately 1 g of root sample was taken from each individual plant, surface sterilized (in 1% NaOCl solution for 2 min, in 70% ethanol for one min, then rinsed 3 times in sterile distilled water) and macerated in mortar and pestle in 1 ml of sterile distilled water. One hundred-εl of this macerated sample was taken and a dilution series performed. These were plated on nutrient agar medium and incubated for 24 h at 28°C to determine bacterial colony growth, and calculate the number of bacterial cell per gram of root weight.

Experimental design and statistical analysis: The experiment had two treatments (with and without artificial inoculations) each with 10 plants and arranged in a completely randomized design. Analysis of variance was determined using Statistics Analysis System (SAS⊇). A t-test was conducted.

Results and Discussion

Brachiaria hybrid CIAT 36061 had indigenous endophytic bacteria that are difficult to eliminate. Because of the difficulty to eliminate these indigenous bacteria, we set out to introduce three different strains of bacteria, originally isolated from *Brachiaria* hybrid CIAT 36062, into CIAT 36061, in addition to the indigenous bacteria that this hybrid already has. In general, the introduction of these bacteria had a positive effect on plant growth and development in the recipient plant CIAT 36061 (Figure 1.14.1). Figure 1.14.2 further demonstrates more tiller and root development in artificially inoculated CIAT 36061 plants than plants containing only indigenous endophytic bacteria.

Figure 1.14.1. *Brachiaria* hybrid CIAT 36061 plants with indigenous endophytic bacteria (1), and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) [2], 65 days after inoculations and maintained under greenhouse conditions with no nutrients.

In nitrogen- and other nutrient-deficient conditions, *Brachiaria* plants inoculated with the three bacterial strains had significantly higher average values in all evaluated parameters (with the exception of soluble proteins in leaves) than those control plants containing just indigenous bacteria (Table 1.14.1).

Table 1.14.1. Average values of various parameters evaluated for endophyte-inoculated and non-inoculated plants of CIAT 36061.

Parameters	Control	Inoculated
Plant height (cm)	103.9b[†]	115.6a**
Leaves/ plant	22.5b	36.9a***
Tillers/ plant	4.1b	7.4a***
Leaf area (cm^2/plant)	993.7b	1430a***
Chlorophyll SPAD	340.3b	433.7a***
Soluble Protein (σg/ cm^2 fresh leaf)	928.93a	1095.42[a]
Stem N content (%)	0.51b	0.67a**
Green leaf N content (%)	1.0 b	1.3a **
Dead leaf N content (%)	0.44b	0.66a**

[†]Each value is the mean of values from 10 plants. Data in each row followed by the same letter are not significantly different (P<0.05) according to t-test.

Figure 1.14.2. A *Brachiaria* hybrid CIAT 36061 with indigenous endophytic bacteria (1), and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) [2], 65 days after inoculations and maintained under greenhouse conditions with no nutrients. Note the difference between the artificially inoculated plant and the one with just indigenous bacteria, in the number of tillers and root growth and development.

Analysis of variance showed that the total biomass production (leaf, stem and root) collected from control Brachiaria CIAT 36061 plants was significantly ($P< 0.05$) less than that from inoculated ones (Figure 1.14.3). The data presented indicate that a close and beneficial interaction existed between the introduced as well as indigenous endophytic bacteria and *Brachiaria* hybrid CIAT 36061, resulting possibly in nitrogen fixation and enhancement of plant growth. Had we managed to eliminate the indigenous endophytic bacteria from control CIAT 36061 plants, the difference between bacteria-containing and control plants would probably have been even more dramatic.

A high correlation value ($r= 0.89$; $P<0.01$) was observed between leaf chlorophyll content and % nitrogen in leaves. Inoculated plants maintained a more profound green color and higher nitrogen content in their leaves than control plants. As expected, bacterial cells were isolated from both control plants containing indigenous bacteria and those inoculated with the 3 bacterial strains with values that are similar (6.56 $\log_{10}$ CFU/g of fresh root tissue of inoculated plants vs 6.53 $\log_{10}$ CFU/g of fresh root tissue of non-inoculated control plants). These endophytic bacterial population data are very similar to the natural endophyte concentrations in alfalfa, sweet corn, sugar beet, squash, cotton and potato, reported to vary between 2.0 and 6.0 $\log_{10}$ CFU/g of tissue (Kobayashi and Palumbo, 2000, *In* Bacon and White, eds. Microbial endophytes. Marcel Dekker, Inc., NY).

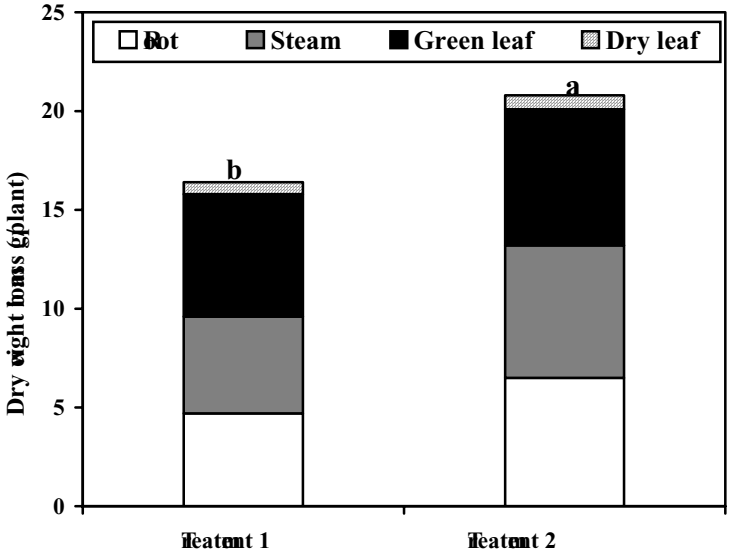


Figure 1.14.3. Total tissue biomass production in *Brachiaria* hybrid CIAT 36061 control plants with indigenous endophytic bacteria (treatment 1), and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) [treatment 2], 65 days after inoculations and maintained under greenhouse conditions with no nutrients. Values are average of 10 plants per treatment.

These preliminary data strongly suggest that endophytic bacteria have a direct beneficial effect on plant growth and development, and possibly on associated nitrogen fixation in *Brachiaria*. More work is needed to further verify these findings preferably after completely removing indigenous bacteria from species of *Brachiaria*.

Activity 1.15. Cloning and characterization of a nitrogen fixation gene (*nif*) sequences from a plant growth promoting bacterium associated with species of *Brachiaria*.

Contributors: P. Fory and S. Kelemu

Rationale

A number of prokaryotes are known to be involved in nitrogen fixation as well as enhancement of plant growth. Nif genes which encode the nitrogenase complex (encoded by approximately 20 different nif genes) and other enzymes involved in nitrogen fixation has consensus sequences identical from one nitrogen fixing bacteria to another, but while the structure of the nif genes is similar, the regulation of the nif genes varies between different diazotrophes (nitrogen fixing organisms).

Previously, we have reported the isolation of three strains of bacteria from *Brachiaria* hybrid CIAT 36062 (BR97-1371) from roots, leaves and stems that were designated 01-36062-R2, 02-36062-H4, and 03-36062-V2, respectively. Using nested PCR and three primers designed for the amplification of the *nifH* gene sequences, amplified products were generated with template DNA from these bacterial strains. We have also reported in 2003 that fatty acid analysis conducted on these 3 strains resulted in matching them

with various bacteria that are known to be nitrogen fixers and/or plant growth promoters (for example with *Flavimonas oryzihabitans*).

This study focused on strain 01-36062-R2. The fatty acid analysis data of the isolate 01-36062-R2 matched it with *Leclercia adecarboxylata*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, at 0.879, 0.841, and 0.820 similarity index, respectively (IP-5 Annual Report 2003). *E. cloacae* has been described as one of the dominant endophytic bacteria isolated from citrus plants.

In this study we cloned and sequenced nested PCR amplified products using primers derived from nif-gene sequences. The objective of this work is to develop a specific primer that will allow us to screen (without using nested PCR) *Brachiaria* and other tropical plants-associated bacteria that contain *nif*-gene sequences.

Materials and Methods

Bacterial isolates: Bacterial strain isolated from roots of *Brachiaria* CIAT 36062 and designated 01-36062-R2 was used for this study. For evaluation of the various primers, strains isolated from stems and leaves of the same *Brachiaria* genotype, and designated 02-36062-H4 y 03-36062-V2, *Bradyrhizobium* species CIAT 2469 isolated from the legume Desmodium species, and a pathogenic bacterium *Xanthomonas campestris* pv. *graminis* (included as a negative control) were used.

Bacterial DNA extractions: DNA extraction was conducted using a modified protocol based on combinations of standard methods, which includes growing bacterial cells in liquid media LB (tryptone 10g, yeast extract 5g, NaCl 10g, 10 ml of 20% glucose in 1 L of distilled water), treatment of cells with a mixture of lysozyme (10 mg.ml in 25 mM Tris-Hcl, ph 8.0) and RNase A solution, and extraction of DNA with STEP (0.5% SDS, 50 mM Tris-HCl 7.5, 40 mM EDTA, proteinase K to a final concentration of 2mg/ml added just before use. The method involves cleaning with phenol-chloroform and chloroform/isoamyl alcohol and precipitation with ethanol. The quality of DNA was checked on 1 % agarose gel.

Nested PCR Amplification: Three primers were used, which were originally designed by Zehr and McReynolds (1989, Appl. Environ. Microbiol. 55: 2522-2526) and Ueda *et al*. (1995, J. Bacteriol. 177: 1414-1417), to amplify fragments of *nifH* genes. Amplification steps described by Widmer *et al* (1999, Applied and Environmental Microbiology 65:374-380) were adopted. The final product of the nested PCR amplification is about 370 bp in size.

Cloning of amplified DNA fragments: Amplified products were eluted from agarose gel using Wizard ® PCR Preps DNA Purification System (Promega) according to instructions supplied by the manufacturer. The purified fragments were ligated to the cloning vector pGEM-T Easy (Promega) and used to transform *E. coli* DH5Ŭusing standard procedures (Sambrook *et al*., 1989. Molecular Cloning: a laboratory manual. 2nd ed. Cold spring harbor laboratory, USA)

Plasmid extraction: Plasmids were extracted from transformed *E. coli* DH5Ŭcells using a Wizard® Plus Mini-preps DNA Purification System (Promega) using the protocol supplied by the manufacturer. To confirm whether the transformants contained the desired size of insert (approximately 370 bp), the plasmid DNA was digested to completion with the restriction enzyme *Eco*RI. The digested products were separated by electrophoresis on a 1% agarose gel (Bio-Rad, NJ), stained with ethidium bromide and photographed under UV light.

Amplification of DNA inserts for sequencing: PCR reactions (25-σl) contained 20 ng/σl plasmid DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, primers T7 (5'-GTAATACGACTCACTATAGGGC-3')

and Sp6 (5' –TATTTAGGTGACACTATAG-3') each at 0.1 σM concentration, 0-5U Taq polymerase and amplified in a programmable thermal controller (MJ Research, Inc, MA) programmed with 35 cycles of a 30 sec (2 min for the first cycle) denaturation step at 94°C, annealing for 30 sec at 50°C, and primer extension for 1 min (4 min in the final cycle) at 72°C. Samples of amplified products were separated on a 2% agarose gel by electrophoresis for further confirmation of the expected size insert.

The ABI prism BigDye terminator Cycle sequencing kit was used to further prepare the samples for sequencing. Sequencing was conducted using ABI PRISM^{TM} 377 DNA sequencer. The sequence data were compared with sequences in databases using the program BLAST version 2.0 or 2.1 (http://www.ncbi.nlm.nih.gov/BLAST/-). The program compares nucleotide sequences to databases and calculates the statistical significance of matches.

Specific primer construction: Based on the sequence data, primers were designed using the program DNA-MAN (version 4:0), and synthesized by Integrated DNA Technologies, Inc. (Coralville, USA). These primers were tested on bacteria that are confirmed positive and negative controls: strains 01-36062-R2; 02-36062-H4 and 03-36062-V2, *Bradyrhizobium*, and *Xanthomonas campestris* pv. *graminis.*

Results and Discussion

Cloning and sequence analysis: A 371 bp nested PCR amplification product using template DNA isolated from the bacterial strain 01-36062-R2 (a strain isolated from *Brachiaria* CIAT 36062 and that tested positive for *nif*-gene sequences) was successfully cloned in the vector pGEM-T Easy (Promega). Figure 1.15.1 shows randomly picked transformants, the majority showing the desired size insert, with the exception of lanes 2 and 7.

The sequence analysis demonstrated the presence of *nif*H gene sequences in these sequenced clones. The deduced amino acid sequence showed a 97% similarity with 120 amino acids that correspond to the *nif*H gene sequence of *Klebsiella pnuemoniae*. These results are in agreement with the fatty acid analysis results of this bacterial strain that matched it with *Klebsiella pnuemoniae* at 0.84 similarity index. *Nif* genes that encode the nitrogenase complex and other enzymes involved in nitrogen fixation have consensus sequences identical in various nitrogen-fixing bacteria.

Klebsiella pneumoniae is a member of the Enterobacteriaceae that has the ability to fix nitrogen, and possesses a total of 20 *nif* genes that are clustered in a 24 kb region of the chromosome and responsible in nitrogenase synthesis and its regulation. Three of these genes, *nif*HDK, code for the three structural nitrogenase subunits. *K. pnuemoniae* has been reported as an endophytic bacterium associated with various plants and involved in nitrogen fixation, including maize (Chelius and Triplett, 2001, Microb. Ecol. 41: 252–263), wheat (Iniguez *et al*., 2004, Molecular Plant-Microbe Interactions 17: 1078–1085) and rice (Dong *et al*., 2003, Plant Soil 257:49-59).

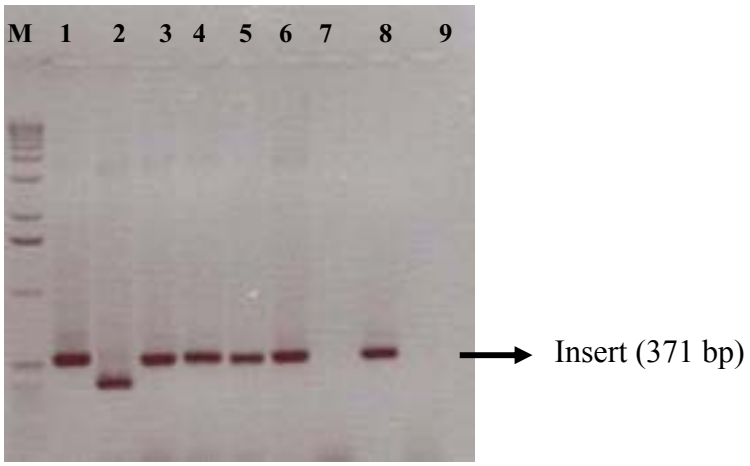


Figure 1.15.1. Amplified products of independent clones using template DNA of the bacterial strain 01-36062-R2 associated with *Brachiaria* CIAT 36062. Amplification conditions were as described in the materials and methods section. M = molecular marker. Lanes 1-8 inserts of clones, lane 9 = negative control. The clones that showed the expected 371 bp size insert were sequenced.

The consensus sequences obtained in this study are listed below as deduced amino acid sequences (**1**) and nucleotide sequences (**2**).

1.
GVIQADSTRLILHAKAQNTIMEMAAEVGSVEDLELEDVLQIGYGGVRCAESGGPE
PGVGCAGRGVITAINLEEEGAYVPDLDFVFYDVLGDVVCGGFAMPIRENKAQEIY
IVCSGEMMALYA

2.
5'-
TGGGTGTGATCCAAGCCGACTCCACGCGTTTGATCCTGCATGCGAAAGCGCAGA
CACCATTATGGAGATGGCCGCCGAAGTCGGCTCCGTCGAAGACCTGGAATTAGA
GACGTGCTGCAAATCGGTTACGGCGGCGTGCGCTGCGCGGAATCCGGTGGCCCG
AGCCAGGTGTGGGCTGTGCCGGTCGTGGCGTGATCACCGCGATTAACTTCCTCG
AGAAGAAGGCGCTTACGTGCCGGATCTGGATTTTGTTTTCTACGACGTGCTGGG
GACGTGGTATGCGGTGGTTTCGCCATGCCGATTCGTGAAAACAAAGCGCAGGAG
TCTACATCGTTTGCTCTGGCGAGATGATGGCCCTCTACGCA-3'

Specific primer development: In this study we developed specific primers that would allow us detect endophytic bacteria associated with species of *Brachiaria* just with one step PCR instead of nested PCR. Based on the consensus sequence listed above (in #2) and using the DNA-MAN program, 9 primers were designed and synthesized. Twenty combinations of these primers were tested on selected positive and negative control bacteria. Of these combinations, a pair of primers with sequences 5'-GTTTGATCCTGCATGCAAAAG-3' and 5'-AGAGCAAACGATGTAGATCTCCTG- 3', produced only one amplification product with a size of approximately 344 bp in bacteria that are used as positive

controls, where as negative controls resulted in no amplified products (Figure 1.15.2). This pair of primers will be tested on various bacteria associated with species of *Brachiaria* and other plants and that are suspected to fix nitrogen. We also want to use this pair of primers to directly detect these bacteria in tissues or soil samples.

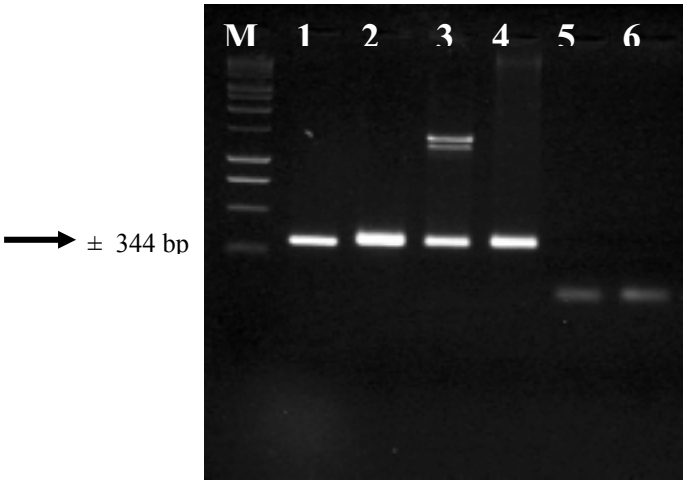


Figure 1.15.2. Specific amplifications of template DNA from lanes 1-4, positive control bacteria *Bradyrhizobium* sp., strains 01-36062-R2; 02-36062-H4 and 03-36062-V2 isolated from *Brachiaria*, respectively; lane 5, negative control *Xanthomonas campestris* pv. *graminis*; lane 6: negative control PCR reaction mixture; M, size marker. Primer combination used 5'-GTTTGATCCTGCATGCAAAAG- 3' and 5'-AGAGCAAACGATGTAGATCTCCTG –3. The PCR reaction (20-σl) contained 16 ng/σl template DNA, 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2-µM dNTPs, 0.2-µM each of the primers, 0.2U Taq polymerase. The amplification was carried out in a programmable thermal controller (MJ Research, Inc, MA) programmed as follows: 94°C for 2 min (94°C for 30 sec, 50°C for 45 sec, 72°C for 30 sec), for 35 cycles; 72°C 8 por min. The products were separated on a 1.2% agarose gel by electrophoresis, stained with ethidium bromide and photographed under UV light.

Activity 1.16. Identification of White Grubs Complex and its Natural Enemies in Antioquia

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Highlights:

- ∉ Key pests and their natural enemies in Antioquia identified
- ∉ Yield losses due to white grub attacks in Antioquia identified

Rationale

Soil pests cause serious economic losses to many important crops in South America. Until about 20 years ago white grubs were not considered important pests in Latin America. However, in the recent past outbreaks are no longer seasonally restricted as before and are present in many agro-ecosystems, such as

hillsides, tropical lowlands, including savannas and forest margins in Central and South America (Posada., 1993, Agricultura Tropical 30: 71-79; Londoño & Pérez, 1994, Rev. Colomb. Entomol. 20: 199-206; Shannon & Carballo, 1996, CATIE Informa Tecnico No. 227).

Inappropriate agricultural methods like inadequate cropping patterns, burning of harvest residues, deforestation, cultivation of marginal land, discontinuance of tillage, loss of floral and faunal biodiversity, reduction of organic material, ill-timed and excessive applications of synthetic pesticides and elimination of natural enemies of pests are considered to be the key factors responsible for the increased pest status of white grubs (Posada, 1993, Agricultura Tropical 30: 71-79; Londoño & Pérez, 1994, Rev. Colomb. Entomol. 20: 199-206). The frequent use of highly toxic soil pesticides can lead to development of resistance in pests and is additionally very often ineffective.

The populations of white grubs have increased in Eastern Antioquia since 1988 and are causing severe damage on a wide range of crops. In 2002 CIAT started collections in Northern and Eastern Antioquia. The quantity of individuals that we have collected is enormous: approximately 190,000 specimens until December 2003 (CIAT, PE-1 Annual Report 2004). In the present study we identified the most important white grubs that are considered as soil pests, described their feeding behavior, and estimated the damage that they caused on potatoes.

Materials and Methods

Description and definition of soil pests (diagnostic): We selected the sites of survey according to records of ICA and the Departmental Secretary of Agriculture. The Umata's helped us to identify potato and pasture farms where white grubs are traditionally a problem. The selected municipalities in Eastern Antioquia were characterized by similar physiographic characteristics and a mean altitude of 2100 m.a.s.l.: Rionegro, Marinilla, San Vicente, and El Carmen de Viboral and La Union (2600 m.a.s.l.). In Northern Antioquia we surveyed potato and pasture fields in San Pedro, Santa Rosa, and Entrerríos, all of them agroecologically similar zones at an altitude of approximately 2700 m.a.s.l. We surveyed the potato fields at harvest in order to avoid damage of the crop. In every field we evaluated 10 randomly selected square meters.

Determine yield losses due to white grub attacks: In order to determine the damage on potatoes and pasture due to attacks of white grubs and other soil pests we tabulated the information obtained from each surveyed square meter in the three agroecological zones of Northern and Eastern Antioquia and La Union. The parameters in potato fields were: number of plants, number o potatoes, number of potatoes damaged by white grubs, and number of potatoes damaged by other soil pests. In pasture we only tabulated the area (m^2) damaged white grubs.

Type of Damage: We set up experiments in the greenhouse for estimating damage on potato tubers in order to define the pest status of the white grub genera *Ancognatha*, *Cyclocephala*, *Ceraspis*, *Astaena*, and *Heterogomphus.* We collected white grubs during seven surveys in the savanna of Northern during August, September, October, and November in 2004 and took them to the greenhouse in the Research Station "La Selva" where the larvae fed on potato tubers. We also received 56 larvae (*Clavipalpus*, *Ancognatha*, *Astaena*, and *Cyclocephala*) from the departments Boyacá and Nariño. We revised the tubers once a week distinguishing the damage in four classes: normal (no damage), rasped, bitten, and burrowed (Figure 1.16.1). We identified the insects after having completed their development as adult.

Results and Discussion

Description and definition of soil pests (diagnostic): We continued the collection of white grubs and adults in Eastern and Northern Antioquia. In the cold climate of Northern Antioquia we collected 8.62±9.65 white grubs in one square meter. The white grub complex of this zone is detailed in Table 1.16.1. *Ancognatha* was by far the most dominant genus, followed by *Cyclocephala*.

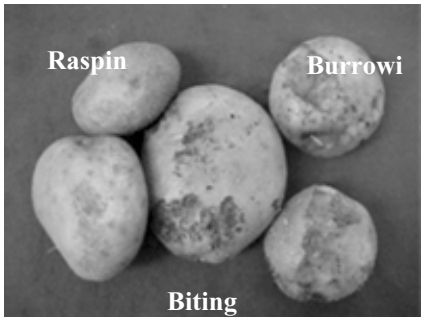

Figure 1.16.1. Types of damage caused by white grubs on potato tubers (Photo: CORPOICA)

Table 1.16.1. Number of collected white grubs from December 2002 until February 2004 in the cold climate of Northern Antioquia.

	Nº of collected specimens				
Municipality	**Entrerríos**	**San Pedro**	**Santa Rosa**		
Altitude ± SD m.a.s.l.	2384 ± 24.6 m.a.s.l.	2600 ± 7.9 m.a.s.l.	2584 ± 130.6 m.a.s.l.	**Total**	**%**
Ancognatha	43	83	275	401	73,6
Not identified	4	7	61	72	13,2
Cyclocephala	2	6	53	61	11,2
Astaena	0	0	7	7	1,3
Phyllophaga	0	1	2	3	0,6
Plectris	1	0	0	1	0,2
Total	50	97	398	545	100,0

In themoderate cold climate of Eastern Antioquia we collected 7.10±11.5 grubs per square meter. The proportion of genera presents differences between the surveyed sites. We collected 489 collected specimens of *Ancognatha* that was the most abundant genus in El Carmen de Viboral (Table 1.16.2). In the municipalities Rionegro and Guarne we found less grubs, however, the dominant genus were *Phyllophaga* and *Anomala*. We hypothesize that these marked differences are due to the elevated altitude of El Carmen favoring members of the subfamily Dynastinae such as *Ancognatha* spp. We observed that soil organic matter (SOM) is abundant in the colder zone and that is this subfamily prefers this food type. This observation was corroborated by the findings by Zuluaga *et al*. (2005, Feeding behavior of three white grub species associated with potato in the Savanna of Bogotá, manuscript in preparation) who report that *Ancognatha* spp. thrives best on SOM. It would be interesting to conduct soil analyses considering variables like SOM, pH, or altitude in order to understand the prevalence of this white grub group in determined areas. In the cold climate of La Union in Eastern Antioquia (2416 m.a.s.l.) we collected

8.5±12.38 grubs/m^2. Similar as in high altitudes of Northern Antioquia dominated the genus *Ancognatha* (Table 1.16.3). Surprisingly, we collected less white grubs in pasture than on potatoes. The number of specimen varied a lot. In the North we found 3.65±8.44 grubs / m^2, in the East 14.27±28.9 and in La Union 0.60±1.04.

Table 1.16.2. Number of collected white grubs from December 2002 until February 2004 in the moderate cold climate of Eastern Antioquia.

