Output 1: Pest and disease complexes described and analyzed.

Activity 1.1. Identification of common bean genotypes and interspecifc lines resistant to *Rhizoctonia solani.*

Contributors: C. Jara, G. Castellanos, and G. Mahuku.

Highlight:

- We identified several interspefic lines, combining resistance to angular leaf spot, anthracnose, ascochyta blight and *Rhizoctonia* root rots. These materials constitute an important set for use in breeding programs intended for multiple constraint improvement.

Rationale

Root rots have become a major bean production constraint, especially for small-holder farmers with limited arable land (declining farm sizes), often do not practice crop rotation resulting in increasing low soil fertility problems. Several pathogen cause root rots, and these often occur in complex. The prevalence of some pathogens is associated with high soil humidity and rain conditions (e.g. *Pythium* spp, *Fusarium* spp), while others are associated with water deficit and drought conditions (e.g. *Macrophomina phaseolina*). Identification of genotypes that are resistant to the largest number of root rot causing pathogens would be a major contribution towards the development of genotypes resistant to multiple constraints. Such materials would be useful as parents in breeding programs, or they can be deployed in areas where these pathogens are a major production constraint. Towards this goal, we have started evaluating genotypes from the international root rot nursery (put together in the 90s) and interspecific lines direved from *P. vulgaris* by *P. coccinrus* or *P. polyanthus* crosss for their reaction to *Rhizoctonia solani*. The interspecific lines have previously been found to have resistance to other bean pathogens, including *Coletotrichum lindemuthianum* and *Phaeoisariopsis griseola*.

Materials and Methods

Forty genotypes representing the international root rot nursery (Table 1.1.1) and 50 interspecific lines (Table 1.1.2) were evaluated under greenhouse conditions for their reaction to a mixture of *R. solani* isolates, previously isolated from Popayán, Pradera, Darien, Pereira and Quilichao, Colombia. Inoculum preparation was done as described by Abawi and Pastor-Corrales (Abawi and Pastor-Corrales, 1990. Diagnosis, research methodologies and management strategies. CIAT.114 pp). For each entry, 10 seeds were planted in 50 cm rows and covered with 100 grams of *R. solani* inoculum. Disease evaluations were done 20 days after planting using the 1-9 scale of Schoonhoven and Pastor-Corrales, (Schoonhoven and Pastor-Corrales, 1987. Standard system for the evaluation of bean germplasm. CIAT).

Results and Discussion

Only 14 genotypes from the international root rot nursery were tolerant or resistant to the new *R. solani* isolates used in this study (Table 1.1.1). In contrast, all the interspecific lines were highly resistant or tolerant to *R. solani*, and none was susceptible (Table 1.1.2). These results point to the potential usefulness of the secondary gene pool as a source of *R. solani* resistance in bean improvement programs.

Table 1.1.1. Reaction of the international *Rhizoctonia* root rot nursery to inoculation with several new isolates of *Rhizoctonia solani* under greenhouse conditions.

Genotype	Disease severity	Genotype	Disease severity
BAT 332	7.3	G003807	5.2
RIZ 21	7.2	EMP 81	5.1
BAT 1753	6.9	A 195	5.1
RIZ 30	6.8	BAT 1297	5.0
A 197	6.7	PORRILLO SINTETICO	5.0
A 107	6.6	AND 313	5.0
A 300	6.4	NEP-2	4.9
AND 286	6.4	ARGENTINO	4.9
SANILAC	6.3	ICA TUI	4.8
BAT 477	6.2	BAT 1385	4.7
LM-21525-0	6.2	NIMA	4.5
RIO TIBAGI	5.7	XAN 112	4.5
ICA PIJAO	5.7	CG/82-131	4.4
DIACOL CALIMA	5.7	DOR 500	4.4
OJO DE CABRA 400	5.6	AFR 159	4.3
CORNELL-49-242	5.3	ROSINHA	4.2
AND 323	5.3	ECUADOR 1056	4.2
G 003719	5.3	CG/82-108	4.1
MORTINO	5.3	CG/82- 77	3.6
A 211	5.2	CG/82- 81	3.5

Table 1.1.2. Response of interspecific lines to inoculation with *Rhizoctonia solani* isolates fromdifferent locations of Colombia under greenhouse conditions.