	Nº of collected specimens					
Municipality	**Rionegro**	**Guarne**	**El Carmen**	**San Vicente**		
Altitude ± SD m.a.s.l.	2175 ± 32.61 m.a.s.l.	2336 ± 76.04 m.a.s.l.	2260 ± 4.57 m.a.s.l.	2200 ± 0.00 m.a.s.l.	**Total**	**%**
Ancognatha	5	8	489	24	526	55,5
Not identified	41	18	0	132	191	20,1
Anomala	6	66	0	23	95	10,0
Phyllophaga	73	2	1	6	82	8,6
Cyclocephala	29	2	0	1	32	3,4
Plectris	0	2	5	6	13	1,4
Astaena	4	0	0	5	9	0,9
Total	158	98	495	197	948	100

Table 1.16.3. White grub complex in the cold climate of Eastern Antioquia (survey from December 2002 until September 2003

	Nº of collected specimens	
Municipality	**La Union**	**%**
Altitude ± SD m.a.s.l.	2416 ± 71.65 m.a.s.l.	
Ancognatha	530	96,9
Cyclocephala	17	3,1
Not identified	10	1,8
Total	557	100,0

Determine yield losses due to white grub attacks: We revised during 14 months 670 m^2, of those 72.8% harbored tubers that presented some type of damage. In Northern Antioquia we revised 12,257 tubers with an average of 49±19.17 tubers per square meter. We found on 10.15% of all revised tubers some damage that was caused by white grubs (7.51%), the potato tuber moth (*Tecia solanivora*) (1.58%), the Black Cutworm (*Agrotis ipsilon*) (0.08%), other insects (0.01%) and diseases (0.97%).

In Eastern Antioquia we revised 8509 tubers. Similarly to the north we found on 9.74% of the tubers symptoms of insect attacks. 5.89% of the damage was caused by white grubs, 3.35% by *T. solanivora*, 0.08% by *A. ipsilon*, 0.11 by other insects, and 0.32% by diseases. In the cold zone of La Union we checked 8289 tubers, of these 7.44% presented symptoms of damage: grubs (3.81%), *T. solanivora* (1.82%). Damage caused by other insects and diseases was about 0.2%.

Type of Damage: Larvae of the genus *Heterogomphus* never attacked potatoes indicating that they feed on decaying organic matter (Figure 1.16.2) confirming the observations by Zuluaga *et al.* (manuscript in preparation). A few *Plectris* larvae rasped the potato surface. *Ceraspis* and *Astaena* prefer to rasp the tuber; however, some larvae of these genera also bit the potato. *Ancognatha* and *Cyclocephala* can cause damage to root by biting, burrowing, and rasping. This corroborates the reports of Zuluaga *et al*. (manuscript in preparation) that *Ancognatha* can be a major pest problem when soil organic matter is not sufficiently available. The observation that *Cyclocephala* feeds on fresh plant material is new; however, it wouldn't be a surprise if these grubs may also act as pests when soil conditions force them to do so. We suggest conducting further experiments in order to understand the feeding behavior of this genus.

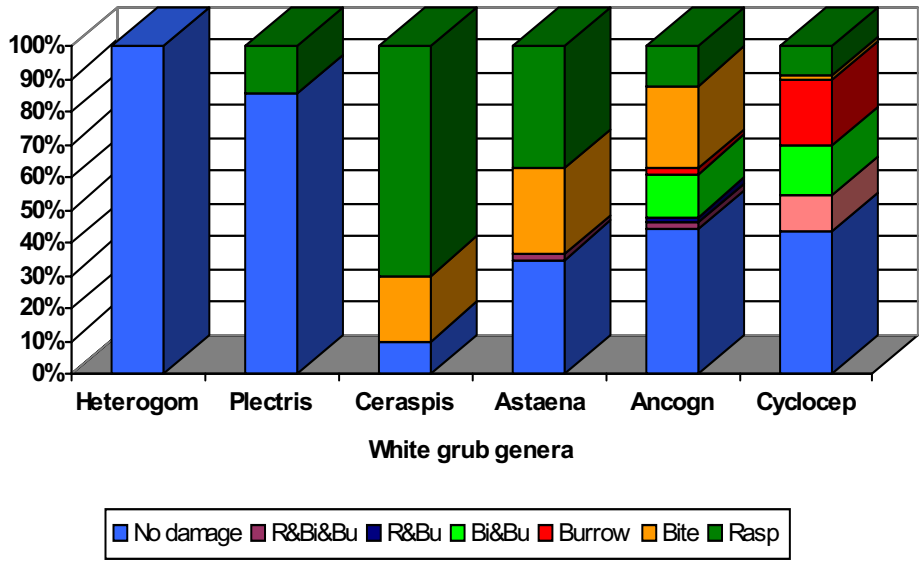

Figure 1.16.2. Feeding behavior of the white grub genera *Heterogomphus* (Heterogom), *Plectris*, *Ceraspis*, *Astaena*, *Ancognatha* (Ancogn), and *Cyclocephala* (Cyclocep). The larvae were collected in the field and the experiment was conducted in the greenhouse. Abbreviations in Legend: R= Rasp, Bi= Bite, Bu= Burrow.

Activity 1.17. Mass rearing *Phyllophaga menetriesi* (Col.: Melolonthidae) under controlled conditions

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Highlight:

∉ Methodology for mass rearing of white grub species developed

Rationale

Numerous insects feed on the foliage or roots of bushy or tree species. Sometimes, the larvae of some are economically significant, as in the case of several species of the family Melolonthidae (or Scarabaeidae Pleurosticti), which can greatly affect the vegetation of their habitats. In tropical and subtropical America,

these white grubs, also known as *coro, joboto, chisa*, or *mojojoy*, number more than 500 species throughout the region. Yet, we are barely learning the basics of their taxonomy and ecology. Colombia presents a great diversity of beetles, some of which are of agricultural interest as adults are defoliators or leaf eaters, and their larvae are root-eating pests. Of the latter, larvae of the genus *Phyllophaga* stand out for their economic importance as they feed on the roots of various tropical and subtropical crops of the Americas, including cassava, maize, and vegetables, reducing their yields by either weakening or killing the plants.

Because this genus has a broad range of hosts, farmers mostly use chemicals to control these pests. However, precisely for being chemical, this type of control has proven inefficient in managing the pests and, above all, has had a severe impact on the environment. The rhizophagous larvae are protected by the soil, making control highly complex. Only by collecting basic information can technologies be developed to adequately and rationally manage the pests in their agroecosystems, thus increasing productivity at minimal economic and environmental cost, and permitting the preservation and use of biodiversity.

To seek the most viable strategy for combating the pests and generating suitable recommendations for their control, methodologies of mass rearing must be developed to obtain disease-free larvae that develop uniformly. Thus, they can be used for testing with entomopathogenic fungi, bacteria, and nematodes (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20)

The research discussed below was carried out at the International Center for Tropical Agriculture (CIAT). Its goal was to mass-rear *Phyllophaga menetriesi* Blanchard, a species belonging to the economically most important white-grub complex in southwestern Colombia. The methodology has been steadily improved and, currently, it can raise 8000 larvae per year.

Materials and Methods

Capturing adults: The site selected for capturing adults was in the Village District of Pescador, Department of Cauca, located at 1500 m above sea level (masl). In this site, 20-W, black-light traps were permanently installed in a cleared and visible place. These traps are in common use and are based on North American models of vertical light. They comprise basically three parts: a source of luminous radiation, a capturing device, and a collection container (Montoya *et al*., 1994, Rev Colomb Entomol 20:130–136). The traps were operated every night from 18:00 to 6:00 during October and November, when adults were most likely to be abundant and diverse (Pardo Locarno, 2002, MSc Thesis, Univ. del Valle, Cali, Colombia).

In Pescador, where nine traps had been set, the farmer's family members believed in the importance of the work, such that they daily surveyed the light traps. Their task was to separate the adults, initially by coloring and size (Ruiz and Posada, 1985, Rev Colomb Entomol 11: 21–26), and then place them in 5-L trays containing substrate that was previously sterilized at CIAT. Pieces of ripe plantain and carrot were used for food. At the same time, traps were set, one for each of three farms, in the Department of Quindío, where the insect's presence had been monitored in previous years (Pardo Locarno *et al*., 2003, *In*: Aragon et al., (eds), Estudios sobre coleopteros del suelo en America, Special publication, Univ. Autonoma de Puebla, Mexico, pp 45-63). The farmers made daily collections from these traps and the collected material was sent weekly to CIAT. As check, two black-light traps were installed in an area from which the chisa complex has not been reported: one trap was set in the Village District of El Olivo (1637 masl) and the other in the Village District of Potrerillo (1420 masl), both of the Municipality of Palmira (Valle del Cauca).

To facilitate different tests with entomopathogens, four light traps were installed at the Experiment Station "Tulio Ospina" of the Instituto Colombiano Agropecuario (ICA) in the Municipality of Bello (Antioquia),

10 km north of Medellín. The traps were set between January and April 2005, when the largest populations of adult Melolonthidae appear in this area (Montoya *et al*., 1994, Rev Colomb Entomol 20:130–136). The two strains were combined into a colony to ensure two periods of flight although, in the field, this species is univoltine only.

Although in eastern Antioquia, *Phyllophaga obsoleta* Blanchard stands out for its economic impact (Pardo Locarno *et al*., 2003, *In*: Aragon et al., (eds), Estudios sobre coleopteros del suelo en America, Special publication, Univ. Autonoma de Puebla, Mexico, pp 45-63; Vallejo *et al*., 1998, Coleopterist's Bull 52:109–117), *P. menetriesi* has also been reported as being present. Thus, traps were set to capture adults and so determine the abundance of this species in the area. Captures of *P. menetriesi* carried out previously at the station increased the number of adults collected for mass rearing. The adults from this area were collected daily and placed in 10-L plastic trays. The number of captured adults was recorded. Because of the efficiency with which this task was carried out, we could obtain two breeding periods at CIAT: October–November and April–May.

The mass-rearing methodology was carried out at the Quindío Laboratory of CIAT's Cassava Entomology Project, located in the Municipality of Palmira (Valle del Cauca; 965 masl). At the same site, a trap was also set to capture adults. However, results were very poor.

Conditions for mass rearing were an average temperature of 23 ± 2 ºC and a relative humidity of 80% ± 10%. The substrate consisted of soil from the organic or humus layer mixed with topsoil in a 1:1 ratio. The mixture was passed through a mesh with a 5-mm pore size (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20). It was then steamed for 4 h in a greenhouse soil sterilizer. The sterilized soil was left to stand for 48 h or more to eliminate the toxic gases that accumulate during the soil's heating and which could kill the insects (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20).

Oviposition: An adult *P. menetriesi* measures 18 to 23 mm, and is dark brown. Its body is robust, being broader towards the rear than at the head. The entire body surface is covered with scorings and dense, brown, and regularly distributed bristles or setas. It is characterized by a sinuate pronotal margin, an erect and somewhat raised clypeus, and metatibial articulated spurs (Figure 1.17.1).

Figure 1.17.1. Typical adults of *Phyllophaga menetriesi*, an economically significant crop pest in Colombia. (A) male, (B) female

The captured adults were separated by sex, according to the external characteristics of their genital orifice (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20), which presents diphormism in the last abdominal segment (Figure 1.17.2). Fifteen couples of adults were placed in 10-L plastic containers, which had covers made of coarse canvas with metal edges to prevent individuals escaping while

permitting air to circulate within the containers. The containers contain about 7 L of sterilized soil, which was watered every 3 days to prevent the soil from drying. Leaves of *Erythrina* spp. (Fabacae) and West Indian cedar or *guácimo* (*Guazuma ulmifolia* Lam.) were also added to feed the adults (Figure 1.17.3). The plant material was constantly replaced and dead adults removed every 8 days to prevent the accumulation of decomposing insects. Genitalia were also extracted from dead males, and mounted on entomological pins for later verification of identification. If the adults were alive, they were returned to the container and the soil watered again.

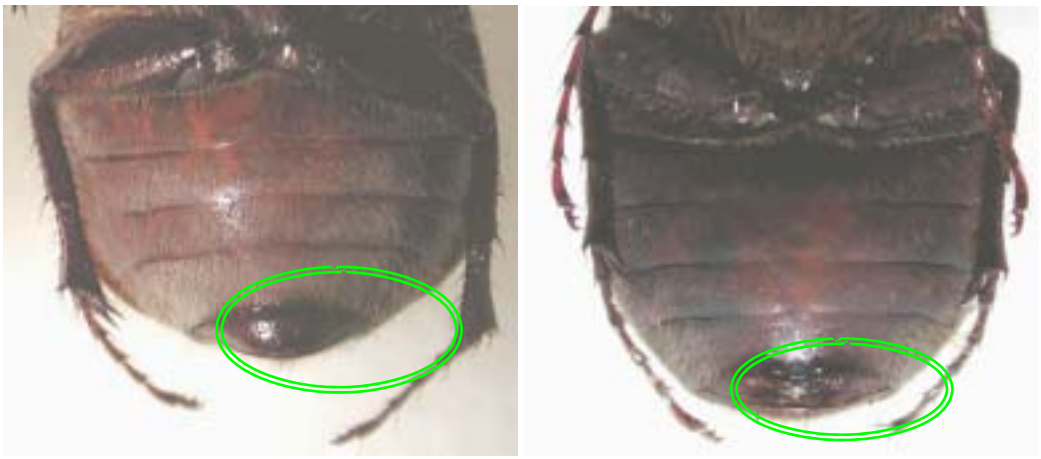

Figure 1.17.2. Diphormism of the last abdominal segment in adult *Phyllophaga menetriesi*. On the left is a male, with a female on the right.

Figure 1.17.3. Ten-liter plastic containers for carrying soil and plant materials for mass-rearing *Phyllophaga menetriesi*. Fifteen couples of adult beetles are placed in each container.

Extracting eggs: Eggs were extracted every 8 days. All the soil was removed from the plastic containers and any eggs removed manually with a spatula, placing them into plastic trays (Hidalgo *et al*., 1993, Rev Man Integ Plagas Hond 56:14–20). They were then separated, 25 eggs per 16-oz plastic container, each of which was duly labeled with the date of placement (Figure 1.17.4). Carrots were also added as food for the

first-instar larvae (Figure 1.17.5), thus preventing cannibalism, which appears from first-instar stage onwards.

The eggs were examined every 8 days to check which ones were close to hatching. Eggs are at first white and round. As they incubate they increase in size, averaging 2.7 mm in length (Ruiz and Posada, 1985, Rev Colomb Entomol 11:21–26). *Phyllophaga menetriesi* females oviposit their eggs individually in a chamber shaped like a clod of earth, according to Ruiz and Posada (1985, Rev Colomb Entomol 11:21–26) when describing *Ancognatha scarabaeoides* Erichson. Each egg appears covered with particles of the substrate used (Figure 1.17.6a). When the egg is fully developed, the jaws that break the chorion can be distinguished.

Figure 1.17.4. Sixteen-ounce plastic containers carrying soil with eggs of *P. menetriesi*.

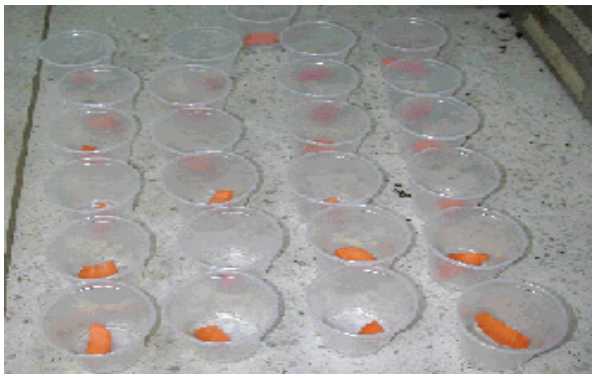

Figure 1.17. 5. Carrot pieces for feeding larvae of *P. menetriesi* are placed in the bottom of 16-oz plastic containers before soil and the pest's eggs are added.

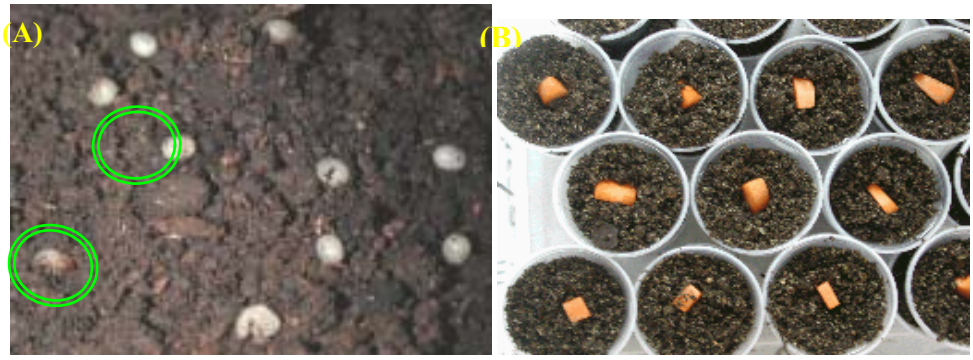

Figure 1.17. 6. (A) Eggs (top circle) and first-instar larvae (bottom circle) of *Phyllophaga menetriesi*, a crop pest. (B) First instars are removed and placed into 4-oz plastic containers carrying soil and carrot pieces

Eclosion and development of the first instar: After eclosion, the larvae were individually placed into 4-oz plastic containers with covers. The soil had the same characteristics as used for oviposition. Carrot pieces were added to feed the larvae (Figure 1.17.6b) and replaced every 15 days, when they were estimated to be totally consumed.

At this stage, care is essential for managing individuals as they are susceptible to any adverse condition occurring during their development. Critical conditions include the presence of dense root masses, loss of soil moisture, and excessive manipulation. A mortality rate of 45%–50% is considered normal for this stage, although the rate for the next stage may drop to 15%.

Second instar: Eighteen to 30 days after eclosion, about 85% of the larvae pass to second instar, developing in the same plastic containers (Figure 1.17.7a). Once they reached the second instar, the soil and food were replaced. Instead of carrots, rice seedlings, which have a larger number of roots that can cater for the larvae's increased consumption, were used. Hence, the larvae could develop without anomalies. To determine if the larvae were in second instar, the cephalic capsule was examined; it should have shown considerable increase, of about 38% (Calberto, 2004, Thesis on Environmental Administration, Faculty of Basic Sciences, Universidad Autónoma de Occidente, Santiago de Cali, Colombia, 65 p) (Figure 1.17.7b).

Third instar: Once they reached third instar (Figure 1.17.8), the larvae, now measuring 34 mm long and with a 5.2-mm-wide thorax, were put in 10-oz plastic containers. The food was changed back to carrot, this time in 5 to 7-g pieces (Calberto, 2004, Thesis on Environmental Administration. Faculty of Basic Sciences, Universidad Autónoma de Occidente, Santiago de Cali, Colombia, 65 p). The change in diet made it possible to reduce observations. The food was changed once a month, as this is the most voracious instar (King, 1984, Trop Pest Man 30: 36–50). The units had to be continually checked to maintain adequate soil moisture and keep the larvae in good condition. During the change from this instar to prepupa, care had to be taken with food spoilage, hastened by the larvae dumping their stomachical contents on the carrot. This dumping is necessary for the pupal chamber's formation because it acts as a compactant, making the chamber solid and giving it an environment that is suitable for the insect's development. At the end of the third instar, the larvae suspended feeding, not requiring plant materials to complete their later stages of prepupa and pupa.

Pupa and imago stages: The pupae stayed in the same containers and under the same conditions in which they developed as larvae. Every 15 days, we checked their progress and looked for precocious adults. Great care was taken to ensure no harm came to the insects while handling them during this delicate stage

of metamorphosing into the adult state. Equally essential was to prevent any damage to the earthen pupal chamber itself. Once damaged, it could not be repaired and would interrupt the insect's development for a considerable time (Figure 1.17.9).

The adults were then placed in 10-L plastic containers carrying sterilized soil and left for 70 to 75 days in their pupal chambers so that their reproductive organs could develop. During this time, they were not given moisture or food. Some adults were mounted on entomological pins to confirm their taxonomic identification and to verify the progeny of parents and children.

After mass rearing, a group of 300 eggs were selected to examine the life cycle of *P. menetriesi* under controlled conditions. Previous experiences with mass rearing led to the use of 400 individuals to compare differences of mortality rates resulting from the use of rice or carrot to feed first-instar larvae. Another important aspect that was compared was differences in percentage of eclosion of eggs oviposited by field females versus those that were mass reared.

Figure 1.17.7. (A) Two technicians individualize first and second-instar larvae of *P. menetriesi*. (B) First (1) and second (2) instars of *P. menetriesi.*

Figure 1.17.8. Third-instar larvae of *P. menetriesi*. (A, left) Early third instar. (B) Prepupal stage.

Figure 1.17.9. From pupa to imago: (A) a third-instar larva had constructed its pupal chamber at the bottom of a 10-oz plastic container (the contents are shown inverted on to the work bench). Pupation takes about 5 weeks. (B) and (C) The fully developed insect ruptures its chamber, pushing the two halves apart, and begins scrambling out. (D) Imago rests beside its pupal molt. It remains underground for about 10 weeks before emerging above ground.

Results and Discussion

Adults captured by region: We collected a total of 9439 adults between October and November 2004, and March and April 2005. Figure 1.17.10 shows the geographic distribution of the captures. Because the largest number of captures was in Cauca, we opted to increase the number of light traps in this Department to more exhaustively collect adults and thus increase the existing breeding stock in the mass-rearing installations.

Monitoring the life cycle of Phyllophaga menetriesi: The study of the insect's life cycle began with 300 eggs, from which we obtained 226 first-instar larvae > 192 second instars > 179 third instars > 160 pupae > 138 adults. The eggs incubated for 10 to 16 days, with an average of 13 days. The first instars developed over 19 days; the second instars took 15 to 32 days, with an average of 27 days, and the third instars took 82 to 201 days, with an average of 175 days. (Table 1.17.1).

As reported by King, (King, 1996, Technical report No. 277 CATIE, p 50-61), we found that, after the third instar, the insects entered a stage of diapause that lasted about 30 days before they became pupae. The pupal stage averaged 34 days. The adults remained in their pupal chamber for about 73 days. Their flight period lasted 15 days. The adult stage therefore lasted 88 days. The entire development of *P. menetriesi* from egg to adult averaged 386 days (Figure 1.17.11).

Table 1.17.1. Life table of the crop pest *Phyllophaga menetriesi*, under mass-rearing conditions.

X	L_x	d_x	q_x	E_x	T_x	e_x
Egg	300	74	0.247	263.0	1045.0	3.97
First instar	226	34	0.150	209.0	782.0	3.74
Second instar	192	13	0.070	185.5	573.0	3.09
Third instar	179	19	0.106	169.5	387.5	2.29
Pupa	160	22	0.137	149.0	218.0	1.46
Adult	138	138	1.000	69.0	69.0	1.00

Where,

X is Age interval in units of time
L_x is Number of live individuals at the beginning of the interval x to x + 1
d_x is Number of individuals that died during the interval x to x + 1
q_x is Mortality rate during the interval x to x + 1
E_x is Average number of live individuals during the interval x to x + 1
T_x is Cumulative sum of L_x to obtain values expressed in number of individuals per unit of time
e_x is Average life expectancy of individuals at the beginning of interval x

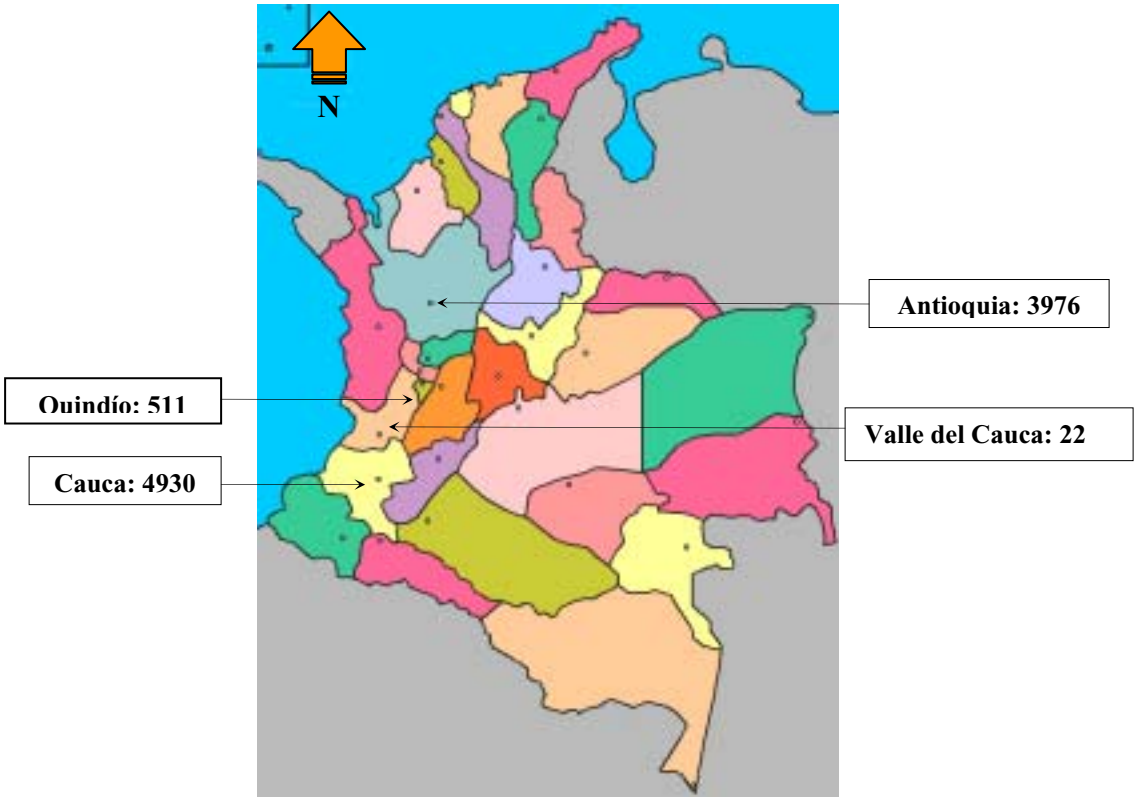

Figure 1.17.10. Colombia and its departments. Numbers indicate the quantity of adult *Phyllophaga menetriesi* captured per department.

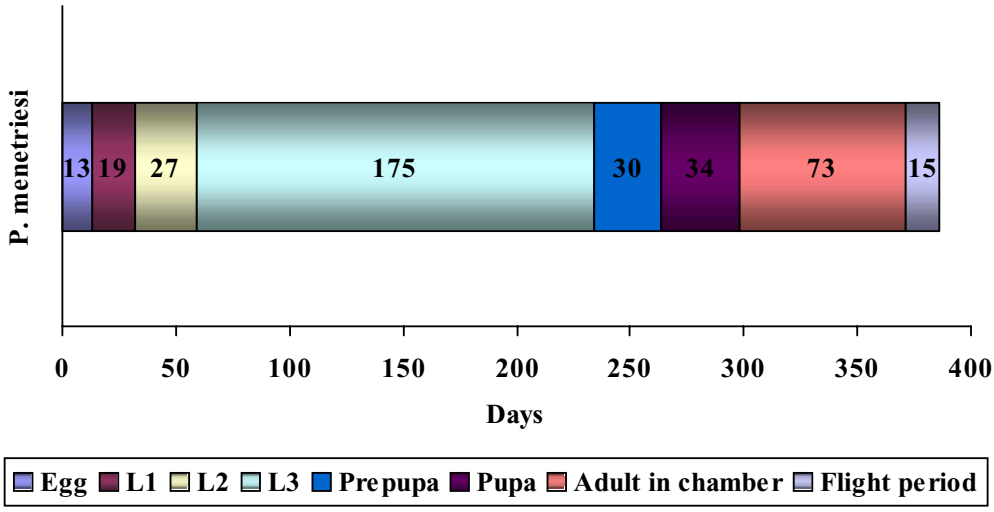

Figure 1.17.11. Average duration of the developmental stages of *Phyllophaga menetriesi*. The total life cycle lasts about 386 days. L1 = first-instar larva; L2 = second-instar larva; L3 = third-instar larva.