	Origin	Identification	Generation	Type of cross	R. solani severity
1	URG	BAT1253 x G35325	F6	Pv x Pp	3.4
2	URG	BAT 338 x G35252	F 11	Pv x Pc	4.4
3	URG	BAT 338 x G35252	F 11	Pv x Pc	3.9
4	URG	BAT 338 x G35252	F 11	Pv x Pc	4.4
5	URG	BAT 338 x G35252	F 11	Pv x Pc	3.9
6	URG	BAT 338 x G35252	F 11	Pv x Pc	4.9
7	URG	BAT 338 x G35252	F 11	Pv x Pc	5.6
8	URG	BAT 338 x G35252	F 11	Pv x Pc	3.3
9	URG	BAT 338 x G35252	F 12	Pv x Pc	2.9
10	URG	BAT 338 x G35252	F 12	Pv x Pc	4.7
11	URG	BAT 338 x G35252	F 12	Pv x Pc	4.6
12	URG	BAT 338 x G35252	F 12	Pv x Pc	5.3
13	URG	BAT 338 x G35252	F 12	Pv x Pc	3.8
14	URG	BAT 338 x G35252	F 12	Pv x Pc	2.9
15	URG	BAT 338 x G35252	F 12	Pv x Pc	4.5
16	URG	BAT 338 x G35252	F 12	Pv x Pc	2.7
17	URG	BAT 338 x G35252	F 12	Pv x Pc	4.2
18	URG	BAT 338 x G35252	F 12	Pv x Pc	3.7
19	URG	BAT 338 x G35252	F 12	Pv x Pc	2.9
20	URG	((G35876 x G 3807)x G35182)x A 114	F 10	((Pcw x Pv)x Pp)x Pv	2.6
21	URG	((G35876 x G 3807)x G35182)x A 114	F 10	((Pcw x Pv)x Pp)x Pv	1.5
22	URG	((G35876 x G 3807)x G35182)x S31003	F 10	((Pcw x Pv)x Pp)x Pv	3.1
23	URG	((G35876 x G 3807)x G35182)x S31003	F 10	((Pcw x Pv)x Pp)x Pv	2.1
24	URG	((G35876 x G 3807)x G35325)x VRA81043	F 6	((Pcw x Pv)x Pp)x Pv	2.3
25	URG	((G35876 x S30985)x G21715)x G35336	F 5	((Pcw x Pv)x Pv)x Pp	2.1
26	URG	((G35876 x S30985)x G35182)x G21715	F 5	((Pcw x Pv)x Pp)x Pv	1.9
27	URG	((S13811 x G 677)x G35023)x BAC 24	F 7	((Pcp x Pv)x Pc)x Pv	1.0
28	URG	(G35649 x G 3807)x BAC 24	F 7	(Pcw x Pv)x Pv	2.3
29	URG	(G35649 x G 3807)x BAC 24	F 7	(Pcw x Pv)x Pv	2.0
30	URG	(G35649 x G 3807)x BAC 24	F 7	(Pcw x Pv)x Pv	1.6
31	URG	(G35649 x L32)x BAC 24	F 9	(Pcw x Pv)x Pv	1.8
32	URG	(G35649 x G 3807)x G35023	F 8	(Pcw x Pv)x Pc	3.2
33	URG	AND 107 x Piloy	F 5	Pv x Pp	2.8
34	URG	AND 279 x G35337	F 5	Pv x Pp	2.1
35	URG	Pasto x G35122	F 11	Pv x Pp	3.0
36	URG	Pasto x G35122	F 12	Pv x Pp	2.6
37	URG	PVA1426 x G35180	F 6	Pv x Pp	2.0
38	MEJ2	(ICA PIJAO X G 35171)F1 X ICA PIJAO/-(NN)P-(NN)P(F8)	F 12	2V1C1_1657	5.3
39	MEJ2	(ICA PIJAO X G 35171)F1 X ICA PIJAO/-(NN)P-(NN)P(F8)	F 12	2V1C1_1662	2.6
40	MEJ2	(ICA PIJAO X G 35172)F1 X ICA PIJAO/-4P-(NN)P(F8)	F 12	2V1C2_1670	3.7
41	MEJ2	(ICA PIJAO X G 35172)F1 X ICA PIJAO/-19P-(NN)P(F8)	F 12	2V1C2_1681	5.0
42	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1702	2.3
43	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1734	2.4
44	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1755	3.9
45	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1756	3.1
46	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1757	3.4
47	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1765	3.1
48	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1775	2.2
49	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1776	2.0
50	MEJ2	ICA PIJAO X (ICA PIJAO X G 35877)F1/-(NN)P-(NN)Q-MP	F 8	2V1L2_1813	1.9

Conclusion: The interspecific lines showed good levels of resistance to *R. solani*, while no resistance was available in the international root rot nursery. The international root rot nursery need to be revised, and include new genotypes that show resistance. A starting point would be to evaluate the core collection for their reaction to the new *R. solani* isolates. The potential of the secondary gene pool as a source of useful traits in common bean improvement was again confirmed. These interspecific lines have previously been found resistant to several races of *P. griseola* and *C. lindemuthianum*. The evaluation of these lines for resistance to other root rot causing pathogens (*Fusarium solani, Fusarium oxysporium, Pythium* spp*, Scletinia rolfsi.* etc) is in progress.

Activity 1.2. Virulence characterization of *Colletotrichum lindemuthianum* isolates collected from different bean growing departments of Colombia.

Contributors: G. Mahuku, C. Jara, G. Castellanos, J. Fory

Highlight:

- Showed that the anthracnose population structure in Colombia has changed, with the disappearance of some previously prevalent pathotypes. However, the population remains exclusively of the Andean lineage.

Rationale

Crop protection strategies that provide stability have two basic components: (1) breeding for stable forms of resistance and (2) deploying varieties in ways to prolong their useful lifetime. Knowledge of pathogen population structure can contribute both to resistance breeding efforts and to the development of strategies for the deployment of resistant genes. By analyzing the structure of pathogen populations and the ways in which populations respond to experimental, agricultural and natural constraints, mechanisms by which pathogen populations change can be understood. This understanding can provide the basis for formulating disease support systems that lead to effective disease management. Monitoring, collection and characterization of the anthracnose pathogen is therefore, an on going process in an effort to understand the distribution and diversity of this pathogen, optimize the use of existing sources of resistance and where necessary, identify appropriate new resistance genes.

Materials and Methods

Samples with typical anthracnose symptoms were collected from Boyaca, Santander, Cundinamarca, Cauca, and Darien – Valle. Single spore isolates were established using standard procedures. The virulence phenotype of these isolates was determined based on their differential interaction with a set of 12 international differential varieties (Schoonhoven and Pastor-Corrales, 1987. Standard system for the evaluation of bean germplasm. CIAT). Plant establishment, growth, inoculation and evaluations were

according to procedures reported by Mahuku et al. (Mahuku et al., 2004. European Journal of Plant Pathology 110: 253-263).

Results and Discussion

A total of 46 monosporic isolates of *C. lindemuthianum* were recovered from 50 samples collected. The majority of the isolates (27) were from Santander, eight from Darien, five from Boyaca and three each from Cundinamarca and Cauca. A total of eight pathotypes (races) were characterized among 46 isolates (Table 1.2.1). Pathotype 3 was the most prevalent, represented by 35 isolates from Santander, Boyaca, Darien and Cauca. In addition to pathotype 3, pathotype 1, 4 and 5 were also detected from Boyaca, pathotypes 0, 7 and 13 from Santander, while pathotypes 0, 1 and 137 were characterized from Cundinamarca (Table 1.2.1). Previous characterization studies identified pathotypes 388, 513 and 9 in Cundinamarca, 263 in Cauca, 261 in Santander that were not detected in the present studies. The rest of the pathotypes have been detected before. The presence of mostly simple pathotypes in these departments is probably a reflection of the cultivation of predominantly of varieties from the Andean gene pool and mostly four varieties, Radical, Floiran, Calima and Cargamanto. These results show that the *C. lindemuthianum* population pathogen structure in Colombia is predominantly Andean and composed of relatively simple pathotypes. Introgressing and pyramiding resistance genes found in the following differential cultivars (Mexico 222, PI 207262, J = Tu, K = AB 136, Widusa, Kaboon and G 2333) will be sufficient to manage the anthracnose disease in Colombia.

Table 1.2.1. Characterization of *Colletotrichum lindemuthianum* isolates collected from different departments of Colombia.

	Anthracnose Differential Cultivars														
Isolate	A	B	C	D	E	F	G	H	I	J	K	L	Race	Origin	Variety
CL-554-COL													**0**	**Santander**	**Radical**
CL-517-COL													**0**	**Cundinamarca**	
CL-521-COL	**a**												**1**	**Boyaca**	
CL-519-COL	**a**												**1**	**Boyaca**	
CL-518-COL	**a**												**1**	**Cundinamarca**	
CL-556-COL	**a**	**b**											**3**	**Santander**	**CALIMA**
CL-555-COL	**a**	**b**											**3**	**Boyaca**	**CERINZA**
CL-553-COL	**a**	**b**											**3**	**Santander**	**Radical**
CL-549-COL	**a**	**b**											**3**	**Santander**	**Calima**
CL-548-COL	**a**	**b**											**3**	**Santander**	**Calima**
CL-547-COL	**a**	**b**											**3**	**Santander**	**Calima**
CL-546-COL	**a**	**b**											**3**	**Santander**	**Radical**
CL-545-COL	**a**	**b**											**3**	**Santander**	**Radical**
CL-544-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-543-COL	**a**	**b**											**3**	**Santander**	**Cargamanto**
CL-542-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-541-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-538-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-537-COL	**a**	**b**											**3**	**Santander**	**Radical**
CL-536-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-535-COL	**a**	**b**											**3**	**Santander**	**Calima**
CL-533-COL	**a**	**b**											**3**	**Santander**	**Radical**
CL-532-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-531-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-530-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-529-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-528-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-527-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-526-COL	**a**	**b**											**3**	**Santander**	**Floiran**
CL-525-COL	**a**	**b**											**3**	**Cauca**	
CL-524-COL	**a**	**b**											**3**	**Cauca**	
CL-523-COL	**a**	**b**											**3**	**Cauca**	
CL-516-COL	**a**	**b**											**3**	**Darien-Valle**	**Linea 7091**
CL-515-COL	**a**	**b**											**3**	**Darien-Valle**	**Linea 7099**
CL-514-COL	**a**	**b**											**3**	**Darien-Valle**	**Linea 7128**
CL-513-COL	**a**	**b**											**3**	**Darien-Valle**	**Linea 7246**
CL-512-COL	**a**	**b**											**3**	**Darien-Valle**	**Linea 7597**
CL-511-COL	**a**	**b**											**3**	**Darien-Valle**	
CL-510-COL	**a**	**b**											**3**	**Darien-Valle**	**Linea 7150**
CL-509-COL	**a**	**b**											**3**	**Darien-Valle**	**Linea 7140**
CL-552-COL			**c**										**4**	**Boyaca**	**Cerinza**
CL-551-COL	**a**		**c**										**5**	**Boyaca**	**Cerinza**
CL-534-COL	**a**	**b**	**c**										**7**	**Santander**	**Jiji**
CL-540-COL	**a**		**c**	**d**									**13**	**Santander**	**Floiran**
CL-539-COL	**a**		**c**	**d**									**13**	**Santander**	**Floiran**
CL-550-COL	**a**			**d**				**h**					**137**	**Cundinamarca**	**Cargamanto**

Differential cultivars: A = Michelite, B = Michigan dark red kidney; C = Perry Marrow; D = Cornell 49242; E = Widusa; F = Kaboon; G = Mexico 222; H = PI 207262; I = To; J = Tu; K = AB 136 and L = G 2333.

Activity 1.3. Identifying and developing molecular markers linked to ALS resistance genes in common bean.

Contributors: G. Mahuku, A.Iglesias, M. Navia, M.C. Hernadez and S. Beebe

Highlight:

- Showed that the $PF9_{260}$ SCAR marker linked to the resistance genes in G10474 and G10909 functions effectively in both Andean and Mesoamerican backgrounds.

Rationale

Angular leaf spot disease (ALS) of common bean, caused by the fungus *Phaeoisariopsis griseola* is the most widely distributed and destructive bean disease in tropical and subtropical countries that can cause yield losses of up to 80% (Stenglein et. al., 2003. Advances in Applied Microbiology 52:209-243.). Stacking or pyramiding resistance genes of Andean and Mesoamerican origin into the same background is most likely to result in stable and durable resistance to this devastating disease. This can be effectively achieved through use of markers tightly associated with the resistance genes of interest. Previously, we have identified molecular markers linked to angular leaf spot resistance genes from the Mesoamerican gene pool. Our focus this year was to (i) identify molecular markers that are linked to one of our best source of ALS resistance genes of Andean origin, G 5686; (ii) identifying additional markers that are linked to the Mesoamerican sources of resistance in the hopes of saturating the resistance locus and improve the efficiency of MAS in bean breeding; and (iii) validating identified markers and developing protocols for their use in MAS.

Materials and Methods

Identifying markers linked to resistance genes in G5686: The Andean genotype, G5686 has been highly resistant to both Andean and Mesoamerican pathotypes of *P. griseola*. Inheritance studies showed that at least two complementary genes condition resistance to *P. griseola* (CIAT, Annual Report Bean Program 2003) in this genotype. The bulk segregant analysis was used to evaluate 59 RAPD primers in an effort to identify markers linked to resistance genes in G5686. DNA extractions, PCR amplifications and gel electrophoresis were conducted as previously described (Mahuku et al., 2004. Crop Science 44: 1817-1824).