Comparing eggs from field females with those from mass-reared females: For this experiment, mass-reared and field individuals were used. Following the proposed methodology for mass rearing, the eggs were separated by origin and placed in 16-oz pots, each with 25 eggs. In all, we compared 2100 eggs distributed in 84 pots.

The percentage of eclosion of eggs obtained from laboratory females was 34.68%, whereas that of field females was 75.30% (Table 1.17.2). We also observed that a laboratory female would oviposit, on the average, 9 eggs per week, with two oviposition events at an interval of 7 days, meaning that she would oviposit 18 eggs during her adult life. In contrast, the field females oviposited, on the average, 2 eggs during a single oviposition event within 7 days.

Table 1.17.2. Oviposition data for *Phyllophaga menetriesi*. Adult females from the field were compared with those reared in the laboratory.

Origin	Eggs (no.)	Days of oviposition	Eclosion (%)
Mass-reared	9	14	34.68
Field	2	7	75.30

Mortality rates of first-instar larvae according to food type: To optimize mass rearing, a sample of 400 first-instar larvae were compared according to the different foods they were fed. Half were given pre-germinated rice and the other half, carrot pieces. The mortality rate from first to second instar was 15.5% with carrot—close to the 15% obtained with the mass-rearing methodology—and with rice, the rate was 45% (Table 1.17.3).

In the containers carrying rice, the roots had proliferated, which led to soil compaction and the consequent drowning of the larvae. In the containers carrying carrot, soil moisture increased only slightly and a fungus also appeared on the food, although it was not detrimental to the larvae's development. Results

therefore suggested that work should continue with carrot as it does not interfere significantly with the larvae's development and is easier to handle.

Table 1.17.3. Mortality rates of first instars of the crop pest *Phyllophaga menetriesi* according to the type of food they received.

Food	Individuals (starting number)	Dead individuals (no.)	Mortality (%)
Carrot	200	31	15.5
Rice	200	90	45

Observations

The laboratory larvae were smaller than the field samples because of being confined throughout their development in the laboratory. The adults were also smaller than their field counterparts. The capacity of the mass-reared insects to oviposit needs to be studied.

The maintenance of adults and larvae in containers with perforated covers that permit the exchange and circulation of air must be considered when selecting materials for use during mass-rearing procedures.

For oviposition, the soil must be rich in organic matter, as preferred by females, so to give them a suitable medium for carrying out this task. At the beginning of each instar, the larvae possess a white head, with the exuviae attached to the lower parts of the body. The exuviae quickly degrade in the soil to the point of being completely removed. Sometimes, however, part of the cephalic capsule can be found during molting.

Care must be taken during first instar with the amount of food placed in the containers because excess food may produce a large number of rootlets that compact the soil, leading to the larvae drowning. Nevertheless, soil moisture must be kept constant throughout the life cycle as all stages are susceptible to unfavorable substrate conditions. Care also needs to be taken with excess moisture, which will weaken the larvae, causing death, and in adults cause inadequate extension of the elytra and sagging of the abdomen.

Larvae of *P. menetriesi* constantly feed until they reach the point of becoming prepupae when, just before becoming immobilized, they expel, in one event, their stomachical contents, thus humidifying the substrate and decomposing the food, which becomes gelatinous. *Phyllophaga obsoleta* and other species studied in Mexico also demonstrate this behavior.

Activity 1.18. Evaluating the Impact of Biotechnology on Biodiversity: Effect of Transgenic Maize on Non-Target Soil Organisms

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Highlights:

- Standardized protocols developed for risk evaluations of GMOs on non-target soil organisms.
- No statistical differences were detected in abundance and diversity of soil organisms in conventional versus genetically modified cotton [Bollgard ® Bt Cry 1A ©] during the 2003-05 period in the Cauca Valley, Colombia.

- Evaluations of the Arthropod family Formicidae and the order Coleoptera during 2004-05 found no significant differences on conventional versus non-conventional cotton (Bt) in the Cauca Valley, Colombia.

- Basic knowledge on biological aspects of the class Collembola, an important soil Arthropod was enhanced. Information on Collembola distribution associated with cotton, maize and pastures in five Colombian Department is available. A references collection for future taxonomic studies has been developed and duplicate collections have been established at the Universidad Nacional en Bogotá and at Cornell University.

Rationale

Despite the controversy over the use of genetically-modified organisms (GMOs), the number of countries with GM commercial crops has grown from one in 1992 to 13 in 1999 (Shelton *et al.,* 2002, Ann. Rev. Entomol. 47: 845-81.). From 1996 to 2000, the global area under GMOs increased 42.5 million ha (James, 2004, www.ISAA.org, cited in May 27, 2004). The countries that reported the greatest increases were USA, Canada and Argentina, with 98% of the total area (Shelton *et al.*, 2002, Ann. Rev. Entomol. 47: 845-81). During 2003/04 67.7 million ha of GMOs were planted worldwide, of which soybean, maize, cotton and colza occupied 99% of the total area. Soybean plantings made up 61%, followed by maize and cotton with 23 and 11%, respectively (ICAC Recorder 2004, www.**icac**.org/**icac**/cotton_info/ cited in July 10, 2004).

For 2003/04, the area planted to GM cotton was 6.8 million ha, representing 21% of the total area planted to cotton worldwide. The countries that currently plant GM cotton are Argentina, Australia, China, Colombia, India, Indonesia, Mexico, South Africa and USA. The proportion of cotton that was GMO in 2003/04 was 77% for USA and 58% for China with an increase of 7% with respect to the previous harvest (ICAC Recorder 2004).

The Ministry of Agriculture and Rural Development, through the Colombian Agricultural Institute (ICA), designed a scheme to determine the viability of incorporating GMOs into the agricultural production system. ICA therefore passed Resolution 03492 in 1998 to establish and regulate the process of introduction, production, liberation and commercialization of GMOs. In two other provisions (Agreements 013/98 and 0002/02) ICA created the National Technical Council of Agricultural Biosecurity (CTN) to function in the assessment and support of GMO technology.

Since the establishment of those regulations, applications have been submitted for *Brachiaria*, carnations, cassava, coffee, cotton (resistance to lepidopterans), maize, rice, *Stylosanthes* and sugar cane. Of these, only four have been approved to date: (i) carnations for cut-flower production, (ii) cotton for commercial production, (iii) rice for small scale field trials, and (iv) maize for biosecurity tests (Díaz, 2003, Informe al Consejo Técnico Nacional de Bioseguridad (CTN), pp 8).

For the period 1991-2002, Colombia experienced a reduction of 83% in the area planted to cotton. The 2001/02 harvest only included 39,000 ha in the two cotton-growing regions of Tolima-Valle and Costa-Meta. One aspect that has greatly influenced the loss of area planted to cotton in Colombia is the high incidence of pests. The greatest losses are caused by the boll weevil (*Anthonomus grandis*, Coleoptera: Curculionidae) that affects 89% of the growing area in the departments of Córdoba, Cesar and Tolima, causing 15% loss of flower heads. *Heliothis virescens* (Lepidoptera: Noctuidae) affects 100% of the cotton planting area of Colombia, causing damage to 15-20% of the flower heads and bolls. Some 10% of the cultivated area is additionally affected by the foliovore "gusano rosado" (*Sacadodes pyralis*, Lepidoptera: Noctuidae) and whiteflies (Homoptera: Aleyrodidae).

Control of these pests is largely based on extensive use of agrochemicals and these represent 23% of the direct costs of the crop to the Colombian producer. In the Atlantic Coast, there was an average of 26 applications of pesticides per crop cycle, with 69.2% of those directed toward the control of lepidopterans. In the Cauca Valley, the number of applications has been reduced 73% to an average of 7 applications per crop cycle, with 57.1% directed towards the control of lepidopterans.

Given this scenario, ICA and the CTN implemented the first studies to determine the effect of the Bollgard® technology (Monsanto) on populations of arthropods and annelids in the cotton zone of Córdoba department in the Caribbean Region. The Bollgard® technology, generated by Monsanto, has the Cry1Ac insert whose target pests include the following lepidopterans: *Alabama argillacea* (Noctuidae, cotton leafworm), *Heliothis virescens* (Noctuidae, tobacco budworm), *Helicoverpa zea* (Noctuidae, corn earworm), *Pectinophora gossypiella* (Gelechiidae, pink bollworm), *Sacadodes pyralis* (Noctuidae, "el gusano rosado colombiano"), *Spodoptera frugiperda* (Noctuidae, fall armyworm), *Trichoplusia* sp. (Noctuidae, looper) and *Bucullatrix* sp. (Lyonetiidae, cotton leaf perforator)

Based on results obtained during the 2001-2002 growing cycle, ICA authorized the first commercial plantings of cotton with resistance to lepidopterans. The department of Córdoba was the first to commercially plant GM cotton with 6,187 ha planted in the second semester of 2003. During the first semester of 2004, 4,495 ha were planted in Tolima-Huila and 696 ha in the Cauca Valley.

Activity 1.18.1. Effect of transgenic cotton (Bollgard® Cry1A(c)) on Non-Target Soil Arthropods in the Cauca Valley of Colombia

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Materials and Methods

In collaboration with ICA's division of Agricultural Regulation and Protection, we initiated field studies for the first cycle of cotton at the ICA research station in Palmira, located at 03°31'N, 76°19'W, 975 m elevation, annual precipitation 1295 mm, mean temperature 24°C, relative humidity 76%, and corresponding to the Holdridge life zone of Dry Tropical Forest.

The evaluations were conducted within the methodology implemented by ICA to evaluate the effect of Bollgard® technology on arthropod populations in the cotton crop in the departments of Tolima, Huila and Valle del Cauca.

The experimental units were plots measuring 225 m^2 (15 m x 15 m) in a completely randomized block design. Each block had 6 plots for a total of 24 plots under evaluation. Plant material was (1) Bollgard® technology represented by the variety NuCont 33B that contains the Cry1A(c), and (2) the conventional technology represented by variety DP 5415 (Figure 1.18.1.1).

Figure 1.18.1.1. The experimental units in the Cauca Valley, Colombia

Sampling: Information was gathered from two types of samples: pitfall traps and berlese funnels. Pitfall traps were located between plants within the rows; eight were put out in each experimental plot (Figure 1.18.1.2). A total of 192 pitfall traps were deployed, and these were opened to sampling for a 24-hour period each week.

Figure 1.18.1.2. (A) Fixed component and removable component and (B) lid of the pitfall traps in the field.

Field samples were brought to the laboratory for their processing on the same day. Larger arthropods were picked out by hand. To recover the microarthropods, the samples were processed in a small funnel lined with a very fine mesh. The field sample was washed into the funnel with water. By capping the end of the funnel, the sample was floated, and the supernatant removed after discarding the larger debris. Then the remaining precipitate was floated again, this time in 35% salt solution and the supernatant removed. Both supernatant samples were then combined and stored in 70% ethyl alcohol until analysis and identification (Figure 1.18.1.3).

Figure 1.18.1.3. Cleaning and storage of samples in the laboratory.

In addition to the pitfall traps, a cup cutter was used to take soil samples every 2 weeks. The cup cutter had a diameter of 10 cm and the sample was taken to a depth of 10 cm in the row between plants (Figure 1.18.1.4). Four samples were taken per plot for a total of 96 samples per evaluation. Samples were placed in berlese funnels for 24 hours after which the samples of separated arthropods were stored in 70% ethyl alcohol until analysis. Because only 48 funnels were available, blocks 1 and 2 were done the first period, followed by blocks 3 and 4 which were maintained at 11^{o}C during the interim 24 hours. Arthropod samples were separated, sorted and processed as in the pitfall trap samples.

Figure 1.18.1.4. Field collection of samples for berlese extraction of arthropods using a "Lever Action Hole Cutter."

Analysis of information: The statistical model used for the analysis of the data was a completely randomized block design. With this design an ANOVA will be used to determine differences in abundance among treatments and determine the effect of their interactions. In addition, for the most abundant groups we will conduct an analysis of the area under the population curve (accumulated insect-

days) to determine differences among treatments during the trial. We will also compare the diversity and abundance among treatments using various indices of taxonomic diversity, dominance and equity.

Results and Discussion

Arthropod Taxonomic Composition: During both cycles of evaluation (2003-2004), 1,167,928 specimens were captured representing 22 orders and 10 taxonomic classes (Tables 1.18.1.1, 1.18.1.2). The most abundant class was Aracnida with 49.8% of total individuals captured (Table 1.18.1.1). Of all individuals captured, 51.9% of those were associated with transgenic cotton and 48.1% with conventional cotton (Table 1.18.1.2, Figure 1.18.1.5). Of the 22 identified orders, the most abundant were Acari and Poduromorpha with 50.3 and 26.7%, respectively (Table 1.18.1.2). Only Chilopoda, Diptera, Neuroptera, Poduromorpha, Symphyla and Symphypleona exhibited a significant difference in abundance between treatments, with Poduromorpha and Symphypleona more abundant in modified cotton. Of taxa identified in the two cycles, only Araneae, Neuroptera, Orthoptera, Strepsiptera and Thysanura did not exhibit a significant difference in abundance between cycles (Table 1.18.1.3).

Table 1.18.1.1. Number of individuals and composition of arthropod classes caught in Pitfall traps and Berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Class	DP5415	%	Nucotn	%	Total	%
Aracnida	295,793	52.7	286,318	47.2	582,111	49.8
Chilopoda	792	0.1	686	0.1	1,478	0.1
Collembola	162,459	28.9	223,465	36.8	385,924	33.0
Diplopoda	382	0.1	422	0.1	804	0.1
Diplura	907	0.2	821	0.1	1,728	0.1
Insecta	95,785	17.1	93,659	15.4	189,444	16.2
Malocostraca	213	0.0	247	0.0	460	0.0
Pauropoda	429	0.1	389	0.1	818	0.1
Protura	14	0.0	12	0.0	26	0.0
Symphyla	4,588	0.8	547	0.1	5,135	0.4
Sum	561,362	**100**	**606,566**	**100**	**1,167,928**	**100**

Pitfall traps: During both cycles (2003-2004), 574,814 individuals were captured, belonging to 19 different taxonomic orders and 3 taxonomic classes (Table 1.18.1.4). Of all individuals captured, 55.7% of those were associated with transgenic cotton and 44.3% with conventional cotton. Of the 19 identified orders, the most abundant were Poduromorpha, Hymenoptera and Acari with 51.2, 23.6 and 21.5%, respectively (Table 1.18.1.4). Abundance, in terms of individuals per order, was 1.3 times greater in NuCotn 33B; of all orders, only 9 were more abundant in DP5415 (Table 1.18.1.4). Only the orders Chilopoda, Isopoda, Neuroptera, Poduromorpha, Psocoptera and Symphypleona exhibited a significant difference in abundance between treatments, were more abundant in NuCotn 33B, excepting to Psocoptera (Table 1.18.1.4).

Given their overall abundance, the class Collembola and the order Hymenoptera were examined in more taxonomic detail. For the class Collembola, seven families were identified belonging to three orders (Table 1.18.1.5). Over both cycles of evaluation (2003 and 2004), Poduromorpha and Simphypleona exhibited statistical differences among treatments, being more abundant in modified cotton (NuCotn 33B) (Tables 1.18.1.3, 1.18.1.4). The order Hymenoptera represented 23.6% of total individuals captured, with 99.7% representing the family Formicidae where 52.1% were captured in DP5415 (Table 1.18.1.4).

Table 1.18.1.2. Number of individuals and composition of arthropod taxa caught in Bt cotton (NuCotn 33B) and conventional cotton (DP 5415), during 2003 and 2004 in the Cauca Valley, Colombia.

Orders	DP 5415	%	NuCotn 33B	%	Total	%
Acari	295,252	52.6	285,708	47.1	580,960	49.7
Aranae	541	0.1	610	0.1	1,151	0.1
Blattaria	57	0.0	43	0.0	100	0.0
Chilopoda[1]	792	0.1	686	0.1	1,478	0.1
Coleoptera	2,337	0.4	2,124	0.4	4,461	0.4
Dermaptera	26	0.0	30	0.0	56	0.0
Diplopoda[1]	382	0.1	422	0.1	804	0.1
Diplura[1]	907	0.2	821	0.1	1,728	0.1
Diptera	1,091	0.2	881	0.1	1,972	0.2
Entomobryomorpha	40,849	7.3	35,590	5.9	76,439	6.5
Hemiptera	144	0.0	119	0.0	263	0.0
Homoptera	4,165	0.7	3,864	0.6	8,029	0.7
Hymenoptera	87,249	15.5	82,754	13.6	170,003	14.6
Isopoda	213	0.0	247	0.0	460	0.0
Lepidoptera	260	0.0	266	0.0	526	0.0
Mantodea	3	0.0	3	0.0	6	0.0
Neelipleona	238	0.0	173	0.0	411	0.0
Neuroptera	9	0.0	1	0.0	10	0.0
Orthoptera	69	0.0	57	0.0	126	0.0
Pauropoda[1]	429	0.1	389	0.1	818	0.1
Poduromorpha	121,254	21.6	187,155	30.9	308,409	26.4
Protura[1]	14	0.0	12	0.0	26	0.0
Psocoptera	82	0.0	57	0.0	139	0.0
Strepsiptera	1	0.0	0	0.0	1	0.0
Symphyla[1]	4,588	0.8	3,701	0.6	8,289	0.7
Symphypleona	118	0.0	547	0.1	665	0.1
Thysanoptera	134	0.0	142	0.0	276	0.0
Thysanura	1	0.0	1	0.0	2	0.0
Unidentified	157	0.0	163	0.0	320	0.0
Sum	**561,362**	**100**	**606,566**	**100**	**1,167,928**	**100**

[1]Taxonomic Class (Including for analysis)

In the analysis of the area under the curve for Poduromorpha, Hymenoptera and Acarina, they presented the same behavior during the two evaluation cycles (Figures 1.18.1.5, 1.18.1.6, 1.18.1.7). Only Poduromorpha presented a change during the 2004, where more area under the curve was accumulated in NuCotn 33B.

Berlese funnels: Over both cycles of evaluation (2003-2004), 593,114 individuals were captured, representing 22 orders and 8 taxonomic classes of arthropods (Table 1.18.1.4). The 51.7% of individuals captured were associated with conventional cotton and 48.3% with transgenic cotton. The most abundant order was Acarina, with 77.2% of total captures and 1.1 times more abundant in DP 5415. Only the orders Acarina, Chilopoda, Coleoptera, Diptera, Entomobryomorpha and Symphyla exhibited a significant difference in abundance between treatments, were more abundant in DP5415 (Table 1.18.1.4).

Table 1.18.1.3. Abundance of arthropods (mean ± S.E. number of individuals caught per evaluation date) associated with cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Taxa	Between treatments		Between samples		Between cycles	
	DP 5415	NuCotn	Pitfall	Berlese	2003	2004
Acarina	50.4±149.9a	67.9±162.9a	18.4±52.7b	264.8±280.3a	54.1±104.4b	82.7±210.4a
Aranae	0.1±0.7a	0.1±2.4a	0.2±2.0a	0.1±0.3b	0.1±0.3a	0.2±2.5a
Blattaria	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.2a	0.0±0.2a	0.0±0.lb
Chilopoda[1]	0.1±0.7a	0.2±0.8b	0.0±0.1b	0.8±1.6a	0.1±0.4b	0.3±1.0a
Coleoptera	0.4±1.5a	0.5±1.7a	0.2±0.5b	1.9±3.3a	0.7±2.1a	0.4±1.2b
Dermaptera	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.0b	0.0±0.1a
Diplopoda[1]	0.1±0.4a	0.1±0.5a	0.0±0.2b	0.4±1.0a	0.1±0.4b	0.1±0.6a
Diplura[1]	0.2±0.7a	0.2±0.9a	0.0±0.0b	1.0±1.7a	0.1±0.5b	0.3±1.1a
Diptera	0.2±1.0a	0.2±1.6b	0.0±0.6b	0.7±1.6a	0.3±1.3a	0.1±1.5b
Entomobryomorpha	7.0±27.7a	8.4±26.7a	1.6±6.8b	38.0±55.3a	11.0±34.1a	7.2±24.5b
Hemiptera	0.0±0.2a	0.0±0.2a	0.0±0.2a	0.0±0.2a	0.0±0.3a	0.0±0.1b
Homoptera	0.7±3.4a	0.9±2.8a	0.9±3.0a	1.2±4.6a	0.9±4.0b	1.0±2.7a
Hymenoptera	14.9±55.5a	19.6±64.0a	20.2±60.1a	20.0±78.0b	39.2±86.6a	1.9±14.7b
Isopoda	0.0±0.4a	0.1±0.5a	0.0±0.3b	0.2±0.9a	0.1±0.6a	0.0±0.1b
Lepidoptera	0.0±0.3a	0.1±0.4a	0.0±0.0b	0.2±0.6a	0.1±0.4a	0.0±0.2b
Mantodea	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0a
Neelipleona	0.0±0.8a	0.0±0.7a	0.0±0.0b	0.2±1.8a	0.1±1.2a	0.0±0.1b
Neuroptera	0.0±0.0a	0.0±0.0b	0.0±0.0a	0.0±0.0a	0.0±0.0a	0.0±0.0a
Orthoptera	0.0±0.1a	0.0±0.1a	0.0±0.1a	0.0±0.1b	0.0±0.1a	0.0±0.1a
Pauropoda[1]	0.1±0.6a	0.1±0.6a	0.0±0.0b	0.5±1.3a	0.1±0.7a	0.1±0.5b
Poduromorpha	20.7±201.7b	44.3±316.2a	43.8±312.8a	8.0±21.0b	56.6±346.5a	17.3±193.0b
Protura[1]	0.0±0.1a	0.0±0.1a	0.0±0.0b	0.0±0.1a	0.0±0.0b	0.0±0.1a
Psocoptera	0.0±0.2a	0.0±0.1a	0.0±0.1b	0.0±0.3a	0.0±0.1b	0.0±0.2a
Strepsiptera	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0a	0.0±0.0a	0.0±0.0a
Symphyla[1]	0.8±3.3a	0.9±3.3b	0.0±0.1b	4.8±6.6a	1.2±3.9a	0.8±3.2b
Symphypleona	0.0±0.2b	0.1±2.4a	0.1±1.9a	0.0±0.2b	0.2±2.4a	0.0±0.1b
Thysanoptera	0.0±0.2a	0.0±0.5a	0.0±0.2b	0.1±0.8a	0.0±0.4b	0.0±0.4a
Thysanura	0.0±0.0a	0.0±0.0a	0.0±0.0b	0.0±0.0a	0.0±0.0a	0.0±0.0a
Unidentified	0.0±0.3a	0.0±0.3a	0.0±0.0b	0.2±0.7a	0.1±0.5a	0.0±0.0b

[1]Taxonomic Class (Including for analysis)

For each row, means followed by different letters are statistically different at P<0.05 (Tukey-Kramer test for multiple comparisons)

The same as for the pitfall traps, the class Collembola and the order Hymenoptera were examined in more taxonomic detail. For the class Collembola, four orders were identified only genus Cyphoderus was exclusive for this sampling type (Table 1.18.1.5). Over both cycles of evaluation (2003 and 2004), only Entomobryomorpha exhibited statistical differences among treatments, being more abundant in conventional cotton (DP 5415) (Table 1.18.1.4). The order Hymenoptera represented 5.8% of total individuals captured, with 47.8% representing the family Formicidae where 54.2% were captured in DP5415 (Table 1.18.1.4).

During two evaluation cycles, the area under the curve showed significant differences in favor of the conventional cotton for Acari and Entomobryomorpha. Only Acari exhibited changes of a cycle to other, accumulating more area during the 2004 in modified cotton.

Arthropod Taxonomic Diversity: The species richness and Shannon indices were not significantly different between the treatments NuCotn 33B and DP 5415. The Simpson index showed dominance for

one species, presenting values of 0.65 and 0.66 for DP5415 and NuCotn 33B, respectively (Table 1.18.1.6).

Table 1.18.1.4. Number of individuals and composition of arthropod orders caught in Pitfall traps and Berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

	Pitfall traps				Berlese funnels			
	DP 5415	**NuCotn**	**Total**	**%**	**DP 5415**	**NuCotn**	**Total**	**%**
Acarina	59,668a	63,693a	123,361	21.5	235,584a	222,015b	457,599	77.2
Aranae	468a	555a	1,023	0.2	73a	55a	128	0.0
Blattaria	44a	36a	80	0.0	13a	7a	20	0.0
Chilopoda[1]	8b	20a	28	0.0	784	666b	1,450	0.2
Coleoptera	615a	641a	1,256	0.2	1,722a	1,483b	3,205	0.5
Dermaptera	19a	24a	43	0.0	7a	6a	13	0.0
Diplopoda[1]	61a	72a	133	0.0	321a	350a	671	0.1
Diplura[1]	0	0	0	0.0	907a	821a	1,728	0.3
Diptera	343a	335a	678	0.1	748a	546b	1,294	0.2
Entomobryomorpha	4,946a	5,841a	10,787	1.9	35,903a	29,749b	65,652	11.1
Hemiptera	104a	94a	198	0.0	40a	25a	65	0.0
Homoptera	3,025a	2,903a	5,928	1.0	1,140a	961a	2,101	0.4
Hymenoptera	70,603a	64,877a	135,480	23.6	16,646a	17,877a	34,523	5.8
Isopoda	48b	113a	161	0.0	165a	134a	299	0.1
Lepidoptera	55a	49a	104	0.0	205a	217a	422	0.1
Mantodea	3a	2	5	0.0	0a	1a	1	0.0
Neelipleona	0	0	0	0.0	238a	173a	411	0.1
Neuroptera	8a	1b	9	0.0	1a	0a	1	0.0
Orthoptera	65a	50a	115	0.0	4a	7a	11	0.0
Pauropoda[1]	0	0	0	0.0	429a	389a	818	0.1
Poduromorpha	114,462b	180,067a	294,529	51.2	6,792a	7,088a	13,880	2.3
Protura[1]	0	0	0	0.0	14a	12a	26	0.0
Psocoptera	42a	22b	64	0.0	40a	35a	75	0.0
Strepsiptera	0	0	0	0.0	1a	0a	1	0.0
Symphyla[1]	30a	27a	57	0.0	4,558a	3,674b	8,232	1.4
Symphypleona	89b	525a	614	0.1	29a	22a	51	0.0
Thysanoptera	87a	70a	157	0.0	47a	72a	119	0.0
Thysanura	0	0	0	0.0	1a	1a	2	0.0
Unidentified	2a	2a	4	0.0	155a	161a	316	0.1
Sum	**254,795**	**320,019**	**574,814**	**100**	**306,567**	**286,547**	**593,114**	**100**

[1]Taxonomic Class

For each row, means followed by different letters are statistically different at P<0.05 (Tukey-Kramer test for multiple comparisons)

Finally, in the comparison of diversity between the two cycles of evaluation (2003 and 2004), no significant differences were observed in richness based on the taxa identified. The Simpson index did not differ between the two cycles of evaluation. The value of the equality index showed a tendency for greater diversity in the surveys conducted in 2003 (Table 1.18.1.6).