*Validation of SCAR markers linked to resistance genes in G10909***:** Three previously described SCAR markers ($OPE4_{709,}$ $PF9_{260}$, and $PF13_{310}$) were polymorphic when evaluated on the G10909 x Spite population. The markers $OPE4709_{,}$ $PF9_{260}$ were developed for Mexico 54 and G 10474 respectively (CIAT, Annual report Bean Program 2004). These markers were used to amplify several genotypes that have been used in crosses with G10909 (Table 1.3), in order to validate the utility of these markers in MAS breeding.

*Evaluation of common bean microsatellite markers***:** A total of 11 SSR markers that have been developed for the common bean (Yu et al., 1999 Journal of Heredity 91 (6): 429-434.) were used to amplify DNA from G10909, G10474, G5686, G10613 Mexico 54, VAX 6, MAR 1 and Sprite, in the hopes of identifying markers that segregate either in coupling or repulsion with the resistance genes in these genotypes. PCR amplification,

polyacrylimaide gel electrophoresis and visualization were done as described previously (Mahuku et al., 2004 Crop Science 44: 1817-1824).

Results and Discussion

*Identifying markers linked to resistance genes in G5686***:** Of the 59 RAPD primers evaluated, 15 were polymorphic in the parental genotypes, G5686 and Sprite, the resistant and susceptible bulks. When these markers were evaluated on an additional 8 resistant and susceptible individuals, only three RAPD primers were polymorphic. These primers were evaluated on an F_2 population of 139 individuals. However, none of these markers were closely linked to the G5686 resistance genes.

*Validation of SCAR markers linked to resistance genes in G10909***:** The three SCAR markers (SCAR_PF9$_{260}$, SCAR_OPE4$_{709}$, and SCAR_PF13$_{310}$) segregated in coupling with the resistance genes in G10909. SCAR_PF9$_{260}$ was located 9.9 cM from the resistance gene, while markers SCAR_OPE4$_{709}$, and SCAR_PF13$_{310}$ were located 7.4 cM and 5.5 cM respectively, from the resistance gene (Figure 1.3.1). When tested on genotypes that are commonly used in crosses with G10909, SCAR_PF9$_{260}$ was only detected in G10474, but was absent from all other genotypes, irrespective of whether they were of Andean or Mesoamerican origin (Table 1.3.1). These results show that SCAR_PF9$_{260}$ can effectively be used to introgress resistance genes from G10909 or G 10474 into any background (Table 1.3.1). The presence of SCAR_PF9$_{260}$ in G10474 was expected, as this marker was originally found to be linked in coupling and located at 3.0 cM from the resistance gene in G 10474.

Therefore, the resistance genes in G10909 and G10474 maybe be closely linked and might be members of a cluster of ALS resistance genes. Mapping of these markers to the bean core map are in progress and might reveal the relationship of these genes. However, from phenotypic evaluations, the spectrum of activity of these genes is different. We have characterized some *P. griseola* pathotypes that overcome the G10909 resistance gene, while they are unable to overcome the G10474 resistance gene.

Table 1.3.1. Response of bean genotypes used in crosses with G10909 to inoculation with *Phaeoisariopsis griseola* pathotype 63-63 and evaluation with SCAR_PF9$_{260}$, SCAR_OPE4$_{709}$, and SCAR_PF13$_{310}$ markers.

	Genotype	Pg 63-63	PF9$_{260}$ [a]	OPE4$_{709}$	PF13$_{310}$		Genotype	Pg 63-63	PF9$_{260}$	OPE4$_{709}$	PF13$_{310}$
1	A 247	7	-	+	-	30	GLP 2	2.5	-	-	-
2	A 686	8	-	+	+	31	GLP 585	8	-	+	+
3	A 811	8	-	+	+	32	MA 23-24 BRASIL	8	-	-	+
4	AFR 699	6.7	-	-	-	33	MAM 38	1	-	+	+
5	AND 279	6	-	-	-	34	MAR 1	4	-	-	+
6	AND 1055	4	-	-	-	35	MAR 2	6.7	-	+	+
7	AND 1064	3.7	-	-	-	36	MAR 3	3.7	-	-	+
8	BEAVER 4	6.3	-	-	+	37	MD 23-24	8	-	-	+
9	BRB 198	3	-	-	-	38	MEX 54	8	-	+	-
10	CAL 96	7	-	-	-	39	MLB 40-89A	8	-	+	+
11	CAL 96_ss	7	-	-	-	40	MLB 49-89A	1	-	+	+
12	CAL 143	8	-	-	-	41	Montcalm	2	-	-	
13	DOR 390	8	-	-	+	42	MR 12439-18	7	-	-	+
14	DOR 500	8	-	-	+	43	MR 13304-74	8	-	+	+
15	FEB 212	8	-	+	+	44	MR 13363-14	8	-	+	+
16	FEB 214	8	-	+	+	45	PARAGACHI	8	-	-	-
17	G 685	1.3	-	+	+	46	TIO CANELA	7	-	+	+
18	G 3353	8	-	-	+	47	Ruda	8	-	+	+
19	G 4090	8	-	-	+	48	RWR 719	8	-	-	+
20	G 4691	4.7	-	+	+	49	RWR 1092	1	-	-	+
21	G 5207	1	-	+	+	50	SAM 1	7	-	-	+
22	G 9603	2.7	-	-	-	51	SCAM 80-CM/15	2	-	-	-
23	G 10909	1	+	+	+	52	SUG 137	6	-	-	-
24	G 10613	1	-	-	+	53	POA 12	7	-	-	-
25	G 13910	7	-	-	-	54	URUGEZI	6	-	+	
26	G 15430	7	-	-	-	55	VAX 1	5.7	-	+	+
27	G 19833	2	-	-	-	56	VAX 2	5	-	+	+
28	G 21212	8	-	-	+	57	G10474	1	+	+	+
29	G 23070	1	-	-	-	58	Sprite	8	-	-	-

[a] + = presence of marker; - = absence of marker

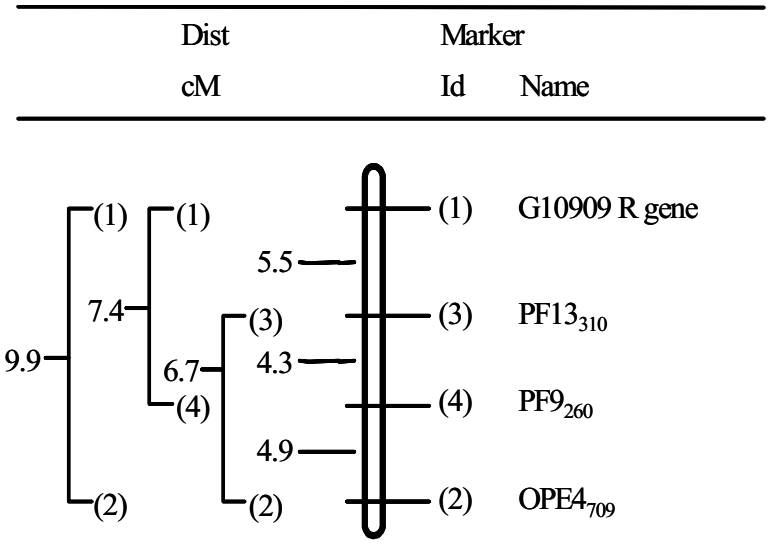


Figure 1.3.1. Organization of three SCAR markers relative to the resistance gene in the Mesoamerican genotype G10909.

SCAR markers (SCAR_OPE4$_{709}$, and SCAR_PF13$_{310}$) were present in some genotypes and absent in others, revealing that a parental survey is necessary before these markers can be used in a MAS breeding program.

Identification of common bean microsatellite markers linked to ALS resistance in common bean: Eight microsatellite markers were identified that segregated with the resistance gene in G5686 (Table 1.3.2). One of these markers, was evaluated on a population of 139 F_2 individuals derived from crossing G5686 x Sprite. The marker was found to be located at 19.8 cM from the resistance gene in G5686. The marker is co-dominant, and the resistant and susceptible fragments have been cloned and are currently being sequenced. New markers will be developed based on the differences between the fragments from resistant and susceptible individuals and it is hopped that a tight association between marker and phenotype will be obtained. Work is in progress to evaluate the other 7 markers on the 139 F_2 populations. Other SSR markers that have been identified for other genotypes are shown in Table 1.3.2. These markers are currently being evaluated on the entire F_2 population. The association of the markers with the resistance genes in these genotypes, and their utility for MAS breeding will be known once evaluations have been completed.

Table 1.3.2. Common bean microsatellite markers that are linked to angular leaf spot resistance in the common bean genotypes G5686, G10909, G10474, MAR 1 and Mexico 54.

	G5686 x Sprite		G10909 x Sprite		G10474 x Sprite		MAR 1 x VAX 6		Mex 54 x Sprite	
SSR marker	Parents	F_2 plants	Parents	F_2 plants	Parents	F_2 plants	Parents	F_2 plants	Parents	F_2 plants
PV-ctt001	+	+	-		-		-		-	
PV-atgc001	+	ns	-		+	ns	-		+	ns
PV-atgc002	+	ns	+	+	+	+	+	ns	+	ns
PV-ag001	-	-	+	ns	+	ns	-		-	
PV-ag004	+	+	-		+	ns	-		-	
PV-gaat001	-	-	+	ns	+	ns	-		+	+
PV-gaat002	+	ns	-		-		-		-	
PV-at003	-	-	+	ns	+	ns	+	ns	-	
PV-at004	+	-	-		-		-		-	
PV-cct001	+	-	+	ns	+	ns	+	ns	+	ns
PV-at007	+	+	+	ns	+	ns	+	ns	+	ns

+ represents polymorphic in parental and F_2 individuals (5 resistant and 5 susceptible F_2 plants).
- represents no polymorphism
ns = pending evaluation in F_2 individuals.

Conclusion: The results presented here show that SCAR_PF9$_{260}$ is a more reliable and useful marker that can be used to introgress resistance from G10909and G10474 into both Andean and Mesoamerican genotypes. A protocol for the use of this marker in MAS breeding was developed and this marker is currently being validated in Kawanda, Uganda. For the other markers, SCAR_OPE4$_{709}$, and SCAR_PF13$_{310,}$ a parental survey is necessary before they can be effectively used in a MAS breeding program. The identification of molecular markers linked to G5686 is crucial for efficient use of resistance genes in this genotype. G5686 is a crucial source of ALS resistance of Andean origin. These genes need to be stacked with Mesoamerican ALS resistance genes from Mexico 54, G10909 and G10474, for stable and durable ALS resistance. Additional markers that have been identified need to be tested in the entire mapping population and validated outside the mapping population. However, it is crucial to saturate the resistance loci of these genotypes so as to increase the efficiency of MAS.

Activity 1.4. Identifying and developing molecular markers linked *Pythium* root rot resistance.

Contributors: G. Mahuku, M. Navia, A. Matta, R. Buruchara, R. Otsyula

Highlight:

- Molecular markers that are linked to *pythium* root rot resistance in RWR 719, MLB 49-89A, and AND 1062 were identified and protocols for two SCARs PYAA19 and PYB08 were developed. These markers are currently being used in MAS fro root rots in Kampala, Uganda.