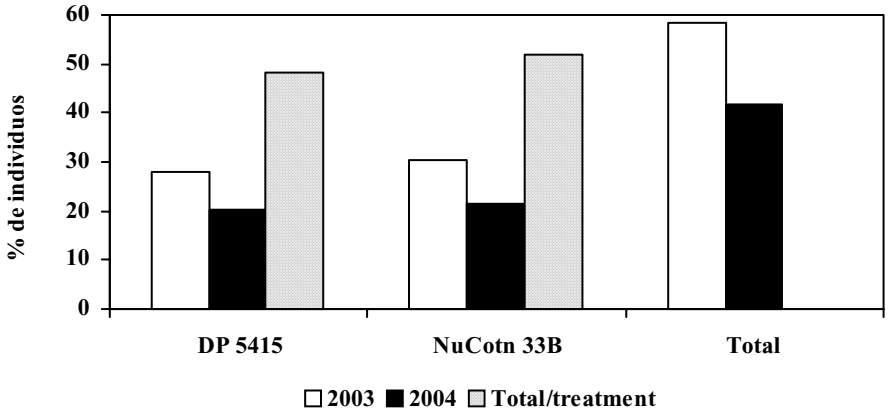

Figure 1.18.1.5. Total abundance of arthropods captured by treatment, during 2003-2004 in the Cauca Valley, Colombia.

Table 1.18.1.5. Collembola families collected from pitfall traps and berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Order	Family	Genus
	Hypogasturidae	*Ceratophysella*
Poduromorpha	Brachystomellidae	*Brachystomella**
	Neanuridae	*Arlesia*
	Cyphoderidae	*Cyphoderus***
	Entomobryidae	*Seira, Lepidocyrtus*
Entomobryomorpha		*Isotoma, Proisotoma,*
	Isotomidae	*Folsomides*
	Paronellidae	*Paronella, Salina*
Symphypleona	Dicyrtomidae	*Calvatomina*
Neelipleona		

*Only on the soil surface (pitfall traps)
**Only in soil (soil cores)

Table 1.18.1.6. Indices of arthropod taxonomic (ordinal level), caught in Pitfall traps and Berlese funnels in cotton, during 2003 and 2004 in the Cauca Valley, Colombia.

Index	Between treatments		Between cycles	
	NuCotn 33B	DP 5415	2003	2004
Species richness (S)	29 a	28 a	28 a	28 a
Equity	0.40 a	0.40 a	0.42 a	0.28 a
Shannon index	1.33 a	1.32 a	1.40 a	0.96 a
Simpson index	0.65 a	0.66a	0.71 a	0.44 a

For each row, means followed by different letters are statistically different at P<0.05 (Tukey-Kramer test for multiple comparisons)

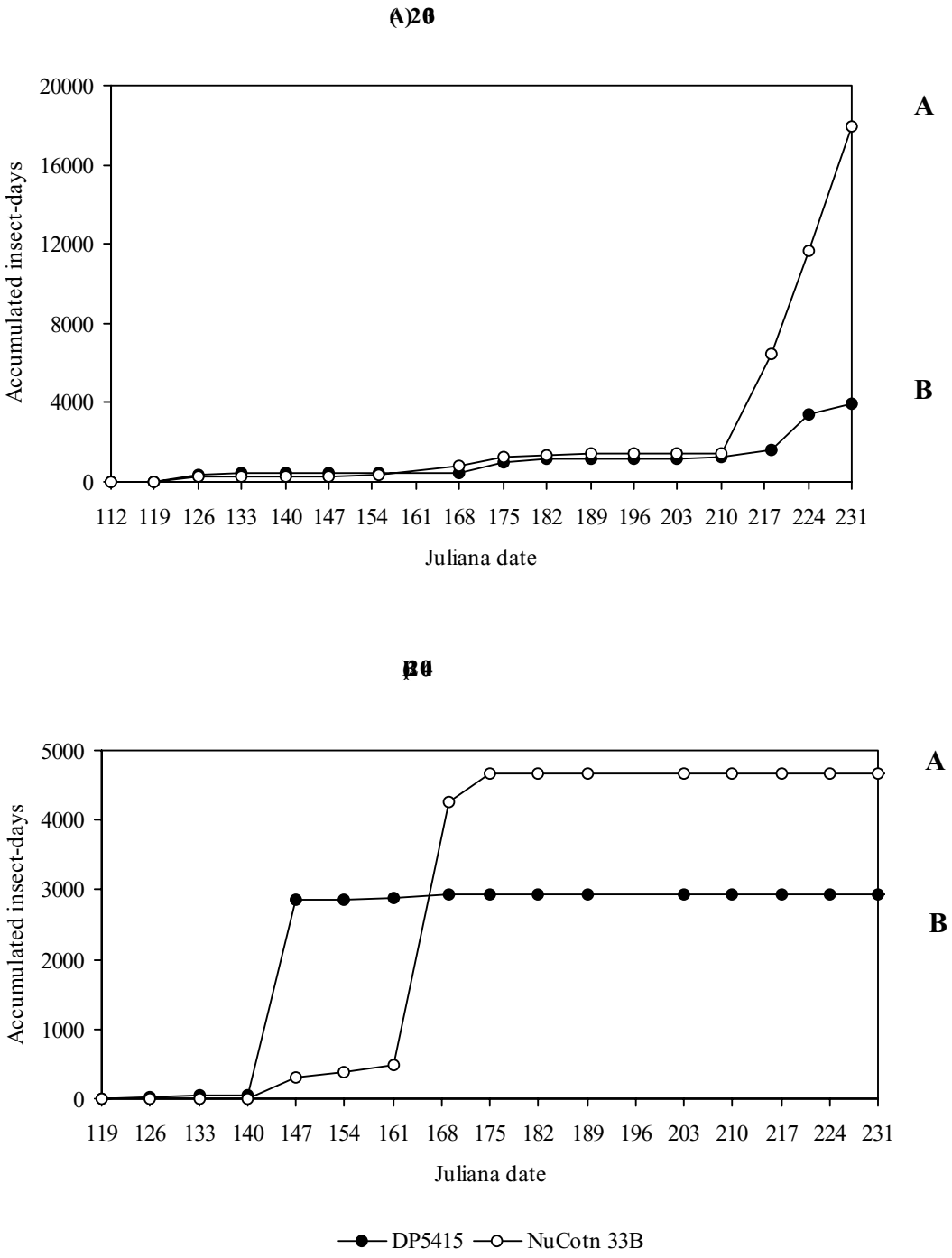

Figure 1.18.1.6 Area under the abundance curve for Poduromorpha caught in pitfall traps in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

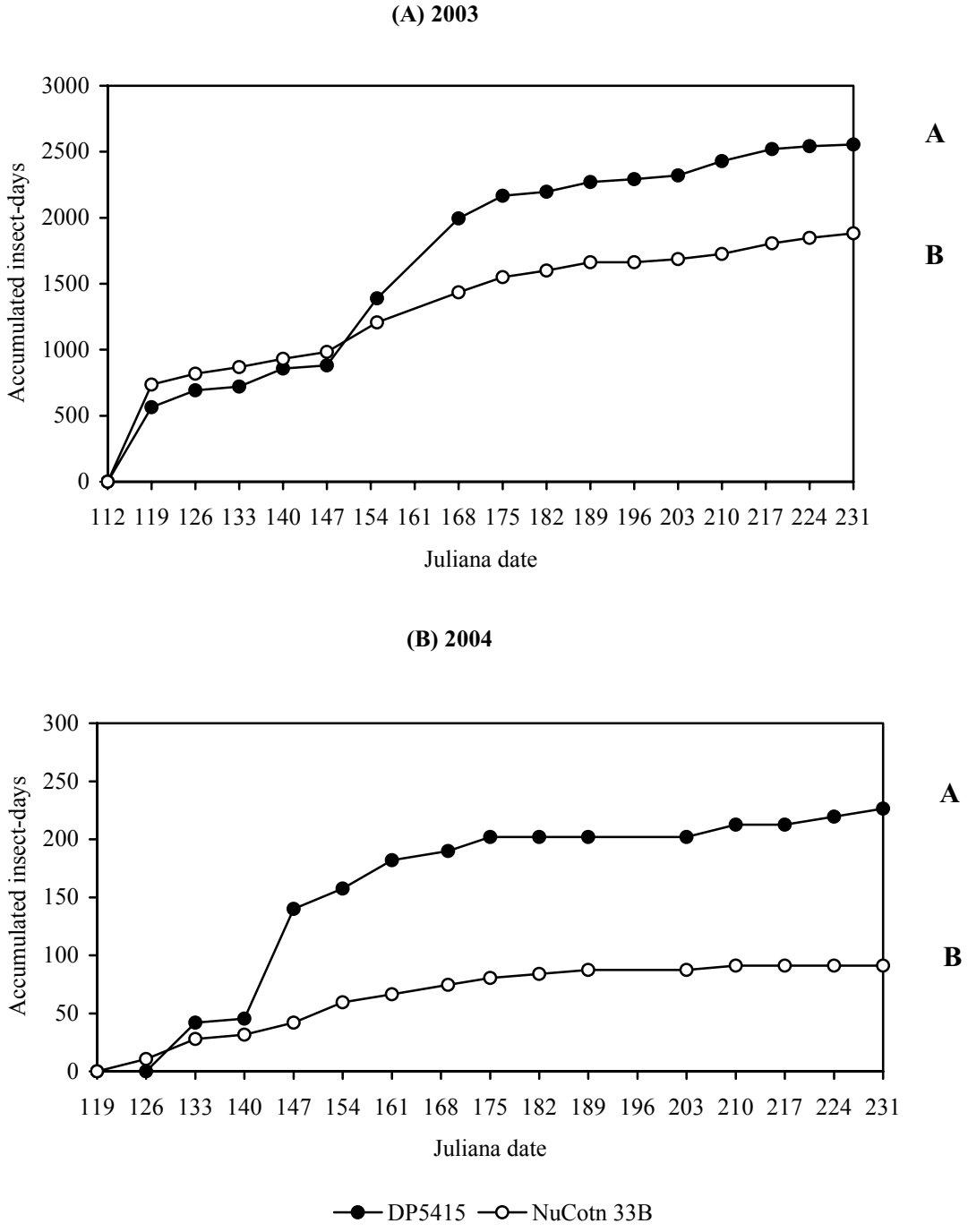

Figure 1.18.1.7. Area under the abundance curve for Hymenoptera caught in pitfall traps in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

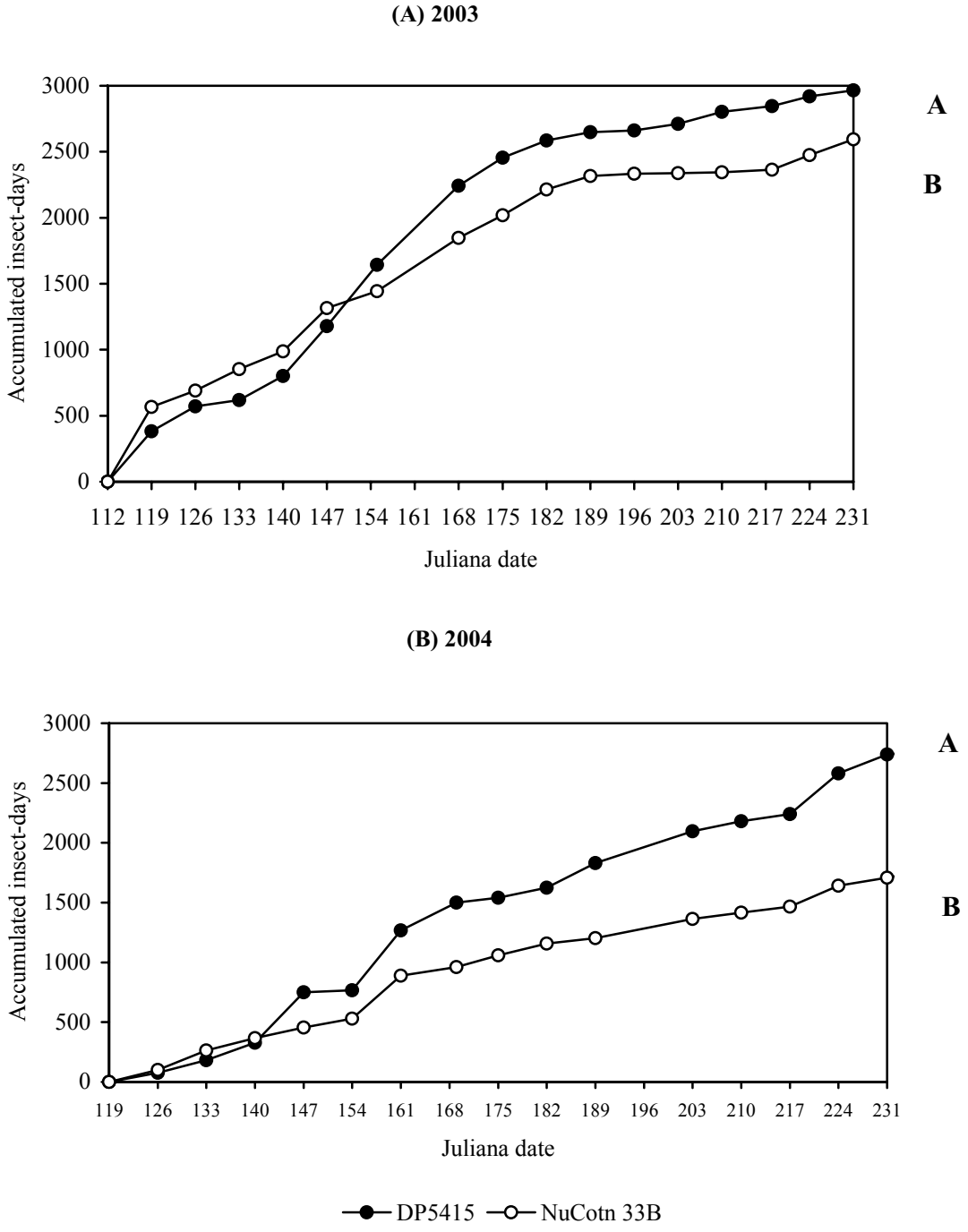

Figure 1.18.1.8. Area under the abundance curve for Acarina caught in pitfall traps in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

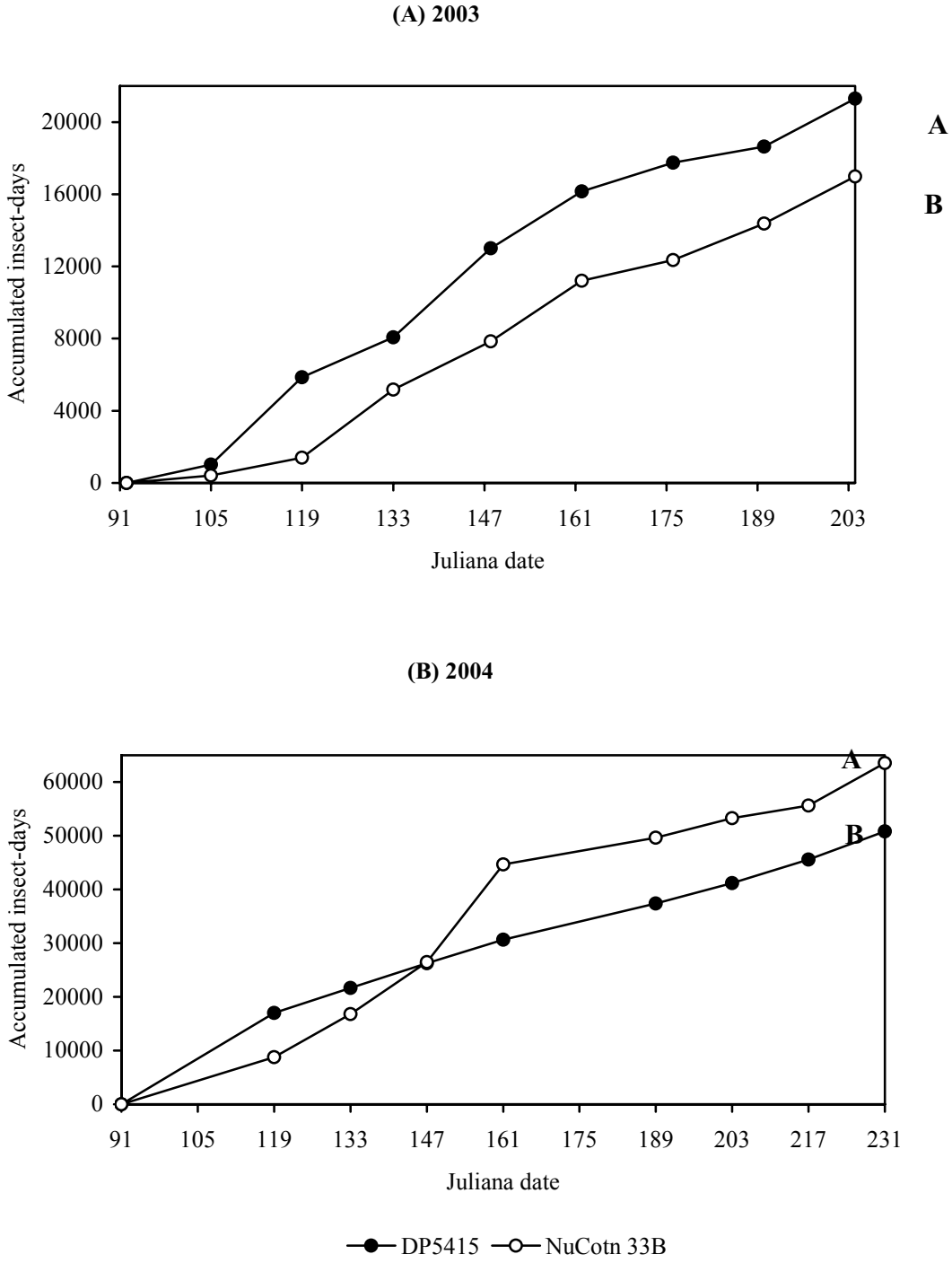

Figure 1.18.1.9. Area under the abundance curve for Acari caught in Berlese funnels in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

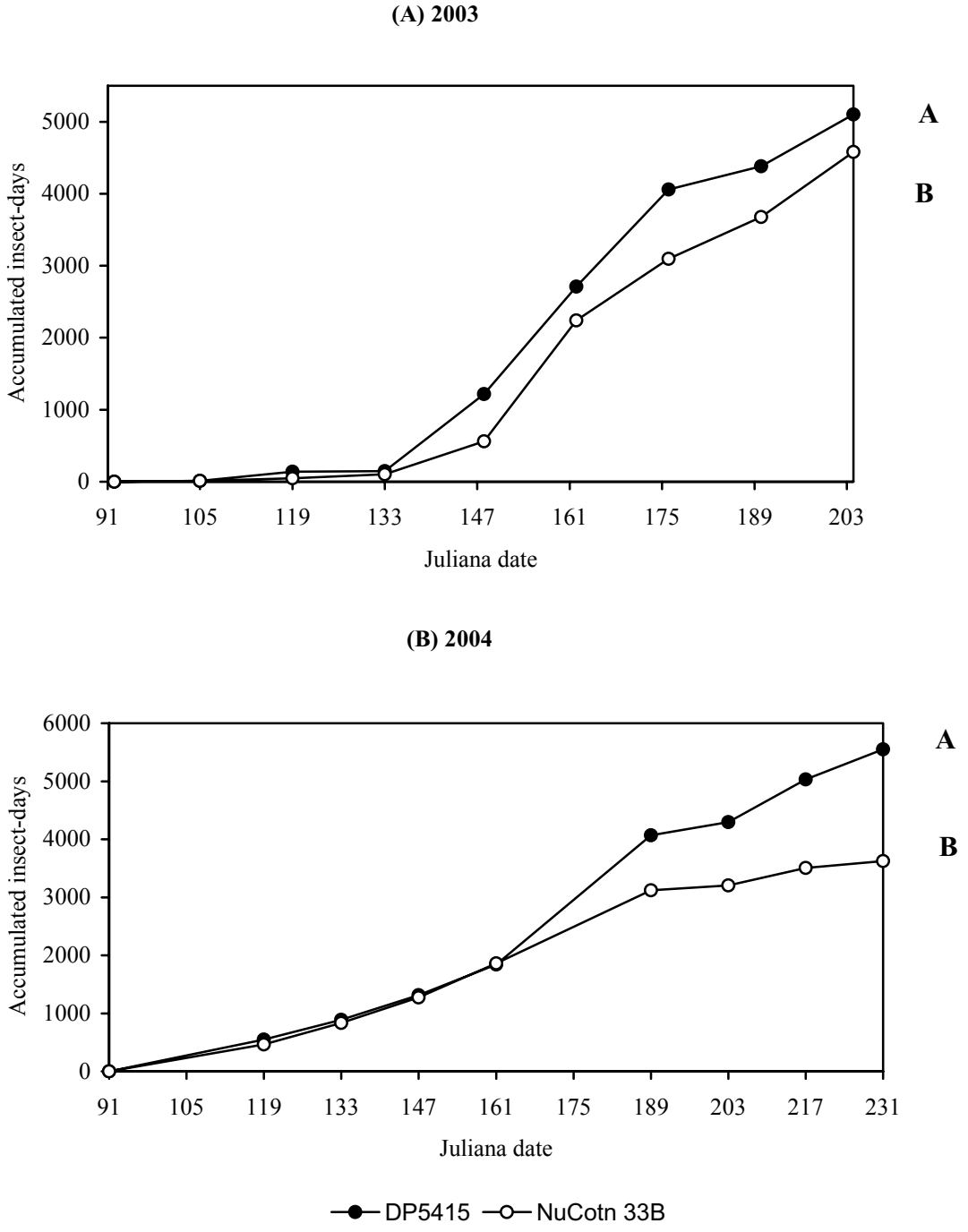

Figure 1.18.1.10. Area under the abundance curve for Entomobryomorpha caught inberlese funnels in cotton, during (A) 2003 and (B) 2004 in the Cauca Valley, Colombia.

Conclusions:

- These studies have identified a high abundance and diversity of soil-active and surface-active fauna associated with the cotton crop under the conditions of the Cauca Valley, Colombia.
- Pitfall traps are an appropriate method for measuring the abundance of surface-active arthropods and comparing their activity and diversity across treatments.
- Extracting soil cores with berlese funnels is an adequate method for measuring the abundance of soil-active arthropods and comparing their activity and diversity across treatments.
- Of the 28 taxa identified during these first two evaluation cycles in pitfall traps and Berlese funnels, only 6 exhibited statistical differences between treatments.
- Five of the identified taxa did not exhibit a significant difference in abundance between cycles (2003-2004) [Figures 1.18.1.6-10]
- Poduromorpha and Acarina were the most abundant groups in pitfall traps and Berlese funnels, respectively.
- There were no differences between treatments or evaluation cycle for any of the diversity indices evaluated.
- The abundance differences observed between treatments in the first two cycles of cotton should be studied in more detail to define how GMOs affect those differences. The protocols established in the two first cycle will therefore be implemented in three two additional cycles to better describe abundance effects over time, and to gather information to compare differences in species composition of key groups such as the springtails and ants.
- Although abundance and diversity differences may exist in response to GMO technology, it is important to determine whether the magnitude of those differences is ecologically relevant, i.e. have an effect on ecological function or overall soil health.

Activity 1.18.2. Evaluation of the ant community (Hymenoptera: Formicidae) in conventional (DP5415) and Bt (NuCotn 33B) cotton in the Cauca Valley, Colombia.

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Rationale

Among the diverse technologies that have been developed with genetically modified crops is theexpression of insecticidal proteins from the bacteria *Bacillus thurigiensis* (Bt), whose target is principally larval lepidopterans. Although it has been argued that the technology is specific to this group of insects, the environmental benefits and risks have been widely argued (Brill, 1985, Science 227: 381-384 ; Colwell *et al*., 1985, Science 229: 111-112 ; Harlander,1990, Cereal Foods World 35: 1106-1109; Boulter, 1993, Phytochemistry 34: 1453-1466;Nottingham, 1998, Eat your genes, Choice Books, Marrickville, New South Wales; Porter, 1999,The good news about GM foods. Intellectual Capital 10/21/99 [On line]; Raybould *et al.,* 1999, New Phythology 141: 265-275; Barton & Dracup, 2000, Agronomy Journal 92: 797-803; NAS 2002 Environmental effects of transgenic plants: The scope and adequacy of regulation.[On line]). Since the plant contains Bt, it can be ingested by nontarget arthropods or the residues and exudates of the plant can be liberated in the soil where they can come into direct contact with nontargets (Shelton *et al*., 2002, Annual Review Entomology 47: 845-881) and generate a potential toxicity (Conner *et al*., 2003, The Plant Journal 33: 19-46). On the other hand, the indirect impact on natural enemies has also been considered given the multrophic relations in which enemies are immersed when they consume prey, parasitize hosts or consume their excretions (Dively & Rose, 2002,

1st. Int'l Symposium on Biological Control of Arthropods : 265-274: Dutton *et al*., 2003, Biocontrol 48: 661-636).

The diverse studies that have been conducted to determine the possible effects of Bt-modified crops on arthropods have focused primarily on springtails, beetles (Carabidae) and spiders. Very few have taken into account the ant community.(Dively & Rose 2002, 1st. Int'l Symposium on Biological Control of Arthropods, pp 265-274; Brooks *et al*., 2003, Philosophical Transactions of Royal Society London 358:1847–1862; Haughnton *et al*., 2003, Philosophical Transactions of Royal Society London 358: 1863–1877).