Rationale

Bean root rot, caused by several *Pythium* species is one of the most destructive diseases affecting common bean (*Phaseolus vulgaris*) in East and Central Africa where beans are grown in intensive agricultural production systems (Buruchara and Rusuku, 1992. CIAT Workshop Series

No. 23. pp. 49-55). Use of resistant cultivars is considered to be the most viable option for controlling bean root rot particularly for small-scale growers (Otsyula et al., 1998. African Crops Science Journal 6:61-67). A few bean genotypes with resistance to Pythium root rot have been identified, among them RWR 719, MLB 49-89A and AND 1062 (Buruchara and Rusuku 1992. CIAT Workshop Series No. 23. pp.49-55). Last year, we reported the identification of molecular markers linked to the resistance genes in RWR 719 and MLB 49-89A, and the conversion of the markers linked to the resistance gene in RWR 719. This year, we report the identification of additional markers for these genotypes, including AND1062, the conversion of these markers to sequence characterized amplified region (SCAR) marker types, and their suitability for marker assisted selection (MAS) breeding.

Materials and Methods

Plant material and evaluations: The resistant varieties RWR 719, MLB49089A, and AND 1062 were crossed to the susceptible commercial cultivar, GLP2 to establish F_1, F_2, and backcross populations to susceptible (BC_S) and resistant progenitors (BC_R). These populations were evaluated under greenhouse conditions using an isolate of *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.). Plant establishment, inoculations and evaluations were done as described previously (CIAT, Annual report 2005). Plants with no or limited symptoms (score 1-3) were rated as resistant, and the rest of the plants as susceptible.

DNA extraction: Young trifoliate leaves were collected from the two parents, and from resistant and susceptible F_2 progenies, and DNA was extracted using the procedure described by Mahuku (2004. Plant Molecular Biology Reporter 22: 71-81.). In addition, DNA was extracted from other varieties that are commonly used as parents in root rot breeding program.

*Marker identification***:** Five resistant and 5 susceptible F_2 plants, including the parents were used to evaluate 300 RAPD, 10 SSR, 40 UBC microsatellites and 50 RAMS primers as previously described (CIAT, 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 1.4.1). The marker scoring data in the F_2 were merged with the disease scoring data for linkage analysis using the computer program MAPMAKER

*Marker development and validation***:** Candidate fragments were excised from agarose gels, cloned and sequenced as described by Mahuku et al.(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 ten resistant and susceptible F_2 individuals. If polymorphism was maintained, the designed SCAR primers were tested in the entire F_2 population. 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 identification: RWR 719***:** A total of 12 markers have been identified that are linked in coupling to the resistance gene in RWR 719. Six of these markers are RAPDs (OPAA19, OPBA08, OPG3, OPH20, OPY20 y B459), one is RAMS $(VHVGT)_5G$) and five are SSR markers previously developed for common bean. Three of the RAPDprimers were successfully converted to SCAR markers at 1.5 cM ($PYAA19_{800}$), 4.0 cM ($PYBA08_{350}$) and 6.0 cM ($PYY20_{1200}$) from the resistance gene. The RAMS derived marker is located 6.3 cM from the RWR 719 resistance gene. The identified SSR markers are currently being evaluated in the entire F_2 population composed of 150 individuals (Table 1.4.1).

MLB 48-89A: Five markers (OPBA08, OPF10, OPG3, OPY20 y UBC 815) have been identified that are linked in coupling to the resistance gene in MLB 49-89A. Linkage analysis showed that the OPF10 marker was located 7.5 cM from the resistance gene, while the OPG3 markers was located at 5.7 cM, and UBC815 was 7.4 cM from the resistance gene. SCAR markers for these fragments have been developed and are currently being evaluated in the entire population. In addition, two SSR markers linked to the pythium resistance gene in MLB 49-89A have been identified (Table 1.4.1). These are currently being evaluated in the entire F_2 population.

Table 1.4.1. Common bean microsatellite markers linked to Pythium root rot resistance in the common bean genotypes RWR 719, MLB 49-89A and AND 1062.

SSR Marker	Pythium root rot resistance sources		
	RWR-719	AND-1062	MLB49-89A
PV-atgc001	+	-	-
PV-ag004	-	-	-
PV-gaat001	-	+	-
PV-ag001	-	-	-
PV-gaat002	-	-	-
PV-cct001	+	+	-
PV-at007	-	-	-
PV-ctt001	+	-	-
PV-atgc002	-	-	+
PV-at004	ns	ns	ns
PV-at003	ns	ns	ns

\+ represents polymorphic in parental and F2 individuals (5 resistant and 5 susceptible F2 plants).
\- represents no polymorphism
ns pending evaluation

AND 1062: Two RAPD primers OPAA19 and OPBA08, located 2.9 cM and 5.5 cM from the pythium resistance gene in AND 1062 have been identified. These fragments have been converted to SCAR markers and are currently being evaluated in the F_2 population. In addition, three SSR markers have been identified linked to this resistant gene (Table 1.4.1). These are currently being evaluated in the F_2 population.

SCAR Markers: A total of eight SCAR markers have been designed. These are derived from the RAPD primers OPAA19, OPBA08, OPY20, B459, OPF10, OPG3, OPR2 and the RAMS (GT)n primers. The RAPD primers OPAA19, OPBA08 are linked to the resistance gene in the three

sources of resistance (RWR 719, MLB 49-89A and AND 1062), probably signifying that a single locus or a cluster of closely associated genes conditions pythium resistance in common bean. These results are in agreement with allelism tests that showed no segregation, thus revealing that a common locus or closely associated loci controls resistance to *Pythium* in common bean. Both SCARs (PYA $A19_{800}$ (Figure 1.4.1a) and PY $BA08_{350}$ (Figure 1.4.1.b) are dominant in nature. The primers designed for OPY20 and (GT)n were monomorphic and new ones have been designed. We are currently testing SCAR primers derived from the RAPD primers B459, OPF10, OPG3 and OPR2 and new ones developed for OPY20 and (GT)n. MAS protocols for PYA $A19_{800}$ and PY $BA08_{350}$ SCAR markers were developed and these primers are currently being validated in Kawanda, Uganda.