Ants are one of the most conspicous and versatile groups in agroecosystems, considering that they are numerically abundant and fulfill a great number of ecological functions, such as when they (a) associate with other groups of organisms, (b) take sugar substances from homopterans and extrafloral nectaries, (c) consume vegetative material and weed seeds, and (d) prey on other groups of arthropods (Way & Khoo 1992, Annual Rev. of Entomology 37: 479-503; Philpott *et al*., 2004, Oecologia 140: 140-149; Armbrecht *et al*., 2005, Conservartion Biology 19: 897-907).

On the other hand, ants are important components in the transformation of soil when they modify the physical and chemical conditions by incorporating organic matter and nutrients (Folgarait, 1998, Biodiversity and Conservation 7: 1221-1244). Studies that have been conducted to date on the behavior of the ant community in Bt-modified crops have not demonstrated any observable effect on this group in particular (Candolfi *et al*., 2004, Biocontrol Science and Technology 14: 129-170). One possible limitation of these studies is the time factor and it would be highly desirable to conduct longer term studies to establish whether there are effects over time. Such studies with ants have only been conducted in Bt-modified maize (Candolfi *et al*., 2004, Biocontrol Science and Technology 14: 129-170) and little emphasis has been given to other crops as evidenced by the absence of information on this group and its behavior in genetically modified crops.

Materials and Methods

This work was based on specimens collected during two years of evaluation (2003 and 2004), in the ICA research station in Palmira, located at 03°31'N, 76°19'W, 975 m elevation, annual precipitation 1295 mm, mean temperature 24°C, relative humidity 76%, and corresponding to the Holdridge life zone of Dry Tropical Forest. Specimens were collected as part of the activities of the project **"**Evaluating the Impact of Biotechnology on Biodiversity: Effect of Transgenic Maize on Non-Target Soil Organisms" where two treatments of conventional cotton (DP 5415) a Bt-transgenic Bollgard® cotton (NuCotn 33B) were evaluated (Activity 1.18.1).

Ants ere separated from the original samples of arthropods that had been collected from cotton during 2003 and 2004 and stored in 70% ethyl alcohol. This was performed by examining the samples under a stereoscope, separating all ants, and returning the remaining arthropods to their original specimen vial.

Analysis of information: To determine whether there were treatment differences in the abundance, richness and composition of the ant community, an ANOVA was performed with a randomized block design followed by the Tukey-Kramer GLM multiple comparison test at $P>0.05$. All analyses were done with the software package SAS 8.1 for Windows (SAS Institute 2000). In addition, estimates and predictions of species richness were calculated on the richness of ant species with the nonparametric Chao 2 using the specially designed software of Colwell (Colwell, 2005, Statistical Estimation of Species Richness and Shared Species from Samples. http://viceroy.eeb.uconn.edu/estimates [on line]). Data on richness in each site were randomized 100 times in order to minimize the sampling error and the

heterogeneity among sampling units (Colwell & Coddington, 1994, Philosophical Transactions of Royal Society London 345: 101-118).

Results and Discussion

Taxonomic composition: Over the two cycles of evaluation a total of 151,528 ants were captured, 94.8% corresponding to to captures in 2003. As a result of evaluations in the first cycle (2003), during 2004 the family Formicidae was included as a group of interest and we managed to identify 18 genera grouped in five subfamilies (Table 1.18.2.1). In 2004 the subfamily Myrmycinae was the most represented with 95.0% of the total capture during that period. In this subfamily the species *Wasmannia auropunctata* was the most abundant with 74.4% (Table 1.18.2.1).

Pitfall traps: For ants captured in pitfall traps, in 2004 we identified 15 genera distributed in five subfamilies (Table 1.18.2.2). Among these subfamilies the species *Wasmannia auropunctata* was the dominant species in the surveys with 93.3% of the total ants captured. This results could be explained by the extremely agressive interspecific behavior of this species influencing the low number of individuals in the other populations.

Atta cephalotes was second most abundant species, observed forraging on cotton plants and indicating that this species is feeding to some degree on Bt-modified cotton. The data show no impact on its populations due to an affect of the the Cry toxin. Ants from the genus *Solenopsis* exhibited statistically significant difference between treatments, being more abundant in Bt-modified cotton. This group consists of a large number of species that are generally omnivorous and they did not evidence a detrimental effect on their abundance as a result of the Bt-modified cotton (Table 1.18.2.2).

Berlese funnels: In the family Formicidae, five subfamilies and 13 genera were identified of which seven were more abundant in conventional cotton (Table 1.18.2.3). In addition, the most important groups in terms of abundance were the omnivorous ants of the genera *Wasmannia* and *Solenopsis*.

Taxonomic Diversity: Over the two consecutive cropping cycles, 845 samples of ants were taken, 446 in modified cotton (NuCotn 33B) and 399 in conventional cotton (DP 5415) (Figure 1.18.2.1). The captures represent five subfamilies, 20 genera and 29 different species. Of the 29 species identified, 24 were common to both modified and conventional cotton, while three were exclusive to modified cotton and one to conventional cotton.

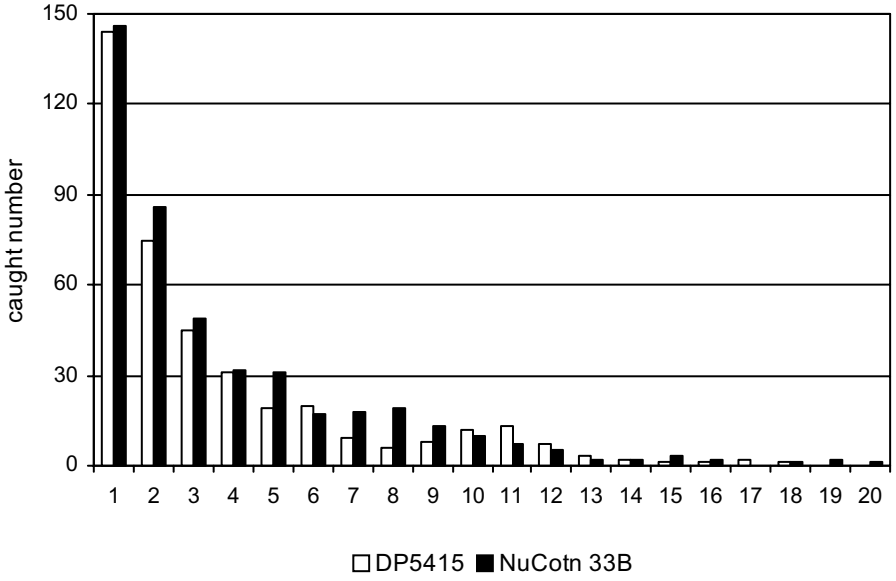

Figure 1.18.2.1. Distribution of the ant genera found in Bt-modified and conventional cotton in experimental plots at ICA, Palmira. 1 = *Wasmannia*, 2 = *Solenopsis*, 3 = *Hypoponera*, 4 = *Monomorium*, 5 = *Ectatomma*, 6 = *Rogeria*, 7 = *Atta*, 8 = *Strumigenys*, 9 = *Pheidole*, 10 = *Brachymyrmex*, 11 = *Cyphomyrmex*, 12 = *Paratrechina*, 13 = *Tapinoma*, 14 = *Neivamyrmex*, 15 = *Tranopelta*, 16 = *Probolomyrmex*, 17 = *Cardiocondyla*, 18 = *Nomamyrmex*, 19 = *Pachycondyla*, 20 = *Linepithema*.

Estimate of richness: Based on the species richness estimate Chao2 (calculated from the presence/absence of species), according to the survey methods implemented in the study, 93.0 y 89.0% of the species were captured in modified and conventional cotton, respectively (Figure 1.18.2.2A and 1.18.2.2B). This effort is considered to have identified a representative richness of ants, taking into account that the curve stabilized in the Bt-modified cotton and tended toward stabilization in the conventional cotton. The same result is complemented by the behavrior of the curves of the "uniques" (species that occur in only one sample) and the "duplicates" (species that occur in two samples which converge before arriving at the end of the two years of evaluation. When the singleton and doubleton curves converge it means that the sampling effort has been sufficiently complete to describe the richness present. McKamey (1999, American Entomologist 45: 213-222) indicates that in inventories of groups of insects, the amount of time and effort invested does not matter because it is possible to never arrive at the real number of species, and therefore these species richness estimates become useful tools.

Richness and abundance: For these indices, no differences were detected between Bt-modified and conventional cotton for the richness and abundance of ants. This is unlike what was observed in comparing abundance between years, where there were differences in richness and abundance. The differnce between years could be explained b a prolonged period of drought during 2004 which may have influenced the number of captures and the ant populations (Figure 1.18.2.3).

Ant guilds: The ant fauna from the cotton treatments were grouped into functional guilds for further analysis. This classification refers to groups of species that obtain their subsistence from the same types of resources and use the same strategies in occupying their ecological niches. In this study, they were grouped into three guilds taking into account the number of occurrences, capture method and based on the

bibliographic references of Andersen (2000, *In*: Agosti D., J.D. Majer, L.E. Alonso, T.R. Schultz (eds.), Standard methods for measuring and monitoring biodiversity, pp 25-34) and Silvestre *et al.* (2003, In: Fernández, F (ed.). Introducción a las hormigas de la región Neotropical. Instituto de Investigaciones Entomológicas Alexander von Humboldt y Smithsonian Institution. Bogotá, Colombia. 424)

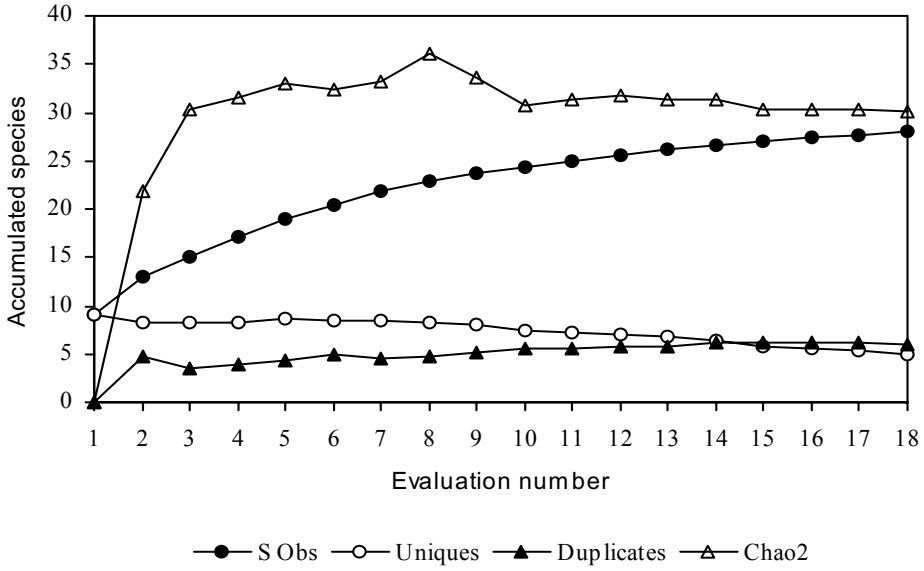

Figure 1.18.2.2A. Species accumulation curves over two years of evaluation in Bt-modified cotton. ICA, Palmira, Valle del Cauca. (Dates 1-9 are 2003, 10-18 are 2004).

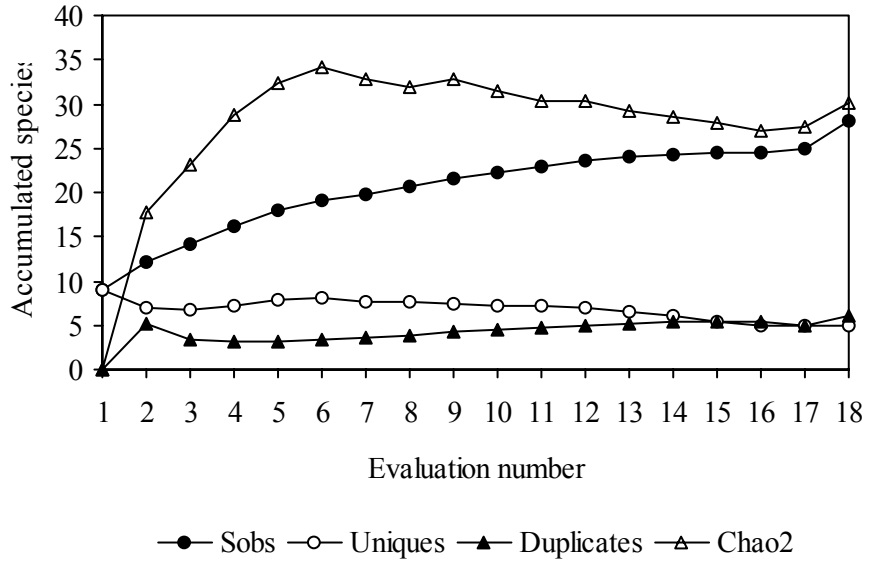

Figure 1.18.2.2B. Species accumulation curves over two years of evaluation in conventional cotton. ICA, Palmira, Valle del Cauca. (Dates 1-9 are 2003, 10-18 are 2004).

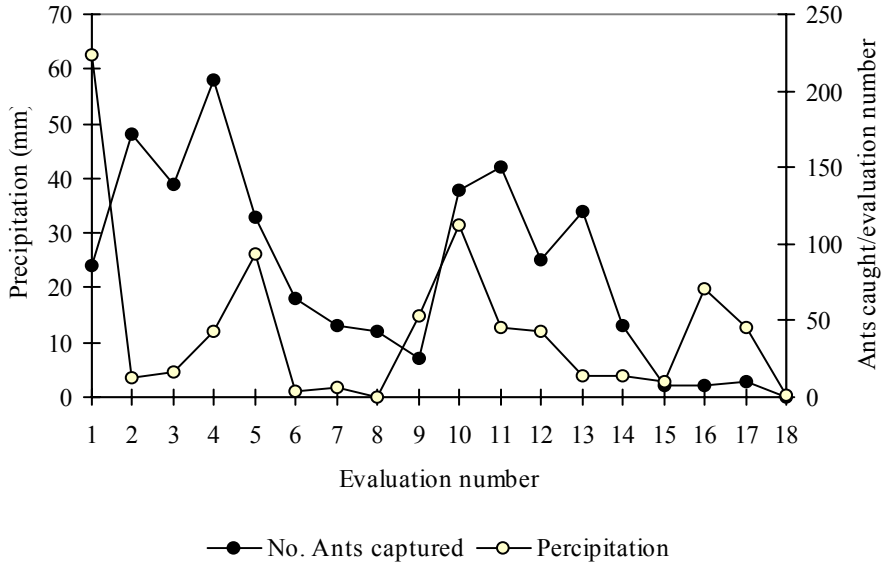

Figure 1.18.2.3. Accumulated weekly Precipitation during 2003-2004 versus the number of ants captured.

1. Omnivores –the majority of ant species; function as opportunistic generalists in their diet; include genera such as *Wasmannia*, *Solenopsis* and *Ectatomma*.
2. Cryptics – represented by species of small size; very few captures in the samples.
3. Specialized cryptic predators – small species that live in the litter; manibles are well-developed for specialized diets that include springtails and diplurans; includes species of the genus *Strumigenys*.

An assessment of guilds will help us understand the structure and function of the ant community according to vegetation and land-use patterns, among others (Andersen, 2000, In: Standard methods for measuring and monitoring biodiversity, 280 p; Silvestre *et al.*,.2003, In : Fernández, F (ed.). Introducción a las hormigas de la región Neotropical. Instituto de Investigaciones Entomológicas Alexander von Humboldt y Smithsonian Institution. Bogotá, Colombia. 424), and in this case the possible impact that could be related to Bt-modified cotton on the soil arthropod communities. In a comparison between treatments, the only significant difference occurred in the specialized cryptic predators, being more abundant in Bt-modified cotton. The possible causes for this include microclimate, distribution of food sources, and nesting resources but not possible exposure to the Cry toxin. Analyses of other insect orders have shown this same tendency. In the case of Coleoptera, Mazo *et al*. (2005, Resumenes XXXII Congreso SOCOLEN, Colombia, 88 p.) found that of 22 families captured, two (Cicindellidae and Lathrididae) were statistically more abundant in Bt-modified cotton than conventional cotton. Similarly, for the class Collembola, the orders Poduromorpha and Entomobryomorpha were more abundant in Bt-modified cotton (Mazo 2005, Efecto del algodón Bollgard ® (Bt) sobre la diversidad y abundancia de artrópodos del suelo durante su segundo año en el Valle del Cauca. Undergraduate Thesis. Universidad del Valle, Facultad de Ciencias, Cali. 67 p)

Conclusions:

∉ Over the two cycles of evaluation in conventional and Bt-modified cotton, no differneces were detected in the ant community. Therefore this study does not implicate the Cry toxin as having any effect on this community. It remains to be seen whether effects may accumulate over th elonger term and therefore

studies should be conducted over consecutive years as complementary tests of possible effects on the soil arthropod community.

∉ It is possible to conduct evaluations in this manner to establish the impact of the introduction of Bt-modified crops on the community of arthropods associated with them, not just soil communities, but foliar communities.

∉ This study offers basic information on how to establish the risk of introduction of genetically modified crops in the Cauca Valley. Nevertheless, this only consititutes a fraction of the full range of organisms present in the cotton crop and of the interspecific plant-arthropod interactions that can occur within. It is necessary to conduct further studies on this community and on other groups of organisms.

Table 1.18.2.1. Comparison of the abundance (number and percentage) of captured ants in pitfall traps and soil samples, during 2004, in C.I. del ICA - Palmira, in cotton.

Taxa	Pitfall traps		Soil samples		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Myrmycinae						
Wasmannia auropunctata	4.448	93.3	1.330	44.3	5.778	74.4
Solenopsis	93	2.0	980	32.7	1.073	13.8
Monomorium	10	0.2	346	11.5	356	4.6
Pheidole	61	1.3	75	2.5	136	1.8
Atta cephalotes	30	0.6	0	0	30	0.4
Cardiocondyla	2	0.0	1	0.0	3	0.0
Cyphomyrmex rimosus	2	0.0	0	0	2	0.0
Strumigenys	1	0.0	1	0.0	2	0.0
Tranopelta	1	0.0	0	0	1	0.0
Formicinae						
Brachymyrmex	6	0.1	20	0.7	26	0.3
Camponotus	0	0	1	0.0	1	0.0
Paratrechina	6	0.1	1	0.0	7	0.1
Dolichoderinae						
Tapinoma melanocephalum	4	0.1	24	0.8	28	0.4
Ecitoninae						
Nomamyrmex esenbeckii	3	0.1	0	0	3	0.0
Neyvamyrmex	0	0	1	0.0	1	0.0
Ponerinae						
Probolomyrmex	0	0	3	0.1	3	0.0
Hypoponera	91	1.9	165	5.5	256	3.3
Ectatoma tuberculatum	10	0.2	0	0	10	0.1
Winged Formicidae	0	0	51	1.7	51	0.7
Total	**4.768**	**100**	**2.999**	**100**	**7.767**	**100**

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Table 1.18.2.2. Comparison of the abundance (number and percentage) of captured ants in conventional (DP5415) and Bt-modified (NuCotn 33B) cotton during 2004in C.I. del ICA, Palmira, from pitfall traps.

Taxa	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Myrmycinae						
Wasmannia auropunctata	1.277 b	90,2	3.171 a	94,6	4.448	93,3
Solenopsis	31 b	2,2	62 a	1,	93	2,0
Pheidole	25 a	1,	36 a	1,	61	1,3
Atta cephalotes	7 b	0,	23 a	0,	30	0,6
Monomorium	9 a	0,	1 a	0,	10	0,2
Cardiocondyla	1 a	0,	1 a	0,	2	0,0
Cyphomyrmex rimosus	1 a	0,	1 a	0,	2	0,0
Strumigenys	0 a	0,	1 a	0,	1	0,0
Tranopelta	0 a	0,	1 a	0,	1	0,0
Formicinae						
Brachymyrmex	5 a	0,	1 a	0,	6	0,1
Paratrechina	3 a	0,	3 a	0,	6	0,1
Dolichoderinae						
Tapinoma melanocephalum	2 a	0,	2 a	0,	4	0,1
Ecitoninae						
Nomamyrmex esenbeckii	2 a	0,	1 a	0,	3	0,1
Ponerinae						
Hypoponera	50 a	3,	41 a	1,	91	1,9
Ectatoma tuberculatum	3 a	0,	7 a	0,	10	0,2
Total	1.416	10	3.352	10	4.768	100

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Table1.18.2.3. Comparison of the abundance (number and percentage) of captured ants in conventional (DP5415) and Bt-modified (NuCotn 33B) cotton during 2004in C.I. del ICA, Palmira, from soil samples.

Taxa	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Myrmycinae						
Cardiocondyla	1a	0,1	0a	0,0	1	0,0
Strumigenys	1a	0,1	0a	0,0	1	0,0
Pheidole	61a	3,5	14b	1,1	75	2,5
Monomorium	14b	0,8	332a	26,6	346	11,5
Solenopsis	543	31,0	437	35,1	980	32,7
Wasmannia auropunctata	982a	56,0	348b	27,9	1330	44,3
Formicinae						
Brachymyrmex	14a	0,8	6a	0,5	20	0,7
Camponotus	0a	0,0	1a	0,1	1	0,0
Paratrechina	0a	0,0	1a	0,1	1	0,0
Dolichoderinae						
Tapinoma melanocephalum	7[a]	0,4	17a	1,4	24	0,8
Ecitoninae						
Neyvamyrmex	0a	0,0	1a	0,1	1	0,0
Ponerinae						
Probolomyrmex	0a	0,0	3a	0,2	3	0,1
Hypoponera	106a	6,0	59b	4,7	165	5,5
Alados Formicidae	24a	1,4	27a	2,2	51	1,7
Total	1753	100	1246	100	2999	100

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons)

Activity 1.18.3. Comparison of the abundance and diversity of Coleoptera in conventional (DP5415) and modified (NuCotn 33B) cotton in the Cauca Valley, Colombia.

Contributors: A. Mazo, J.Rodríguez, D. C. Peck, J. Montoya Lerma, and A. C. Bellotti.

Materials and Methods

This work was based on specimens collected during 2004, in the ICA research station in Palmira, located at 03°31'N, 76°19'W, 975 m elevation, annual precipitation 1295 mm, mean temperature 24^{o}C, relative humidity 76%, and corresponding to the Holdridge life zone of Dry Tropical Forest. Specimens were collected as part of the activities of the project "Evaluating the Impact of Biotechnology on Biodiversity: Effect of Transgenic Maize on Non-Target Soil Organisms" where two treatments of conventional cotton (DP 5415) a Bt-transgenic Bollgard® cotton (NuCotn 33B) were evaluated. (Activity 1.18.1)

Coleoptera were separated from the original samples of arthropods that had been collected from cotton during 2004 and stored in 70% ethyl alcohol. This was performed by examining the samples under a stereoscope, separating all Coleoptera, and returning the remaining arthropods to their original specimen vial.

Analysis of information: The statistical model used for the analysis of the data was a completely randomized block design. With this design an ANOVA was used to determine differences in abundance among treatments and determine the effect of their interactions.

Results and Discussion

Pitfall traps: For the order Coleoptera, in the second evaluation cycle (2004) 748 individuals were captured, distributed in 19 families. Of those families, 10 were more abundant in conventional cotton. Nevertheless, the overall abundance of beetles was 1.1 times higher in Bt-modified cotton. Of the 19 families identified, only Cicindellidae and Lathridiidae exhibited significant differneces between treatments, in both cases being more abundant in Bt-modified cotton.

The most abundant families in 2004 were Carabidae and Scarabaeidae with 36.2 and 17.3% of the total individuals captured. Among the captured carabids were representatives of the genus *Calosoma* (caterpillar hunters), *Galeritula*, *Scarites*, and other unidentified morphospecies.(Figure 1.18.3.1). In terms of abundance, *Galeritula* was the most represented (209 individualscaptured), with 51.2% of captures in conventional cotton. (Candolfi *et al.,* 2004, Biocontrol Science and Technology 14: 129 – 170; Lozzia, 1999, Bollettino di Zoologia Agraria e di Bachicoltura 31: 37-58; Haungton *et al.*, 2003, Philosophical Transactionsof the Royal Society B 358: 1863–1877; Duan *et al.*, 2004, Environmental Entomology 33:275-281; ř ezáļ *et al,* 2005, Proceedings Ecological Impact of GMOs. Int'l org. for biological and integrated control of Noxious Animals and Plants June 1-3. Lleida, Catalonia, España; Turanli *et al*., 2005, Proceedings Ecological Impact of GMOs. Int'l org. for biological and integrated control of Noxious Animals and Plants, June 1-3, Lleida, Catalonia, España) Diverse studies support the observations made on this group in Bt-modified cotton, that no effects are reported that diminish populations of these relatively abundant taxa.

In the Scarabaeidae, four subfamilies were identified (Figure 1.18.3.2). Melolonthinae (*Cyclocephala lunulata*) and Rutelinae (*Leucothyreus femuratus*) represented about 10.0% of the captured individuals. Aphodinae and Scarabaeinae (*Canthon* sp.) were the groups of greatest abundance in this family, representing approximately 90.0% of the captures. No statistical differences were detected in abundance due to treatments. This result is comparable to that reported by Frizzas (2003, MSc Thesis, Escuela superior de agricultura Janeiro, Sao Paulo, Brasil, 192 pp) in conventional and Bt-transgenic maize.

Only one species of Cicindellidae was identified, belonging to the genus *Megacephala* sp. The abundance of this species was 2.1 times higher in Bt-transgenic cotton. In Brazil, Frizzas (2003) obtained similar results with respect to cicindelids in Bt-transfgenic maize, confirming the idea that the Cry toxin does not have a negative effect on this group of predators. The family Lathridiidae could be more exposed to the Cry toxin because of their mycetophaguos habits. Nevertheless, results showed this family to be 2.7 times more abundant in Bt-modified cotton (Table 1.18.3.2)

Berlese funnels: A total of 822 individuals, distributed in 15 families, were captured from soil cores extracted with Berelese funnels. Conventional cotton had 1.3 times more captures than Bt-transgenic cotton, but a statistically significant difference was not detected (Table 1.18.3.1).

Table 1.18.3.1. Comparison of the abundance (in total number and percentage) of Coleoptera captured in pitfall traps in conventional and Bt-modified cotton, during 2004, in C.I. del ICA, Palmira.

Family	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Carabidae	138 a	37,9	133 a	34,6	271	36,2
Scarabaeidae	63 a	17,3	66 a	17,2	129	17,3
Cicindellidae	22 b	6,0	47 a	12,2	69	9,2
Chrysomelidae	28 a	7,7	34 a	8,9	62	8,3
Nitiduliidae	35 a	9,6	22 a	5,7	57	7,6
Staphylinidae	17 a	4,7	23 a	6,0	40	5,4
Coccinellidae	10 a	2,8	16 a	4,2	26	3,5
Lathridiidae	7 b	1,9	19 a	5,0	26	3,5
Curculiionidae	10 a	2,8	8 a	2,1	18	2,4
Scydmaenidae	10 a	2,8	5 a	1,3	15	2,0
Cucujidae	10 a	2,8	4 a	1,0	14	1,9
Anthicidae	3 a	0,8	2 a	0,5	5	0,7
Corylophidae	5 a	1,4	0 a	0,0	5	0,7
Scolytidae	1 a	0,3	3 a	0,8	4	0,5
Histeridae	2 a	0,6	0 a	0,0	2	0,3
Tenebrionidae	1 a	0,3	1 a	0,3	2	0,3
Cantharidae	1 a	0,3	0 a	0,0	1	0,1
Cleridae	0 a	0,0	1 a	0,3	1	0,1
Phalacridae	1 a	0,3	0 a	0,0	1	0,1
Sum	364	100	384	100	748	100

For each row, values followed by distinct letters are statistically different ($P<0.05$) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Of the Coleoptera families identified, Staphylinidae and Scarabaeidae were the most abundant, with 55.2 and 27.4% of captures, respectively (Table 1.18.3.1). In both cases the greatest abundance was associated with conventional cotton with 1.4 and 1.3 times more captures than the Bt-transgenic variety, respectively. In addition, the families Scolytidae and Carabidae also had relatively high captures for the order. Scolytidae was 1.2 times more abundant in conventional cotton while Carabidae was 1.7 times more abundant in Bt-transgenic cotton (Table 1.18.3.1). This information agrees with previous studies that have not identified negative effects associated with Bt-transgenic crops (Candolfi e*t al.,* 2004, Biocontrol Science and Technology 14: 129-170).

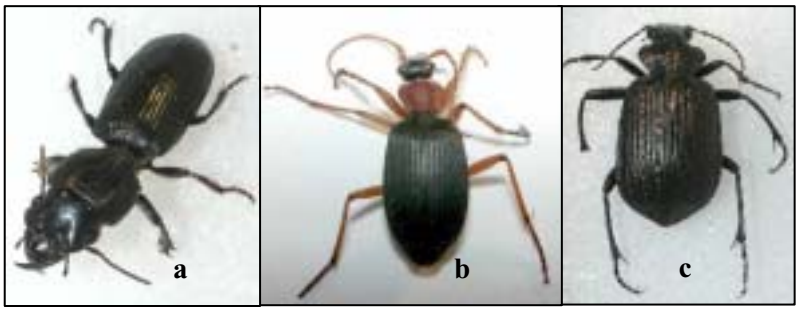

Figure 1.18.3.1. Morphospecies of the family Carabidae found in cotton in C.I. del ICA Palmira, 2004. (a) *Scarites,* (b) *Galeritula*, (c) *Calosoma*

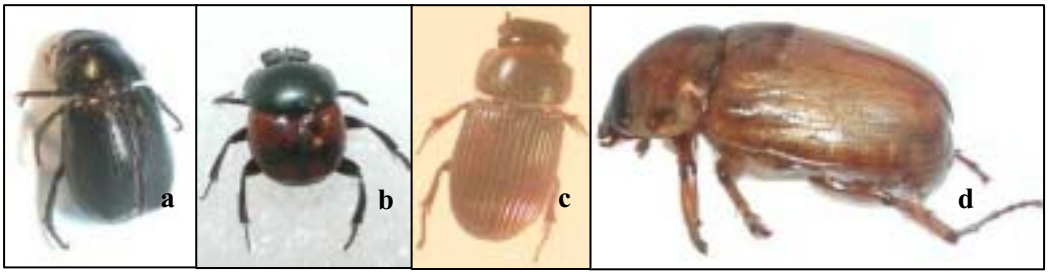

Figure 1.18.3.2. Morphospecies of the family Scarabaeidae found in cotton in C.I. del ICAPalmira, 2004. (a) Rutelinae, (b) Scarabaeinae, (c) Aphodiinae y (d) Dynastinae.

Table 1.18.3.2. Comparison of the abundance (in total number and percentage) of Coleoptera captured in soil cores extracted with Berlese funnels in conventional and Bt-modified cotton, during 2004, in C.I. del ICA, Palmira.

Family	DP5415		NuCotn 33B		Total	
	No. Individuals	%	No. Individuals	%	No. Individuals	%
Staphylinidae	268 a	57,9	186 a	51,8	454	55,2
Scarabaeidae	127 a	27,4	98 a	27,3	225	27,4
Scolytidae	35 a	7,6	30 a	8,4	65	7,9
Carabidae	9 a	1,9	17 a	4,7	26	3,2
Nitiduliidae	7 a	1,5	5 a	1,4	12	1,5
Cucujidae	5 a	1,1	4 a	1,1	9	1,1
Anthicidae	3 a	0,6	5 a	1,4	8	1,0
Curculiionidae	3 a	0,6	3 a	0,8	6	0,7
Chrysomelidae	3 a	0,6	2 a	0,6	5	0,6
Lathridiidae	1 a	0,2	3 a	0,8	4	0,5
Elateridae	1 a	0,2	2 a	0,6	3	0,4
Coccinellidae	0 a	0,0	2 a	0,6	2	0,2
Ptiliidae	0 a	0,0	1 a	0,3	1	0,1
Ptylodactylidae	1 a	0,2	0 a	0,0	1	0,1
Scydmaenidae	0 a	0,0	1 a	0,3	1	0,1
Sum	**463**	**100**	**359**	**100**	**822**	**100**

For each row, values followed by distinct letters are statistically different (P<0.05) (Data transformed Ln(x+1), Tukey Test and GLM for multiple comparisons).

Activity 1.19. Determining the genetic variability of *Ralstonia solanacearum* of plantain, using microsatellite markers (RAMs)

Contributors: E. Álvarez, E. Gómez, J. F. Mejía, G. Llano, and J. Loke

Highlight:

- The first report of the pathogen's genetic diversity as determined by RAM analysis was presented. This analysis permitted identification of infra-subspecific groups of strains that have common biological properties, evolutionary relationships, or geographic origins. *Ralstonia solanacearum* DNA sequences were reported to GenBank.

Rationale

Characterization and knowledge of the genetic structure of pathogen populations have direct applications in disease management. This study therefore aimed to obtain information on the genetic diversity of a population of *R. solanacearum* race 2 from Colombia, causal agent of bacterial wilt of plantain. For the first time a technique based on microsatellites, known as random amplified microsatellites (RAMs), has been used with this pathogen. The main objective of this study was to determine the variability of *Ralstonia solanacearum* from Musaceae crops in different regions of Colombia. Our goal is to develop strategies to improve the acquisition of durable resistance to *R. solanacearum*.

Materials and Methods

We used 59 strains of *R. solanacearum* from tissues of sick plants, infected by bacterial wilt and collected from plantain, banana, and heliconia crops growing in six departments of Colombia, and from soil of infected plantain crops in Quindío. We used, for controls, a strain from eggplant from Kenya and four strains from tobacco (two from Floridablanca, Santander, Colombia; one from Quency, Florida, USA; and one from Japan) (Table 1.19.1). The *R. solanacearum* strains were obtained from the collection held by the Cassava Pathology program at CIAT. This study thus became the most complete on the genetic variability of the causal agent of bacterial wilt of plantain in Colombia. The strains preserved in solution with 60% glycerol were reactivated in a semi-selective medium (SMSA) and then transferred to nutrient agar to obtain pure strains of 24-h growth for later DNA extraction.

Extracting DNA: The protocol described by Boucher *et al.,* (1985, J. Gen. Microbiol. 131:2449-2457) was used to extract DNA from the 59 strains.

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

We evaluated polymorphism among five isolates of R. solanacearum, obtained with seven RAM primers: HVH $(TG)_7T$, DHB $(CGA)_5$, DYD $(CT)_7C$, DBD $A(CA)_7$, VHV $(GT)_5G$, HBH $(AG)_7A$, and DDB $(CCA)_5$, where H is (A,T,C); B is (G,T,C); V is (G,A,C); and D is (G,A,T). We then amplified the 59 strains with the most polymorphic primer (Hantula *et al*., 1996, European Journal of Forest Pathology 26:159-166).

Every PCR-RAM reaction was carried out in volumes of 12.5 µL, made up of dATP, dCTP, dGTP, and

dTTP in proportions of 0.2 mM each; 1.25 µL 10X *Taq* polymerase buffer solution; 1 mM $MgCl_2$; 0.008 U *Taq* polymerase (Promega); 2 ε M primer; and 5 ng total DNA. Amplification was carried out in a MJ Research PTC-100 thermal cycler, programmed at 95 ∀C for 5 min; 37 cycles of denaturation at 95 ∀C for 30 s; annealing at 55 ∀C (TG primer), 61 ∀C (CGA), 41 ∀C (CT), 50 ∀C (CA and AG), and 58 ∀C (GT), all for 45 s, and 55 ∀C (CCA) for 50 s; extension at 72 ∀C for 2 min; and final extension at 72 ∀C for 7 min (Henríquez et al., 2002*,* Phytopathology 92:580-589).

Table 1.19.1. Description of *Ralstonia solanacearum* strains, causal agent of bacterial wilt of plantain, used for the study.

Crop and strain no.	Geographical origin	Source	Crop and strain no.	Geographical origin	Source
Plantain			**Plantain**		
1	Quindío	Rachis	73	Urabá (Antioquia)	Pseudostem
3	Quindío	Petiole	76	Montenegro (Quindío)	Pseudostem
15	Quindío	Soil	78	Montenegro (Quindío)	Rachis
16b	Quindío	Soil	79	Montenegro (Quindío)	Rhizome
17	Jamundí (Valle del Cauca)	Soil	80	Montenegro (Quindío)	Pseudostem
18	Jamundí (Valle del Cauca)	Sucker	83	Quindío	Fruit
32	Caquetá	Pseudostem	84	Quindío	Pseudostem
3	Caquetá	Pseudostem	85	Quindío	Sucker
81	Montenegro (Quindío)	Fruit	86	Calarcá (Quindío)	Rachis
38	Quindío	Soil	88	La Tebaida (Quindío)	Rhizome
39	Quindío	Soil	89	La Tebaida (Quindío)	Pseudostem
40	Quimbaya (Quindío)	Soil	97	Quimbaya (Quindío)	Rhizome
41	Quimbaya (Quindío)	Soil	107	Armenia (Quindío)	Fruit
42	Fuente de Oro (Meta)	Pseudostem	1008	Ibagué (Tolima)	CIAT collection
43	Fuente de Oro (Meta)	Pseudostem			
48	Armenia (Quindío)	Fruit	**Banana**		
54	Fuente de Oro (Meta)	Pseudostem	5	Urabá (Antioquia)	Rhizome
57	Fuente de Oro (Meta)	Pseudostem	6	Urabá (Antioquia)	Fruit
58R	Fuente de Oro (Meta)	Petiole	7	Urabá (Antioquia)	Fruit
59	Fuente de Oro (Meta)	Pseudostem	110	Magdalena	Pseudostem
60	Fuente de Oro (Meta)	Pseudostem	111	Magdalena	Rhizome
63	Granada (Meta)	Pseudostem	112	Magdalena	Sucker
64	Granada (Meta)	Pseudostem			
65	Granada (Meta)	Pseudostem	**Heliconia**		
66	Granada (Meta)	Pseudostem	113	Palmira (Valle del Cauca)	Pseudostem
67	Fuente de Oro (Meta)	Pseudostem	114	Palmira (Valle del Cauca)	Rhizome
71	Urabá (Antioquia)	Rhizome	115	Palmira (Valle del Cauca)	Rhizome
72	Urabá (Antioquia)	Pseudostem			

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

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

Results and Discussion

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

To read the bands, a range between 201 and 1327 bp was selected, observing characteristic bands for the strains from different crops and departments (Figures 1.19.1 to 1.19.3).

By analysis in NTSYS, a dendrogram (Figure 1.19.4) was prepared that generated 12 clusters, having a coefficient of similarity of 0.87 and differentiating according to crop type and geographic location. These were:

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

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

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

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

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

Conclusions:

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

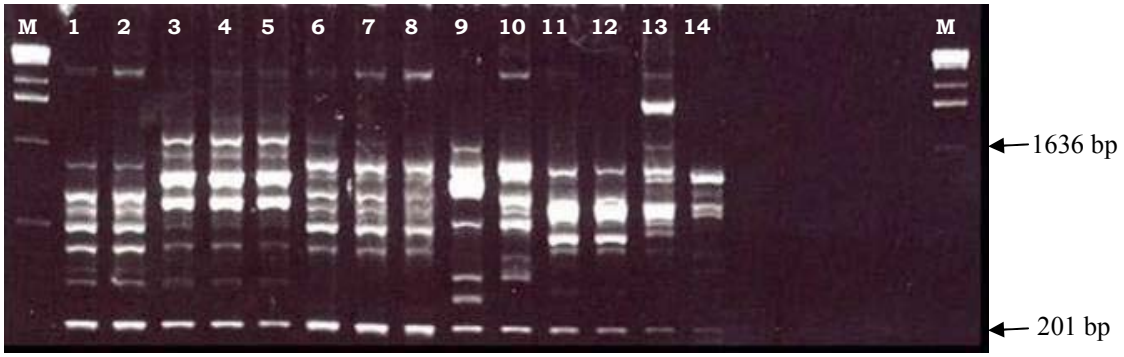

Figure 1.19.1 RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from different plant species. M = 1-kb marker; lanes 1 and 2 = strains from banana, Antioquia, Colombia; lanes 3–5 = strains isolated from banana, Magdalena, Colombia; lanes 6–8 = strains isolated from heliconias, Valle del Cauca, Colombia; lane 9 = strain from eggplant, Kenya; lane 10 = strain from tobacco, Japan; lanes 11 and 12 = strains from tobacco, Santander, Colombia; lane 13 = strain from tobacco, Florida, USA.

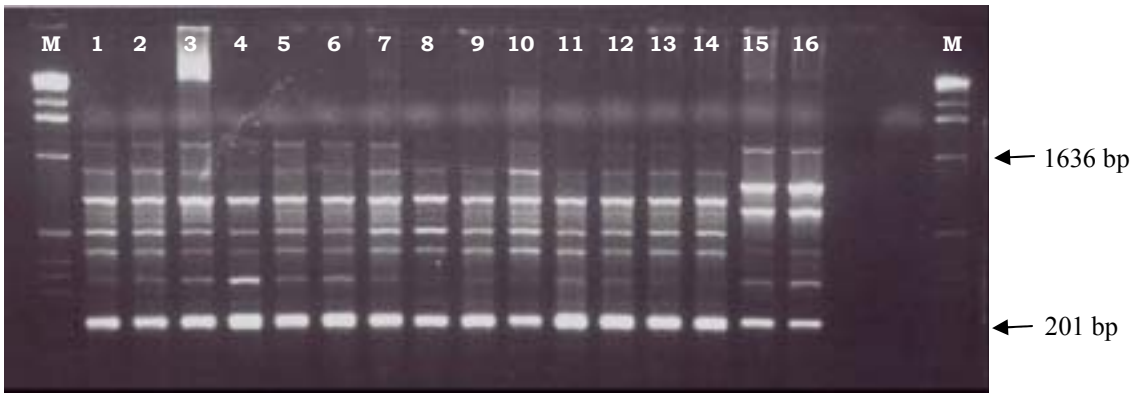

Figure 1.19.2. RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from two different sites in Colombia. M = 1-kb marker; lanes 1–14 = strains isolated from plantain, Quindío; lanes 15 and 16 = strains isolated from banana, Magdalena.

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

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

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

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

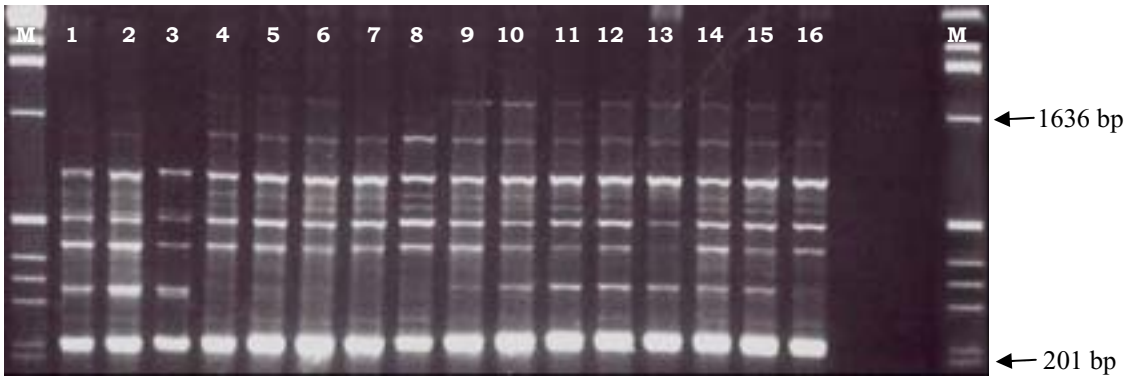

Figure 1.193. RAM patterns obtained with the CGA primer for *Ralstonia solanacearum* strains isolated from plantain in three different sites in Colombia. M = 1-kb marker; lanes 1–3 = strains from Antioquia; lane 4 = strain from Caquetá; lanes 5–16 = strains from Meta.

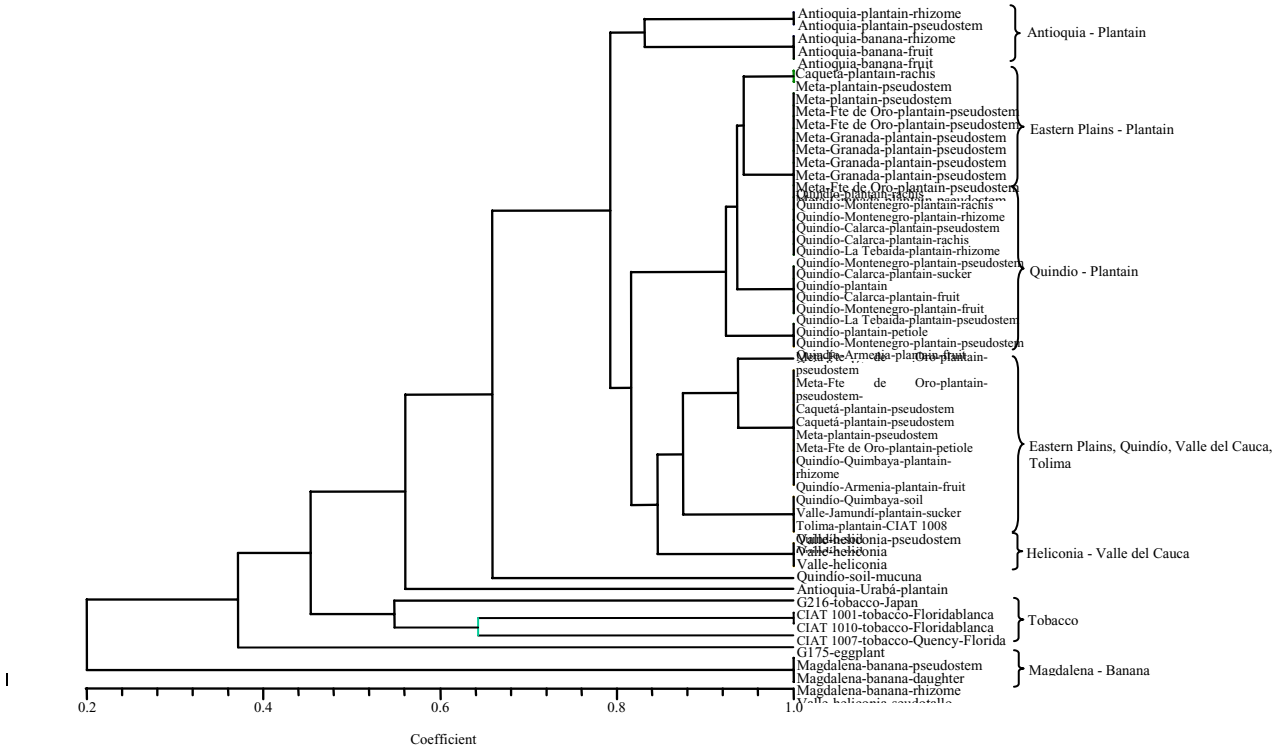

Figure 1.194. Dendrogram of similarity based on the Dice coefficient for 59 strains of *Ralstonia solanacearum*, with the RAM primer CGA.

Activity 1.20. DNA sequence analysis of the ITS region of a phytoplasma obtained from coffee: a collaborative effort between CIAT and CENICAFE

Contributors: E. Álvarez, J. F. Mejía, and C. Gálvis

Highlight:

- We confirm the association of a pathogenic agent to the coffee crispiness or *crespera*, this being the first report of a phytoplasma-caused disease in the *Coffea* genus. As a new member of the 16SrIII group, we propose the name coffee crispiness disease or CCD phytoplasma.

Rationale

Coffee crispiness is a slow-spreading disease that has been reported in Colombian coffee plantations since 1940. It is endemic to limited areas scattered throughout the country. It affects the physiological development of the aerial parts of the plant, especially the leaves and floral buds, causing leaf proliferation and phyllody, and also altering the fruits, increasing the percentages of monospermic berries. The curled leaves and massive vegetative growth that results in the branches gives the local name *crespera* to the disease.

Because no etiology has been clearly associated with coffee crispiness, control measures have been ineffective. Throughout the years, crispiness has been attributed to divergent causes, varying from viral problems, microelement deficiencies, mineral malnutrition (Drosdoff, 1956, Agricultura Tropical 12: 103 – 105), and physical and chemical soil limitations (Valencia, 1993, Fitopatología Colombiana 17:39-45; Carrillo, 1984, Federación Nacional de Cafeteros de Colombia, Informe Trimestral: 20-22). Farmers have also implicated herbicide toxicity. More recently, comparative symptomatology suggested the association of a phytoplasma with *crespera* (Moreira *et al*., 1997, Simposio Latinoamericano de Caficultura.18: 409). No evidence, however, has been provided to support any of these hypotheses.

The present work was aimed to confirme the association of a phytoplasma with coffee crispiness disease in Colombia, and to establish its phylogenetic relationship based on the molecular characterization of the 16S ribosomal DNA. We confirm a phytoplasma as the causing agent of the coffee crispiness disease. This is a necessary step in the process of understanding of the disease epidemiology, vector identification, improved diagnostic methods, and design of strategies to reduce the effect and dissemination of coffee crispiness among plantations. This is also the first report of a phytoplasma causing disease in the *Coffea* genus.

Materials and Methods

Sources of phytoplasmas: Symptomatic branches from *Coffea arabica* L. were collected from the Santa Rosa de Cabal Region (Risaralda, Colombia), in 5-year-old coffee plantations that had been regenerated from stumps. The negative control always used was *C. arabica* var. Caturra, obtained from seeds of healthy plants.

DNA extraction: DNA was totally extracted from leaves according to the procedure described by Bernatzky and Tanksley (1986, Genetics 112:887–898) but with minor modifications. Briefly, 100 mg of leaves, ground in liquid nitrogen, were immersed in 500 µL of extraction buffer (200 mM Tris-HCl, pH 8.0; 70 mM EDTA; 2 M NaCl; 20 mM sodium metabisulfate; 2% CTAB; and 0.2% ß-mercaptoethanol to prevent phenolic oxidation) and incubated in a water bath at 65 ºC for 1 h. Next, one volume of phenol was added and mixed by inversion for 10 min. It was then centrifuged at 13,000 rpm for 5 min, and the supernatant removed to another tube. An extraction with 500 εL of chloroform followed, and after

centrifuging at 6000 rpm, the supernatant was removed to a new tube. DNA was precipitated by adding 250 εL of 5 M ammonium acetate and 750 εL of isopropanol, and spun for 25 min at 14,000 rpm. After rinsing with 1 mL of 70% ethanol and a final centrifuging at 12,000 rpm for 2 min, the supernatant was discarded and, once dry, the pellet was resuspended in 50 εL of TE buffer (10 mM Tris-HCl, pH 8.0; and 0.1 mM EDTA) and treated with 5 εg of SIGMA RNAase per tube.

PCR amplification: Three primer pairs from the 16S rDNA were synthesized by Operon Technologies (Alameda, CA):

P1 (5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3') and
P7 (5'-CGT CCT TCA TCG GCT CTT-3')

R16F2 (5'-ACG ACT GCT AAG ACT GG-3') and
R16R2 (5'-TGA CGG GCG GTG TGT ACA AAC CCC G-3'); and

FU5 (5'- CGG CAA TGG AGG AAA CT-3')
rU3 (5'-TTC AGC TAC TCT TTG TAA CA-3').

A nested-PCR reaction was performed as follows: a first amplification was carried out, using Promega reagents for a Master Mix with 0.1% PCR buffer, 25 mM $MgCl_2$; 2 mM dNTPs; and 0.1 U/εL of *Taq* polymerase, in addition to 0.2 μM of primers P1 and P7; 5 εL of 20 ng/εL coffee DNA; and distilled water to complete a volume of 25 εL. An amplification program was run on an MJ Research thermal cycler, consisting of an initial denaturation at 94 ºC for 2 min, and 35 cycles of 94 ºC for 1 min, 55 ºC for 2 min, and 72 ºC for 3 min. A final extension cycle was performed at 72 ºC for 10 min.

A second amplification was accomplished with 1 εL of a 1/30 dilution in sterilized distilled water, in a similar Master Mix, containing either the primer pair R16F2/R16R2 or FU5/rU3 and running the same thermal cycler program, except that the annealing temperature was changed to 50 ºC. PCR amplification products were separated in 1% agarose gels in TBE buffer at 60 V, stained with ethidium bromide, and recorded under UV light with a digital camera.

Molecular detection in grafted coffee plants: Leaf tissue was obtained from five plants grafted with diseased branches and from six 3-year-old plants from a commercial plot in the Santa Rosa de Cabal Region. They were then compared with healthy leaf tissue by amplifying the 16S rDNA region of the phytoplasma. Primer pairs P1/P7 and FU5/rU3 were used for the nested-PCR reaction, using coffee genomic DNA.

Cloning the PCR products and DNA sequencing: Amplified fragments in PCR by FU5/rU3 were cloned in *Escherichia coli* JM109, using the pGEM®-T Easy Vector System (Promega) according to manufacturer's instructions. 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 DNA sequencing at the Cornell Biotechnology Resource Center (Ithaca, NY). Plasmid sequences were removed from each sequence and high-quality sequence data collected. The sequence results were assembled and analyzed, using BLAST®n at NCBI (http://www.ncbi.nlm.nih.gov). The nucleotide sequences determined in this study were deposited in the GenBank data library (NCBI, MD).