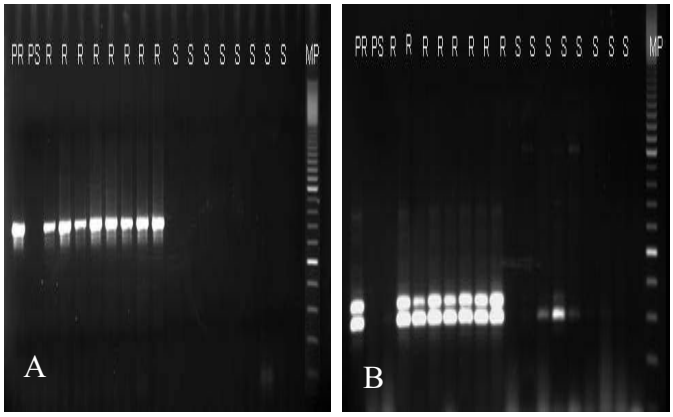


Figure 1.4.1. Validation of the SACR markers (A) PYAA19 and (B) PYBA08 for their utility in marker assisted selection breeding. The markers are linked in coupling with the resistance gene and are dominant in nature.

Conclusion: Several marker types have been identified that segregate in coupling phase with pythium resistance in RWR 719, MLB 49-89A and AND 1062. A total of eight SCAR markers have been developed and two of these (PYA $A19_{800}$ and PY $BA08_{350}$) are currently being validated in Kawanda. In addition, several SSR markers have been identified and these are being evaluated in the mapping population. More information on marker quality will be obtained once all these markers have been tested in the F_2 mapping population. Our objective is to saturate the *pythium* resistance loci with several marker types so as to increase the efficiency of marker assisted selection for this very important disease.

Activity 1.5. Identification of molecular markers linked to rice blast resistance genes

Contributors: J.L. Fuentes, F. Correa-Victoria, F. Escobar, G. Prado, G. Aricapa, M.C. Duque & J.Tohme

Highlight

- The present work evidenced the usefulness of combining near-isogenic progeny analysis with rice genome information available in public databases to identify molecular markers highly linked to blast resistance genes in rice. Although a limited number of polymorphic markers can be expected when near-isogenic lines are used as progenitors, here we found six polymorphic markers in a region of only 13 cM surrounding the blast resistance gene *Pi-1(t)*. Additionally, two of these markers (RM1233*I and RM224) were closely linked to the gene. Our results support the utility of these DNA markers in MAS and gene pyramiding rice breeding programs addressing the improvement of blast resistance in rice cultivars; and eventually to map based cloning of the gene. The speed, simplicity and reliability of PCR based approaches make microsatellite analysis on agarose gels an attractive tool for marker-assisted selection in rice breeding programs aiming at developing durable rice blast resistant cultivars.

Rationale

Rice blast caused by *Pyricularia grisea* (Cooke) Sacc., the anamorphous state of *Magnaporthe grisea*, is the most limiting biotic factor for rice production in the world. The use of resistant cultivars is the most effective and economical way of controlling blast disease, therefore, breeding efforts for developing resistant cultivars continue to be a priority of rice breeding programs. One way to improve the durability of blast resistance is to "pyramid" resistance genes by crossing rice varieties with complementary genes to provide multigenic resistance against a wide spectrum of blast races. Combining these resistance genes broadens the number of races that a variety can resist, and there is evidence that multiple resistance genes make it more difficult for virulent races to evolve (Correa-Victoria et al., 2002. Fitopatología Colombiana 26: 47-54). Unfortunately, pyramiding genes is difficult using conventional greenhouse screening procedures because blast races carrying individual avirulence genes to be used in inoculations for the identification of the corresponding resistance gene are normally not present in nature. As a result, accumulation of several resistance genes in a common background cannot be easily distinguished without a test cross. Recent advances in molecular marker technology, such as development of tightly linked molecular markers, has made it possible to pyramid major genes and QTL's into one genotype and to simultaneously select several complex characters.

The blast resistance gene *Pi-1(t)*, originally identified in the cultivar LAC23, an upland cultivar from Liberia confers complete resistance to several blast populations from Latin America when combined with the blast resistance genes *Pi-2(t)* and *Pi-33(t)* (Correa-Victoria et al., 2002. Fitopatología Colombiana 26: 47-54). The Pi-1 gene confers resistance to all races present in one of the most predominant genetic lineages (SRL-4) from Colombia, while the other two genes confer resistance to all races within two other predominant lineages (SRL-5 and SRL-6), respectively. Mapping studies showed that the *Pi-1(t)* gene is located near the end of

chromosome 11, linked to the Npb181 and RZ536 RFLP markers at a distance of 3.5 and 14.0 cM, respectively. However, RFLP approaches are expensive and laborious limiting their use in applied breeding programs, where a considerably high number of samples need to be analyzed. Convenient and cost-effective microsatellite markers, particularly those that can be scored on agarose gels, seem to be promising for the identification of blast resistance genes and for pyramiding or introgression of these genes into rice commercial varieties and elite lines. Microsatellite markers are hypervariable, abundant and well distributed throughout the rice genome and they are now available through the published high-density linkage map or in the public database (www. gramene.org).

We have designed a molecular marker-assisted breeding program in rice aiming at developing durable blast resistance in elite rice lines and cultivars by pyramiding the resistance genes *Pi-1(t)*, *Pi-2(t)* and *Pi-33(t)*; which are potentially useful to control blast pathogen populations in the Latin American region (Correa-Victoria et al., 2002. Fitopatología Colombiana 26: 47-54). Here we report new microsatellite markers that cosegregate with the blast resistance gene *Pi-1(t)*, using sequences available in a public database. These markers can be potentially used in MAS to introduce this gene into blast susceptible varieties, and provide the basis for map based cloning of this blast resistance gene.