Phylogenetic análisis: 16S rRNA gene sequences (941 pb) from coffee phytoplasma and 42 other strains

(representing 12 16S rRNA phytoplasma groups) were obtained from the GenBank, and a primary alignment performed with ClustalX (Thompson *et al*., 1997, Nucl Acids Res 24:4876–4882), with the flanking non-consensus regions removed by editing with BioEdit (Hall, 1999, Nucl Acids Symp Ser 41:95–98). A phylogenetic tree was constructed by the neighbor-joining method of the ClustalX program. The tree was viewed by using TreeView ([Page 1996, Computer Appl Biosci 12:357–358.])

Results and Discussion

Molecular detection in coffee plants: Clear amplifications of the 941-bp band were obtained from infected field plants. Samples from grafted tissue produced weaker amplifications, but still detectable. Negative controls and healthy tissues resulted in no amplifications (Figure 1.20.1). By nested PCR, a 941-bp fragment was amplified from DNA of infected tissues (GenBank Accession Number AY525125). When a common region for the 16S rDNA of each of the 12 phytoplasma groups was aligned, the one amplified from coffee clustered with the 16SrIII group (Figure 1.20.2), described as the X-disease group by Lee *et al.*(1994, Phytopathology 84:559-66).

Further analysis with the 42 sequences described as belonging to the 16SrIII family or as having close similarity, produced a radial tree for the family, with four main branches. The coffee crispiness phytoplasma was one of the most distant sequences of the whole group, with the phytoplasma causing *machorreo* in *lulo* (or naranjilla; *Solanum quitoense*: Asteridae: Solanaceae) being the closest. It was in a cluster that also contained the phytoplasmas causing chayote witches' broom (in *Sechium edule* (Jacq.) Sw., Dilleniidae: Cucurbitaceae) and garlic wilt (in *Allium sativum* L., Lilidae: Liliaceae) (Figure 1.20.3).

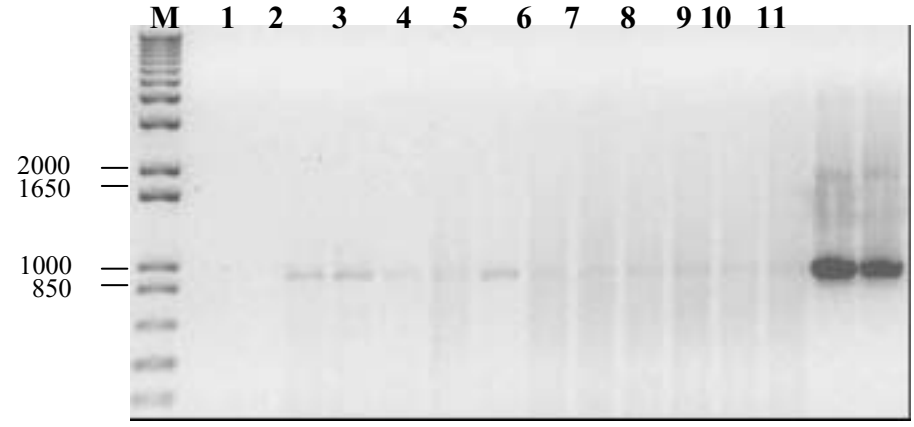

Figure 1.20.1. Agarose gel separation of PCR products from nested amplification of a 941-bp band corresponding to the 16S rDNA. M = molecular weight marker; lane 1 = PCR control; lane 2 = healthy coffee tissue; lanes 3 to 7 = field plant tissue exhibiting crispiness; lanes 7 to 13 = rootstock from grafted tissue, with symptoms; lanes 14 and 15 = cloned and sequenced crispiness phytoplasma rDNA.

The possible taxonomic association between the *machorreo* and coffee crispiness phytoplasmas suggests the presence of derivatives from a common ancestor in South America. However, while the Andes are considered as the geographic origin of Solanaceae, coffee was introduced only within the last 2 centuries. This suggests the hypothesis that coffee became an alternative host for a phytoplasma that more efficiently affects other plant species. In fact, the limited distribution, and moderate dispersion and virulence of crispiness agree with pathogen behavior and may provide alternatives for disease control. Moreover, the

same clade carries the China-tree phytoplasma, reported in Bolivia, the chayote witches' broom from Brazil, and the garlic decline phytoplasma from Argentina (Galdeano *et al*., 2004, Journal of Phytopathology, 152: 174-181).

We confirm the association of a pathogenic agent to the coffee crispiness or *crespera*, this being the first report of a phytoplasma-caused disease in the *Coffea* genus. As a new member of the 16SrIII group, we propose the name coffee crispiness disease or CCD phytoplasma.

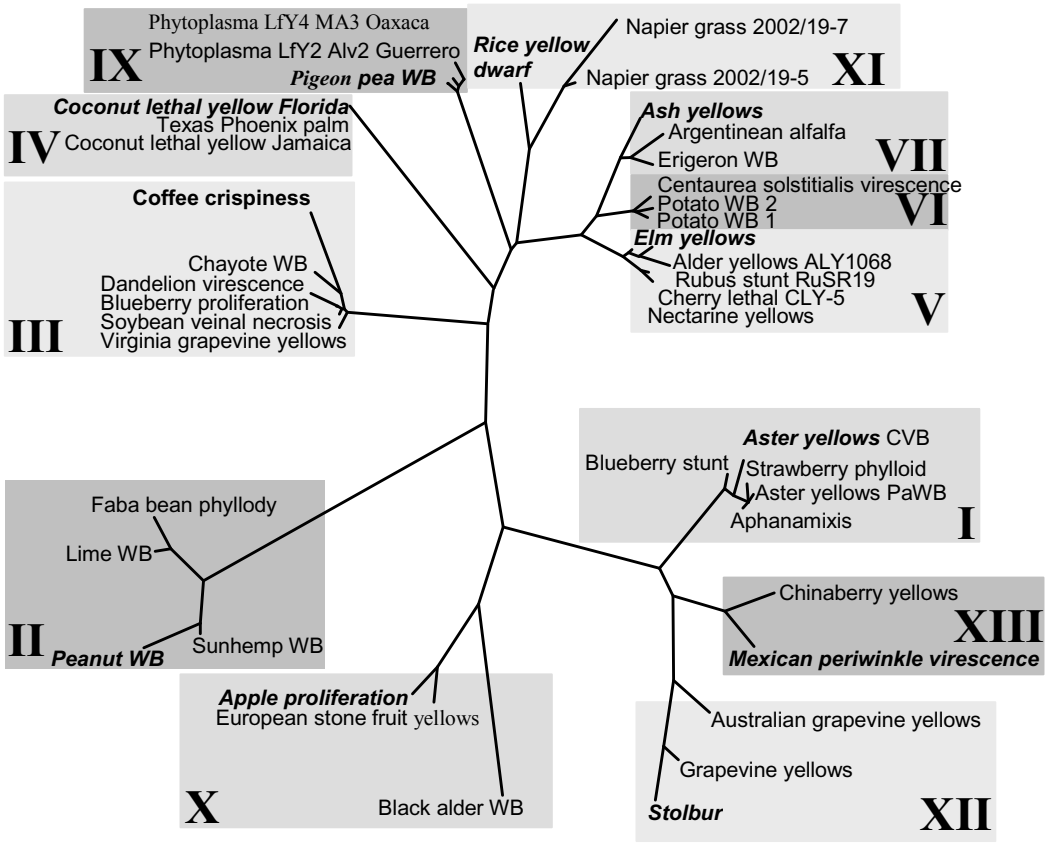

Figure 1.20.2. Dendrogram based on the 16S rDNA sequences of representative phytoplasmas retrieved from GenBank. The coffee crispiness phytoplasma groups with the 16SrIII (X-disease) group.

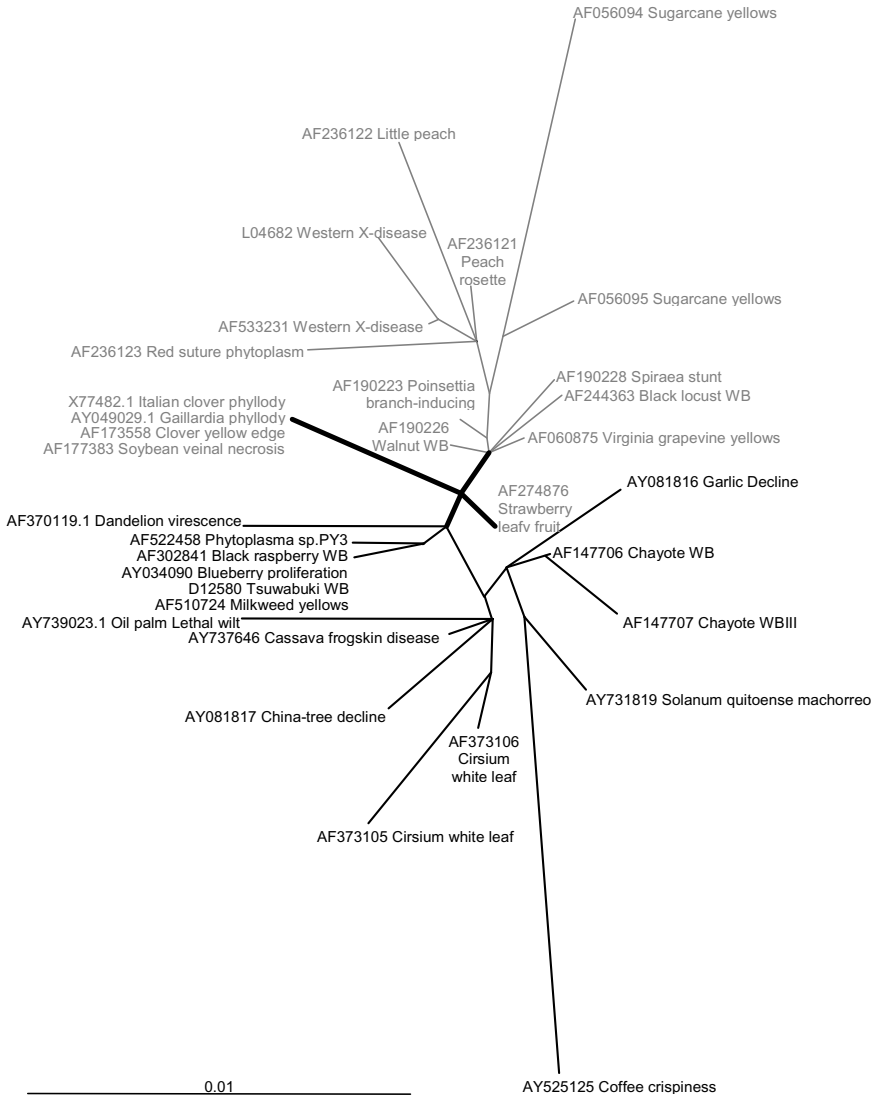

Figure 1.20.3. Detailed organization of the 16SrIII (X-disease) phytoplasma group. The closest relative to the coffee crispiness phytoplasma is the Solanum quitoense *machorreo* phytoplasma, also isolated in Colombia. (WB = witches' broom; thick black lines = major branches)

Activity 1.21. Identifying and Characterizing Strains of *Ralstonia solanacearum* Race 2, Causal Agent of *Moko* of Plantain in Colombia

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Highlight:

∉ A BIO–PCR technique was developed for detecting *R. solanacearum*. This technique had increased sensitivity, and detected only live cells of the pathogen in soil and plant tissue.

Rationale

Moko, *maduraviche*, or *ereke* is a bacterial wilt of plantain and banana caused by *Ralstonia solanacearum* E.F. Smith race 2 (Yabucchi *et al*., 1995, cited by Ito *et al*., 1998, In: Journal of Phytopathology 146: 379 – 384). It is the most important bacterial disease of these crops in Colombia, affecting possibly 125,000 families who depend directly on them for their livelihoods. Currently, despite dissemination of preventive measures and disease management, the disease is spreading, to the point where 95% of plantain fields have at the least one plant with *moko* (personal communication, Galindo 2004, ICA, Bogotá).

The BIO-PCR technique developed by Schaad *et al.* (1995, Phytopathology 85:243-248) improves efficiency in detecting viable cells of this pathogen, especially in soil. This technique consists of isolating colonies in semi-selective medium, South Africa (SMSA). It shows higher sensitivity and specificity than does triphenyltetrazolium chloride (TTC) (Kelman, 1954, Phytopathology 44: 693-695) with later amplification by polymerase chain reaction (PCR). *Ralstonia solanacearum* is variable in the range of hosts it attacks, geographical distribution, pathogenicity, epidemiological relationships, and physiological properties. Hence, in the last three decades, races and biovars have been used informally to classify the pathogen at the infra-subspecific level (not governed by the *International Code of Nomenclature of Bacteria*).

The main objective of our studies is to isolate *R. solanacearum* from soil and from infected plant tissue, using BIO-PCR, culture medium SMSA, specific primer OLI 1, and nonspecific primer Y2 in order to improve efficiency in detecting the pathogen. We also characterized the pathogen with the objective to select plantain cultivars with more durable resistance to *moko* disease and to develop better control practices.

Materials and Methods

Sample sources: We processed 134 samples of infected plant tissue from pseudostems, rachis, fruits, and rhizomes of selected plantain, banana, and heliconia plants that had presented typical symptoms of the disease. We also processed soil samples from farms affected by moko and located in the production areas of the Departments of Valle del Cauca, Quindío, Antioquia, Caquetá, Meta, and Magdalena in Colombia (Table 1.21.1).

Processing plant-tissue simples: The bacterium was extracted from selected infected tissue fragments, which had been washed, disinfected, and macerated in a mortar containing a buffer solution of 10 mM Tris-HCl and 1 mM EDTA at a pH = 7.6. This suspension was cultured, using a sterilized micro-spade, in petri dishes containing the semi-selective culture medium, South Africa (SMSA), a modification of the medium triphenyltetrazonium chloride (TTC). The SMSA medium contained 10 g/L peptone, 5 mL/L glycerol, 1 g/L casamino acids, 18 g/L agar, antibiotics (100 mg/L, i.e., 600,000 U polymyxin η sulfate; 25 mg/L bacitracin; 0.5 mg/L, i.e., 82.5 U penicillin, and 5 mg/L chloramphenicol), 50 mg/L of 2,3,5-TTC, and 5 mg/L crystal violet (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria (APS): 151-173 ; Englebrecht 1994). The dishes cultured with the suspension were incubated at a temperature of 28 ºC for 3 to 5 days, depending on when the colonies appeared.

Processing soil simples: With soil taken from around plantain plants infected by the bacterium, suspensions were prepared by adding 3.3 g soil to 30 mL TE buffer at pH = 7.6. Serial dilutions were carried out in TE buffer. We then took 100 µL of each of the dilutions 10^{-1}, 10^{-2}, and 10^{-3}, culturing into petri dishes containing SMSA medium. The dishes were incubated at 28 ºC for 3 to 5 days, depending on when the colonies first appeared.

Isolating and testing the bacterium: From the samples, we selected bacterial colonies that showed similar

growth patterns to those of the *R. solanacearum* control strain CIAT 1008. The control strain came from Ibagué (Tolima, Colombia) and was held at the strain bank in the Cassava Pathology Laboratory, CIAT.

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

The oxidase test was carried out by placing two drops of a 1% aqueous solution of dichlorohydrate of tetra-methyl-p-phenylenediamine on a strip of filter paper, which was then rubbed over a colony. The reaction was considered positive when the solution in the paper turned from colorless to dark purple within the next 30 to 60 s. The strain CIAT 1008 was included as check for both tests.

Extracting DNA and conducting PCR: Genomic DNA was extracted from pure colonies of selected strains with a 36-h growth in nutritive agar (Seal *et al*., 1999, Plant Pathology 48: 115 – 120). Each colony was suspended in vials containing 100 σL of sterilized distilled water, and heated in a bain-marie at 96 ºC for 5 min. The vials were then centrifuged at 12,000 rpm for 2 min and 2.5 σL of the supernatant taken as DNA mold for the polymerase chain reaction (PCR).

The volume of the cocktail for amplification was 9.98 µL, which contained a 1.25X buffer for *Taq* polymerase; 0.012 mM of each dNTP; 1.87 mM $MgCl_2$; 0.25 U *Taq* polymerase; and 0.16 σM of each of the primers OLI 1 (5'-GGGGGTAGCTTGCTACCTGCC-3') and Y2 (5'-CCCACTGCTGCCTCCCGTAGGAGT-3'), (Martins, 2000, Thesis: Polymerase Chain Reaction in the diagnosis of Bacterial Wilt caused by *Ralstonia solanacearum* Georg-August University, Gottingen, Germany, p127; Seal *et al*., 1999, Plant Pathology 48: 115 – 120).

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

Pathogenicity test and confirmation of race 2: The reaction of hypersensitivity obtained in tobacco leaves 48 h after inoculation indicated that 63 of the 72 strains caused a typical hypersensitivity reaction in tobacco leaves (Figure 1.21.2). The remaining 9 strains induced yellowing, an atypical reaction of hypersensitivity for this race. Eight of these strains came from the Colombian Atlantic Coast (Urabá).

Seventy-one strains were pathogenic when inoculated into plantain plants, confirming that they belonged to race 2. Only one strain, which came from Urabá, was not pathogenic (Figure 1.21.3). The separation-of-means test, estimated through MSD (ζ = 5%), led to grouping the strains in three categories according to their pathogenicity (AUDPC). The highly pathogenic strains had AUDPC values between 45.13 and 73.38; the strains with moderate pathogenicity showed values between 18.00 and 43.13; and the strains with low pathogenicity had values between 0 and 15.75 (Table 1.21.2)

The DNA was amplified in an MJ Research PTC-100 thermal cycler, using the following program: initial denaturation for 2 min at 96 ∀C; 50 denaturation cycles, e ach for 20 s at 94 ∀C; annealing for 20 s at 62 ∀C; extension for 30 s at 72 ∀C; and a final extension of 5 min at 72 ∀C (Seal *et al*., 1999). The PCR products were separated in 1.5% agarose gels, dyed with 0.001% ethidium bromide, and visualized under ultraviolet light. Evaluations were based on the presence of a band, 287–288 base pairs long, from the 16S rRNA fragment generated by amplification with the specific primer OLI 1 and the nonspecific primer Y2 (Seal *et al*., 1999, Plant Pathology 48: 115 – 120).

Table 1.21.1. Samples of plant tissue used to isolate *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt) of plantain, according to origin by department, crop, and source of isolate.

Sample No	Origin			Sample No	Origin		
	Department/ Locality	Crop	Source		Department/ Locality	Crop	Source
1	Quindío	Plantain	Rachis	79	Montenegro (Quindío)	Plantain	Rhizome
2	Quindío	Plantain	Petiole	80	Montenegro (Quindío)	Plantain	Pseudostem
3	Quindío	Plantain	Petiole	81	Montenegro (Quindío)	Plantain	Fruit
4	Urabá (Antioquia)	Banana	Pseudostem	83	Quindío	Plantain	Fruit
5	Urabá (Antioquia)	Banana	Rhizome	84	Quindío	Plantain	Pseudostem
6	Urabá (Antioquia)	Banana	Fruit	85	Quindío	Plantain	Sucker
7	Urabá (Antioquia)	Banana	Fruit	86	Calarcá (Quindío)	Plantain	Rachis
15	Quindío	Plantain	Soil	88	LaTebaida (Quindío)	Plantain	Rhizome
17	Jamundí (Valle)	Plantain	Soil	89	LaTebaida (Quindío)	Plantain	Pseudostem
18	Jamundí (Valle)	Plantain	Sucker	90	Montenegro (Quindío)	Plantain	Petiole
32	Caquetá	Plantain	Pseudostem	91	Montenegro (Quindío)	Plantain	Rachis
33	Caquetá	Plantain	Pseudostem	92	Quimbaya (Quindío)	Plantain	Petiole
34	Caquetá	Plantain	Rachis	94	Quimbaya (Quindío)	Plantain	Rachis
38	Quindío	Plantain	Soil	95	Quimbaya (Quindío)	Plantain	Rhizome
39	Quindío	Plantain	Soil	96	Quimbaya (Quindío)	Plantain	Pseudostem
40	Quimbaya (Quindío)	Plantain	Soil	97	Quimbaya (Quindío)	Plantain	Rhizome
41	Quimbaya (Quindío)	Plantain	Soil	98	Quimbaya (Quindío)	Plantain	Rachis
42	Fuente de Oro (Meta)	Plantain	Pseudostem	99	Quimbaya (Quindío)	Plantain	Pseudostem
43	Fuente de Oro (Meta)	Plantain	Pseudostem	100	Armenia (Quindío)	Plantain	Sucker
48	Armenia (Quindío)	Plantain	Fruit	101	Armenia (Quindío)	Plantain	Pseudostem
54	Fuente de Oro (Meta)	Plantain	Pseudostem	102	Quimbaya (Quindío)	Plantain	Petiole
55	Fuente de oro (Meta)	Plantain	Pseudostem	104	Armenia (Quindío)	Plantain	Fruit
57	Fuente de oro (Meta)	Plantain	Pseudostem	106	Armenia (Quindío)	Plantain	Pseudostem
58	Fuente de oro (Meta)	Plantain	Pseudostem	107	Armenia (Quindío)	Plantain	Fruit
59	Fuente de oro (Meta)	Plantain	Pseudostem	109	Armenia (Quindío)	Plantain	Petiole
60	Fuente de oro (Meta)	Plantain	Pseudostem	110	Magdalena	Banana	Pseudostem
63	Granada (Meta)	Plantain	Pseudostem	111	Magdalena	Banana	Rhizome
64	Granada (Meta)	Plantain	Pseudostem	112	Magdalena	Banana	Sucker
65	Granada (Meta)	Plantain	Pseudostem	113	Palmira (Valle)	Heliconia	Pseudostem
66	Granada (Meta)	Plantain	Pseudostem	114	Palmira (Valle)	Heliconia	Rhizome

Table 1.21.1, (cont'd)

Sample No	Origin			Sample No	Origin		
	Department/ Locality	Crop	Source		Department/ Locality	Crop	Source
69	Granada (Meta)	Plantain	Pseudostem	160	Quindío	Plantain	Soil
70	Granada (Meta)	Plantain	Pseudostem	161	Quindío	Plantain	Soil
71	Urabá (Antioquia)	Plantain	Rhizome	588	Fuente de Oro (Meta)	Plantain	Petiole
72	Urabá (Antioquia)	Plantain	Pseudostem	CIAT 1008[4]	Ibagué (Tolima)	Plantain	Sin Inf.
73	Urabá (Antioquia)	Plantain	Pseudostem				
76	Montenegro (Quindío)	Plantain	Pseudostem				
78	Montenegro (Quindío)	Plantain	Rachis				

Biovar determination: Ralstonia solanacearum strains can be classified into different biovars according to Hayward (1964, Annual review of phytophatology 29: 64-87) by their production of acids from the disaccharides cellobiose, lactose, and maltose and by their oxidation of the hexose alcohols sorbitol, dulcitol, and mannitol in base medium (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria., (APS), 151-173).

The base medium contained (per liter) 1 g $NH_4H_2PO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2O$, 1.0 g Bacto™ Peptone, 3.0 g agar, and 80.0 mg bromothymol blue with a pH between 7.0 and 7.1, becoming green olive. The medium was then sterilized by autoclaving at 121 ºC, with a 20-lb pressure, for 20 to 30 min (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria, (APS), 151-173).

We also prepared 10% aqueous solutions of each of the test carbohydrates and sterilized them by filtration, using Millipore® filters with a pore size of 0.22 µm. These solutions of carbohydrates were added to the sterilized base medium when the temperature was between 55 and 60 ºC, obtaining a final concentration of 1%. After mixing the base medium with each sugar, about 5 mL of the liquid medium placed within tubes containing sterilized cultures (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria, (APS), 51-173).

The medium was inoculated with the bacterium by means of puncturing as deep as three quarters of the medium, using colonies with a 24-h growth in nutritive agar. Reactions were assessed after 1, 3, 7, 14, and 28 days of incubation at 28 ºC. The color changed from green olive to yellow as acids were produced from the disaccharides and the hexose alcohols were oxidized (Denny and Hayward, 2001, Laboratory guide for identification of plant pathogenic bacteria, (APS), 151-173).

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

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

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

suspension was determined by absorbance readings in a spectrophotometer and adjusted to 0.1 with a wavelength of 600 nm. This corresponded to about 1×10^{-8} cfu · mL^{-1} (He *et al*., 1983, Plant Disease 67: 1357-1361).

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

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

Hypersensitivity test: The capacity of the strains to induce a hypersensitivity reaction was tested in leaves of tobacco (*Nicotiana tabacum*). From pure cultures, a suspension was prepared in sterilized deionized water, using colonies with 24 h of incubation in nutritive agar and an absorbance of 0.1 with a 600-nm wavelength, thus corresponding to a concentration of about 1×10^{8} cfu · mL^{-1} (He *et al.* ,1983, Plant Disease 67: 1357-1361)

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

The reaction was evaluated, beginning 16 h after inoculation, for symptoms corresponding to hypersensitivity to race 2 of *R. solanacearum*. These are chlorosis in infected cells of the parenchyma; wet tissue limited by a defined margin of uninoculated tissue; the area of infiltrated leaf becoming, between 36 and 60 h later, necrotic and dry from water loss; and, finally, the affected area becoming thin, white, and translucent (Lozano and Sequeiro, 1970, Phytopathology 60: 833-838).

Data analysis: An analysis of variance was carried out for the variable AUDPC. A test for the separation of means by minimum significant difference (MSD; $\zeta = 5\%$) was also conducted to separate the strains into groups according to their levels of pathogenicity (Table 1.21.2).

Results and Discussion

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

Analysis through the polymerase chain reaction (PCR): In a 1.5% agarose gel, a band with a molecular weight of 288 bp was detected. For 106 of the 189 strains obtained, the fragment was located in gene 16S rRNA, which enabled us to identify them as *R. solanacearum* (Figure 1.21.1).

Table 1.21.2. Origin and pathogenicity of 72 strains of *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt), isolated from banana, plantain, and heliconias.

Strain No	Origin		Path'y			Hypers.[4]	Strain no.	Origin		Path'y			Hypers.[4]
	Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]			Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	
1	Quindío	Pl.	R.	18,00	2	+	79	Montenegro (Quindío)	Pl.	Rh.	66,88	1	+
2	Quindío	Pl.	Pe.	69,38	1	+	80	Montenegro (Quindío)	Pl.	Ps.	67,88	1	+
3	Quindío	Pl.	Pe.	49,00	1	+	81	Montenegro (Quindío)	Pl.	F.	10,88	3	+
4	Urabá (Antioquia)	B.	Ps.	43,13	2	-	83	Quindío	Pl.	F.	55,00	1	+
5	Urabá (Antioquia)	B.	Rh	38,75	2	-	84	Quindío	Pl.	Ps.	61,00	1	+
6	Urabá (Antioquia)	B.	F.	31,83	2	-	85	Quindío	Pl.	Su.	68,38	1	+
7	Urabá (Antioquia)	B.	F.	62,17	1	-	86	Calarcá (Quindío)	Pl.	R.	59,75	1	+
15	Quindío	Pl.	S.	37,63	2	+	88	La Tebaida (Quindío)	Pl.	Rh.	61,75	1	+
17	Jamundí (Valle)	Pl.	S.	69,50	1	+	89	La Tebaida (Quindío)	Pl.	Ps.	60,38	1	+
18	Jamundí (Valle)	Pl.	Su.	42,50	2	+	90	Montenegro (Quindío)	Pl.	Pe.	19,25	2	-
32	Caquetá	Pl.	Ps.	33,88	2	+	91	Montenegro (Quindío)	Pl.	R.	19,38	2	+
33	Caquetá	Pl.	Ps.	40,38	2	+	92	Quimbaya (Quindío)	Pl.	Pe.	63,13	1	+
34	Caquetá	Pl.	R.	27,63	2	+	94	Quimbaya (Quindío)	Pl.	R.	47,25	1	+
38	Quindío	Pl.	S.	59,50	1	+	95	Quimbaya (Quindío)	Pl.	Rh.	65,63	1	+
39	Quindío	Pl.	S	62,00	1	+	96	Quimbaya (Quindío)	Pl.	Ps.	33,00	2	+
40	Quimbaya (Quindío)	Pl.	S.	15,75	3	+	97	Quimbaya (Quindío)	Pl.	Rh.	28,63	2	+
41	Quimbaya (Quindío)	Pl.	S.	56,25	1	+	98	Quimbaya (Quindío)	Pl.	R.	59,50	1	+
42	Fuente de Oro (Meta)	Pl.	Ps.	28,00	2	+	99	Quimbaya (Quindío)	Pl.	Ps.	40,25	2	+
43	Fuente de Oro (Meta)	Pl.	Ps.	20,75	2	+	100	Armenia (Quindío)	Pl.	Su.	71,88	1	+
48	Armenia (Quindío)	Pl.	F.	37,13	2	+	101	Armenia (Quindío)	Pl.	Ps.	58,50	1	+
54	Fuente de Oro (Meta)	Pl.	Ps.	36,25	2	+	102	Quimbaya (Quindío)	Pl.	Pe.	1,38	3	+
55	Fuente de oro (Meta)	Pl.	Ps.	55,25	1	+	104	Armenia (Quindío)	Pl.	F.	41,25	2	+
57	Fuente de oro (Meta)	Pl.	Ps.	45,63	1	+	106	Armenia (Quindío)	Pl.	Ps.	24,75	2	+
58	Fuente de oro (Meta)	Pl.	Ps.	56,63	1	+	107	Armenia (Quindío)	Pl.	F.	68,25	1	+
59	Fuente de oro (Meta)	Pl.	Ps.	0,00	3	+	109	Armenia (Quindío)	Pl.	Pe.	45,13	1	+
60	Fuente de oro (Meta)	Pl.	Ps.	47,25	1	+	110	Magdalena	B.	Ps.	63,25	1	+

Table 1.21.2 (cont'd)

Strain No	Origin		Path'y				Strain No.	Origin		Path'y			
	Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	Hypers.[4]		Dep't or locality	Crop	Source[1]	AUDPC[2]	Group[3]	Hypers.[4]
63	Granada (Meta)	Pl.	Ps.	50,69	1	+	111	Magdalena	B.	Rh.	34,38	2	+
64	Granada (Meta)	Pl.	Ps.	46,13	1	+	112	Magdalena	B.	Su.	29,50	2	-
65	Granada (Meta)	Pl.	Ps.	47,63	1	+	113	Palmira (Valle)	H.	Ps.	40,50	2	+
66	Granada (Meta)	Pl.	Ps.	69,75	1	+	114	Palmira (Valle)	H.	Rh.	40,38	2	+
67	Fuente de oro (Meta)	Pl.	Ps.	41,63	2	+	115	Palmira (Valle)	H.	Rh.	33,63	2	+
69	Granada (Meta)	Pl.	Ps.	27,00	2	+	160	Quindío	Pl.	S.	12,38	3	+
70	Granada (Meta)	Pl.	Ps.	5,75	3	+	161	Quindío	Pl.	S.	1,75	3	+
71	Urabá (Antioquia)	Pl.	Rh.	21,25	2	-	588	Fuente de Oro (Meta)	Pl.	Pe.	71,25	1	+
72	Urabá (Antioquia)	Pl.	Ps.	10,75	3	-	CIAT 1008[5]	Ibagué (Tolima)	Pl.	No date.	65,13	1	+
73	Urabá (Antioquia)	Pl.	Ps.	10,75	3	-							
							DMS ζ= 5%, 28,33						
76	Montenegro (Quindío)	Pl.	Ps.	61,88	1	+							
78	Montenegro (Quindío)	Pl.	R.	73,38	1	+							

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

Of seven strains isolated from banana, five were moderately and two were highly pathogenic. Three strains from heliconias showed moderate levels. Differences in pathogenicity were found among strains isolated from soil or various plant tissues, for example, those from rhizomes and rachis were more highly pathogenic than those from other tissues. No relationship was found between pathogenicity and geographical origin (Table 1.21.2).

Conclusions

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

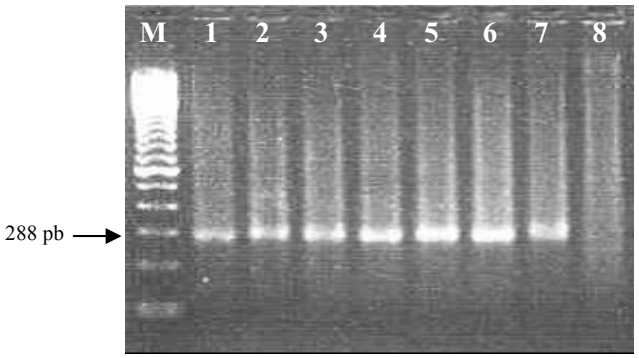

Figure 1.21.1. The figure shows the characteristic band of the bacterium *Ralstonia solanacearum*, causal agent of *moko* (bacterial wilt) in plantain. The product, measuring 288 bp, was amplified with primers OLI 1 and Y2 in the 16S rRNA region. M = 100-bp marker; lanes 1 to 3 = strains from Quindío, Colombia; lanes 4 to 6 = strains from Urabá, Colombia; lane 7 = CIAT 1008; lane 8 = negative control.

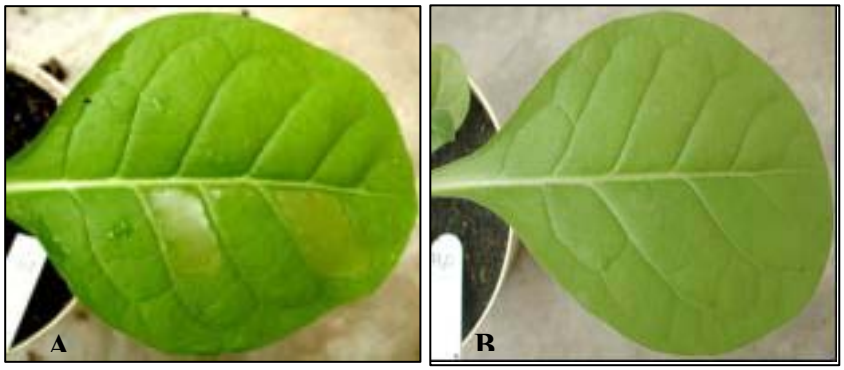

Figure 1.21.2. (A) Typical reaction of hypersensitivity in tobacco leaf, 48 h after inoculation with strain 79 of *Ralstonia solanacearum* race 2, causal agent of *moko* (bacterial wilt) in plantain. (B) Control inoculated with sterilized distilled water.

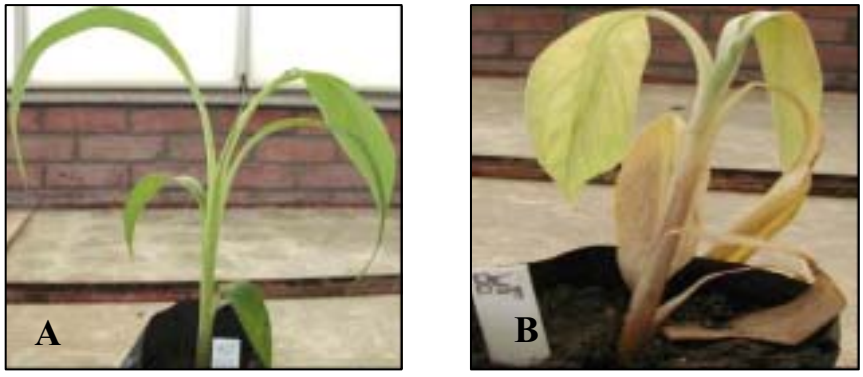

Figure 1.21.3. Plantain inoculated the center of the pseudostem at 2 cm above the soil surface, under greenhouse conditions: (A) Control whit water. (B) Wilt and yellowing of leaves caused by strain 85 of *R. solanacearum* race 2.

Activity 1.22. Molecular characterization of isolates of *Colletotrichum* spp. infecting tree tomato, mango and lemon Tahiti in Colombia.

Contributors: M. Cadavid, J. Osorio (CORPOICA) and S. Kelemu

Rationale

Colombia dedicates approximately 42,000 hectares of land to production of citrus, and 13,500 and 7,500 hectares to tree tomato and mango, respectively (Páez, 1995, ASCOLFI-Informa 21:36-39). The disease anthracnose caused by the fungal pathogen *Colletotrichum* spp. is a major production constraint resulting in losses in the range of 50-100% in various production zones. Anthracnose disease symptoms include fruit rots and blights in shoots, leaf and flowers. The disease can cause up to 50% yield loss in citrus in areas such as Valle del Cauca, Piedemonte and some areas in Magdalena (Osorio, 2000, unpublished results). In tree tomato, the disease directly affects the fruit causing total losses in areas such as Antioquia, Caldas, Risaralda, Cesar, Cundinamarca, Boyacá, Huila, Magdalena, Nariño, Tolima, Cauca andValle del Cauca, in the absence of control measures are taken, and losses between 10-25% under continuous use of fungicides. Mango anthracnose symptoms include blossom and leaf blight, fruit lesions and in severe cases tree dieback.

Effective control measure of the disease in various fruit crops is complicated by the complexity of the pathogen population structure and high variability. The high variability in morphology of *Colletotrichum* spp. in culture and the wide host range makes it difficult to use these criteria for taxonomic purposes. Molecular tools have been used for a more reliable species identification method. The objectives of this study are: 1) to characterize the pathogen population structure infecting mango, tree tomato and lemon tahiti, and 2) to use molecular approach and determine the species infecting these fruit crops. We report here very preliminary results of this work.

Materials and Methods

Fungal isolates: Thirty-five monoconidial isolates of *Colletotrichum* spp. that are maintained at the Integrated Disease and Pest Management Program of CORPOICA were used (Table 1.22.1). The isolates were obtained from naturally diseased tissues in various regions of Colombia. The isolates were grown on oatmeal agar at 28 ∀C for 5-8 days for DNA isolation. For DNA isolations, fungal cultures were grown in V-8 tomato juice broth supplemented with 10 σg/ml of streptomycin and incubated at 28∀C for 8 days in the dark and in a shaker at 130 rpm. A *C. gloeosporioides* isolate CIAT 16100 was included as a control.

DNA extraction: DNA was isolated using methods described previously (Kelemu *et al*., 1999, European Journal of Plant Pathology 105: 261-272). DNA concentration was quantified using DyNA QUANT 200⊇, aliquot at concentrations of 20ng/σl, and stored at –80 ∀C for further analysis.

Polymerase chain reaction (PCR) amplifications: For random amplified polymorphic DNAs (RAPD) analysis and primer screening, several arbitrary 10-base, oligonucleotide primers from Operon Technologies (Alameda, CA) were used for polymerase chain reaction (PCR) amplification. Amplification conditions were as described earlier (Kelemu *et al*., 1999, European Journal of Plant Pathology 105: 261-272). PCR primers for taxonomic purposes included internal transcribed spacer, ITS4 (5'-TCCTCCGCTTATTGATATGC-3'),*C.gloeosporioides* (CgInt) [5'-GGCCTCCCGCCTCCGGGCGG-3'] and *C. acutatum* (CaInt2) [5'- GGGGAAGCCTCTCGCGG-3'] To determine *C. acutatum*, the primers ITS4 and CaInt2 were used. PCR reactions were conducted in a total volume of 20 σl, containing 40 ng of DNA, 1.5 mM $MgCl_2$, 200 σM each of dNTP, 0.3 σM each of the primers, 1 unit of Taq Polimerasa Promega⊇ (Promega Corp, Madison, WI), and 1X buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1%

Triton⊇ X-100). Amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc, Watertown, MA) beginning with a 5 min of denaturation step at 95∀C, followed by 40 cycles consisting of 30 seconds at 95∀C, 30 seconds at 60∀C and 1 min at 72∀C (final for 7 min).

To determine the species *C. gloeosporioides*, the primers ITS4 and CgInt were used. PCR reactions were conducted in a total volume of 20 σl, containing 40 ng of DNA, 2.0 mM $MgCl_2$, 200 σM each of dNTP, 0.5 σM each of the primers, 1 unit of Taq Polimerasa Promega⊇ (Promega Corp, Madison, WI), and 1X buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.1% Triton⊇ X-100). Amplifications were carried out in a PTC-100 thermal cycler (MJ Research, Inc, Watertown, MA) beginning with a 5 min of denaturation step at 95∀C, followed by 40 cycles consisting of 30 seconds at 95∀C, 30 seconds at 65∀C and1 min at 72∀C (final for 7 min). Amplification products were resolved by electrophoresis in a 1.2 % agarose gel, stained with ethidium bromide, and photographed under UV lighting.

Results and Discussion

The DNA extraction protocol used generated high quality DNA (Figure 1.22.1)

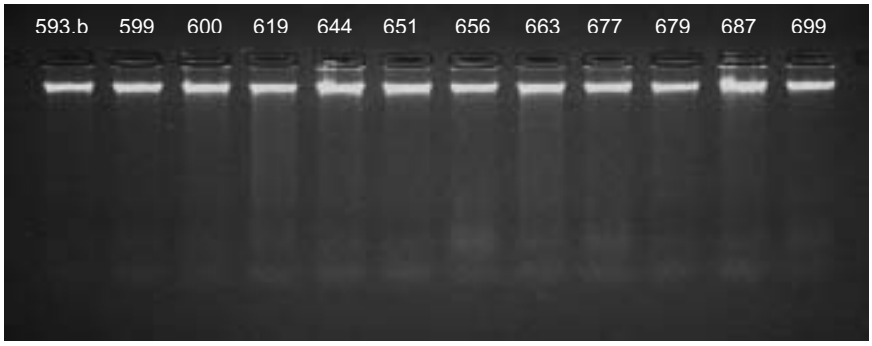

Figure 1.22.1. DNA samples isolated from isolates of *Colletotrichum* spp**.** originated from anthracnose lesions of citrus fruits in Colombia. The numbers at each lane are isolate numbers indicated in Table 1.22.1.

PCR amplifications: Many of the arbitrary primers tested so far resulted in limited polymorphisms (Figure 1.22.2). We are currently screening 40-50 more primers in an attempt to identify those that would generate polymorphism.

Amplifications with the primers CaInt2 – ITS4, indicated that all the isolates tested, with the exception of isolates 656, 677, 699 and the control isolate *C. gloeosporioides* (Cg), amplified a product with a 490 bp size that indicates that the species is *Colletotrichum acutatum*. On the other hand amplifications with the primers CgInt – ITS4 resulted isolates 656, 677, 699 as well as the control isolates generating a DNA product of 450 bp indicating that they all belong to the species *Colletotrichum gloeosporioides* (Figure 1.22.3). The results of the molecular identification of the isolates tested are presented in Table 1.22.1.
In addition to screening more arbitrary primers, we are currently working on implementing the amplified fragment length polymorphism (AFLP) technique for characterization of the pathogen population infecting the three fruit crops in this study.

Table 1.22.1. Isolates of *Colletotrichum* spp infecting citrus fruits used in this study.

	Isolate No.	Zone	Farm	Host	Host tissue	*Colletotrichum spp.*
1.	3	Armenia	El Piñal	Limón Tahití	Flower	
2.	5	Caicedonia	Danubio	Limón Tahití	Flower	*C. acutatum*
3.	6	Manizales	La Bejuca	Limón Tahití	Bud	*C. acutatum*
4.	14	Caicedonia	Danubio	Limón Tahití	Bud	*C. acutatum*
5.	55	Pereira	Catalina (FEDECAFË)	Limón Tahití	Bud	*C. acutatum*
6.	83	Pereira	Catalina (FEDECAFË)	Limón Tahití	Bud	*C. acutatum*
7.	100	Caicedonia	Maracaibo	Limón Tahití	Flower	*C. acutatum*
8.	107	Pereira	Catalina (FEDECAFË)	Limón Tahití	Bud	*C. acutatum*
9.	212	Caicedonia	Maracaibo	Limón Tahití	Flower	*C. acutatum*
10.	269	Pereira	Yarima	Limón Tahití	Bud	*C. acutatum*
11.	275	Pereira	Yarima	Limón Tahití	Bud	*C. acutatum*
12.	589	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
13.	590	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
14.	592	Cumaral	Las Brisas	Limón Tahití	Flower	*C. acutatum*
15.	593.a.	Cumaral	Las Brisas	Limón Tahití	Flower	*C. acutatum*
16.	593.b.	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
17.	594	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
18.	595	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
19.	596	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
20.	597	Cumaral	Las Brisas	Limón Tahití	Flower	*C. acutatum*
21.	599	Cumaral	Las Brisas	Limón Tahití	Bud	*C. acutatum*
22.	600	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
23.	611	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
24.	619	Villavicencio	El Refugio	Limón Tahití	Flower	*C. acutatum*
25.	644	Zona Bananera	La Inmaculada	Limón común	Flower	*C. acutatum*
26.	647	Zona Bananera	La Inmaculada	Limón común	Bud	
27.	651	Ciénaga	Las Margaritas	Limón común	Bud	*C. acutatum*
28.	654	Ciénaga	Las Margaritas	Limón común	Bud	
29.	656	Ciénaga	Las Margaritas	Limón común	Flower	*C. gloeosporioides*
30.	663	Ciénaga	Las Margaritas	Limón común	Flower	*C. acutatum*
31.	677	Zona Bananera	La Inmaculada	Naranja Tangelo	Leaf	*C. gloeosporioides*
32.	679	Zona Bananera	La Inmaculada	Limón común	Bud	*C. acutatum*
33.	681	Ciénaga	Las Margaritas	Limón común	Flower	
34.	687	Zona Bananera	La Inmaculada	Limón común	Bud	*C. acutatum*
35.	699	Montenegro	Estancia	Naranja Valencia	Flower	*C. gloeosporioides*

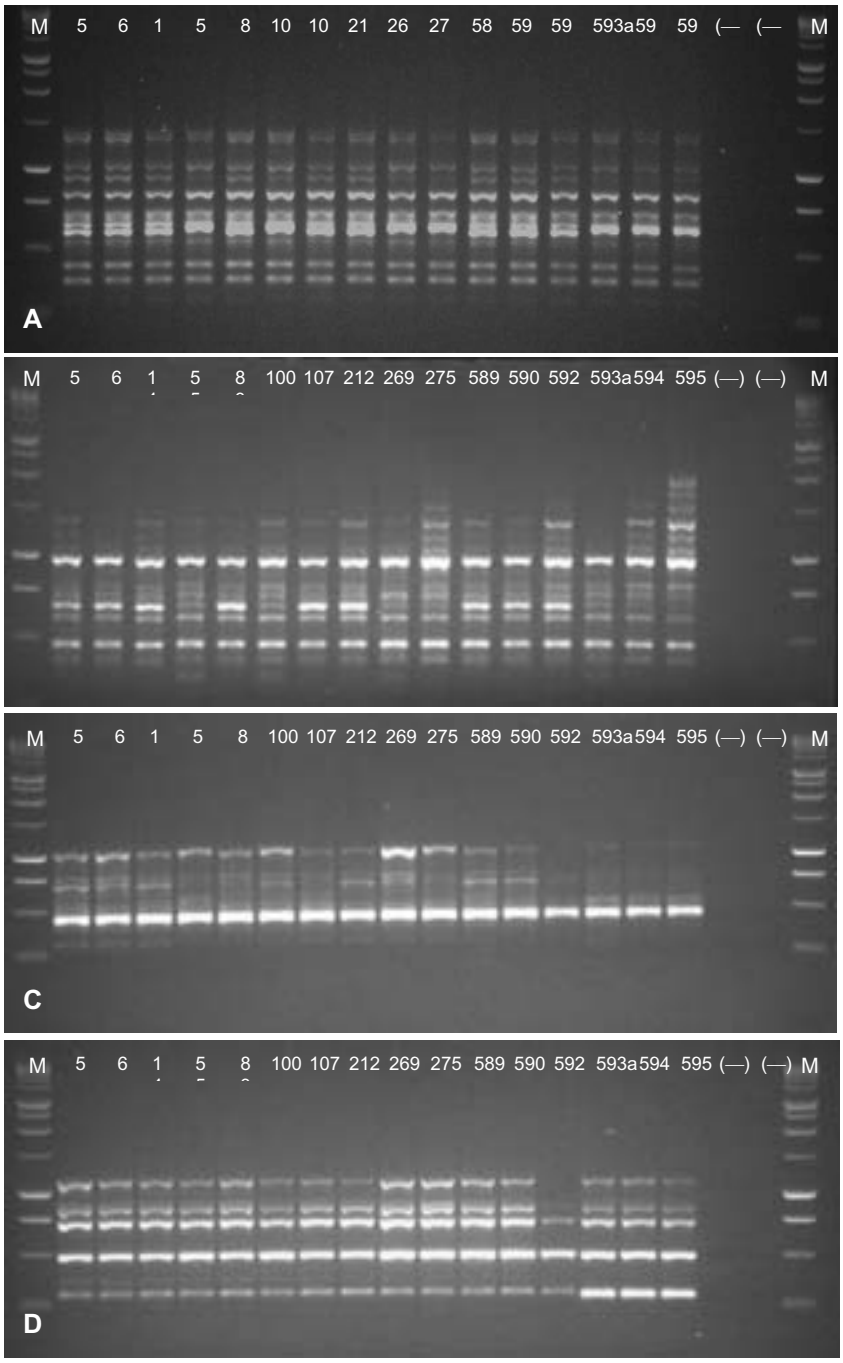

Figure 1.22.2. DNA samples from isolates of *Colletotrichum* spp**.** amplified with arbitrary primers **A,** primer A-02; **B,** primer AK-09; **C,** primer A-03; **D,** primer C-02. The numbers at each lane are isolate numbers described in Table 1.22.1. Lanes (∝), negative control without sample DNA; M, 1 kb marker.

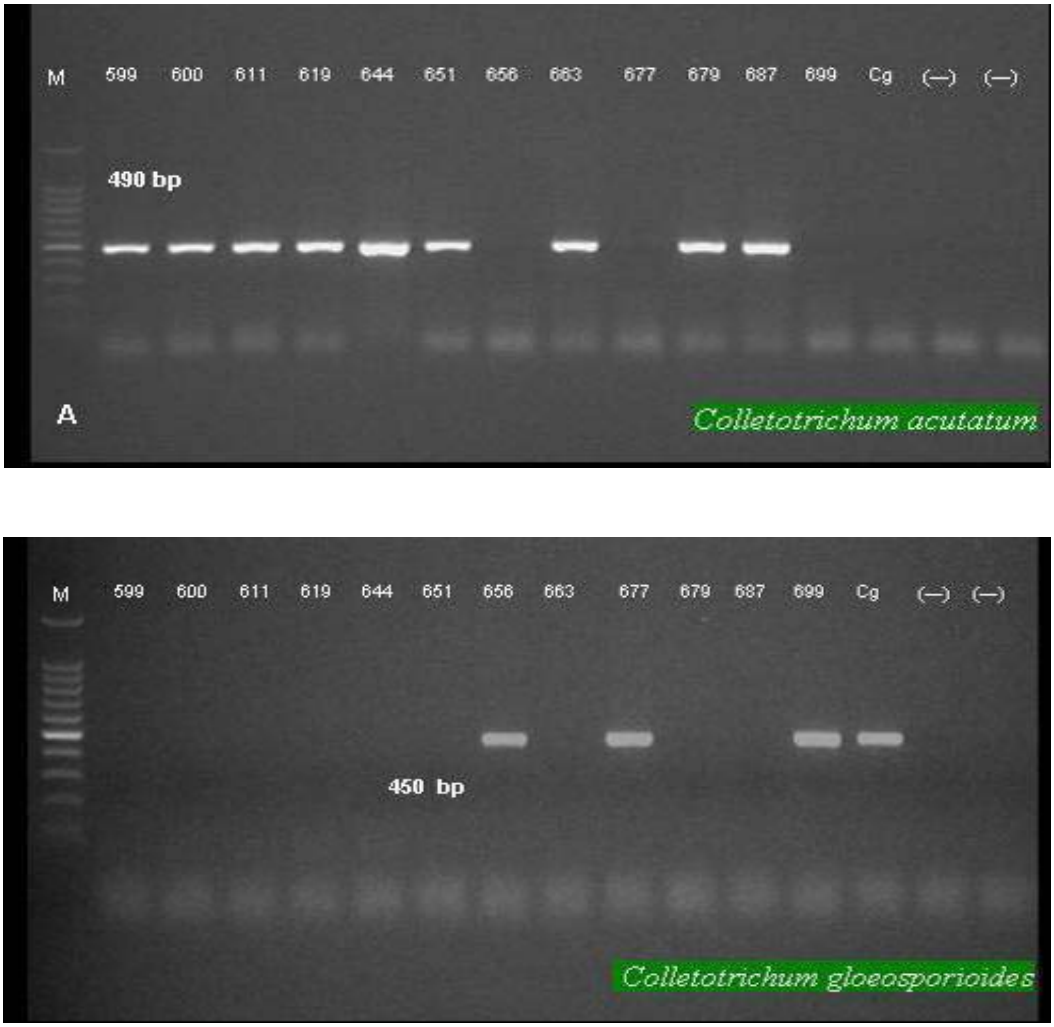

Figure 1.22.3. Taxonomic identification of isolates of *Colletotrichum* spp. using PCR. **A,** *Colletotrichum acutatum* (using primers CaInt2 and ITS4); **B,** *Colletotrichum gloeosporioides* (with primers CgInt and ITS4). The numbers at each lane are isolate numbers described in Table 1. Lanes (∝), negative control without sample DNA; M, 1 size marker; **Cg**, positive control *C. gloeosporioides*