Materials and Methods

The near-isogenic lines C101LAC (resistant line to isolates carrying avr *Pi-1(t)*) and C101A51 (susceptible line) developed at IRRI were crossed (cross CT 13432) and F_1 seeds generated. The F_2 progeny, resulting from self-pollination of F_1 individuals, were self-pollinated to generate 283 CT13432 F_3 lines. Rice varieties from Latin America were obtained from CIAT's rice germplasm bank. Ten rice seedlings 21 days old per pot were sprayed with 2.0 ml of blast inoculum suspension ($5x10^5$ spores/ml of isolate Oryzica Yacu 9-19-1 carrying avr *Pi-1(t)*) and incubated in the greenhouse at a temperature of 24-28^{o}C and relative humidity above 85 %. Plants were evaluated 15 days (two life cycles of the pathogen) after inoculation and scored for resistance and susceptibility in two replications as described by Correa-Victoria and Zeigler (Correa-Victoria and Zeigler, 1993. Plant Disease 77: 1029-1035.). Resistant genotypes exhibit complete resistance with no lesions or few non-sporulating lesions type 1 or 2, and susceptible genotypes exhibit typical sporulating blast lesions type 3 or 4 covering more than 1 % of leaf area.

DNA concentrations were determined in a TKO 100 minifluorometer with the DNA-specific fluorescent dye. DNA bulks were prepared from 13 resistant and 13 susceptible lines within the CT13432 F_3 families evaluated for their blast reaction using the blast isolate Oryzica Yacu 9-19-1. Polymerase chain reaction (PCR) was conducted in a final volume of 20 σl containing between 25-50 ng of template DNA, 0.5 σM of each primer, 200 σM of each dNTP, 3.1 mM $MgCl_2$ and 1 unit of Taq DNA polymerase. For the majority of microsatellite markers studied the reaction was processed as follow: 94^{o}C for 1 min, followed by 40 cycles consisting of 94^{o}C for 30 sec, 50 and/or 55^{o}C for 30 sec and 72^{o}C for 30 sec and a final extension step of 72^{o}C for 10 minutes. After the PCR reaction, 5 σl of blue juice (30 % glycerol, 0.25% bromophenol blue) was added to the amplification product and 20 σl per sample were loaded on high-resolution agarose gels prepared mixing 1.5 % Sinergel (Diversified Biotech) and 0.7 % molecular grade products and containing 0.5mg/mL of ethidium bromide.

Twenty-four primer pairs corresponding to nineteen microsatellite loci (Figure 1.5.1.B) were selected from the Gramene database (www. gramene.org) considering their relative proximity to the *Pi-1(t)* gene in the current rice genetic map (Figure 1.5.1.A). The isogenic lines C101LAC and C101A51 and their common genetic background, the susceptible recurrent parent CO39, were used to identify microsatellite polymorphisms associated to the blast resistance genes. Polymorphic markers identified above were assayed by bulked segregant analysis (BSA).

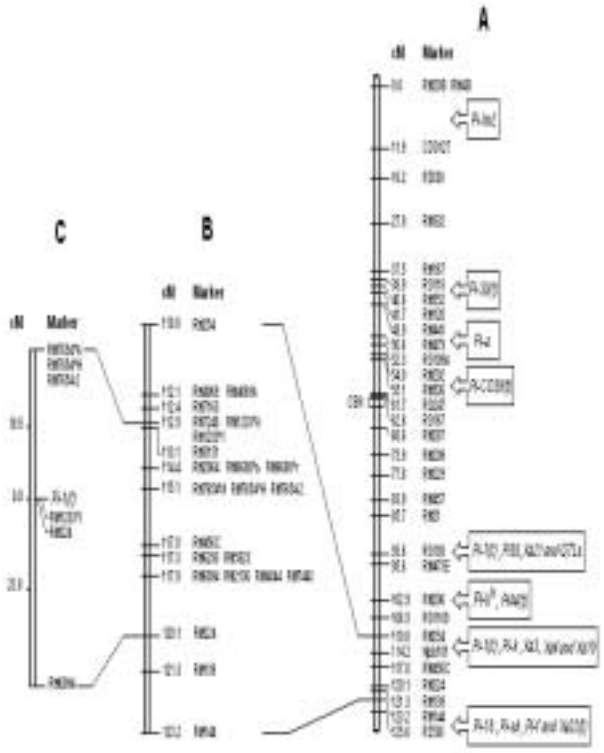


Figure 1.5.1. Genetic map of rice chromosome 11 (A) as indicated by Temnykh et al. (2001) and by McCouch et al. (2001). Region between the 110.0 and 123.2 cM (B) was complemented with public information available at Gramene database (www.gramene.org). Information about position of the resistance genes on chromosome 11 was obtained as follow: *Pi-1(t)* (Yu et al. 1996; Hittalmani et al. 2000), *Pi-7(t)* and quantitative trait locus (QTL) to partial resistance to blast (Wang et al. 2001; Zenbayashi et al. 2002), *Pi-CO39(t)* (Chauhan et al. 2002), *Pi-18(t)* (Ahn et al. 2000), *Pi38* (Gowda et al., 2006), *Pi-44(t)* (Chen et al. 1999), *Pi-a, Pi-k, Pi-sh, Pi-f, Pi-lm2 and Pi-30(t)* (Sallaud et al. 2003), *Pi-k^h* (Sharma et al., 2005), *Xa3, Xa4, Xa10, Xa21* and *Xa22(t)* (Causse et al. 1994; Mackill and Ni, 2001). Chromosome 11 generated through linkage analysis (C).

Genetic analysis of the resistance was conducted measuring the goodness-of-fit to the expected ratio for a single gene model using a chi-square test. For this purpose, we used 283 F_3 near-isogenic lines derived from 283 F_2 plants with no selection. Molecular markers that resulted positive in BSA for the *Pi-1(t)* gene were used in linkage progeny analysis using 158 F_3 near-isogenic lines. Associations between markers and the resistance gene were demonstrated using a chi-square test. Linkage analysis was performed using the software MAPMAKER/EXP V 3.0 on the segregation data obtained from markers and blast resistance scoring of the CT13432 F_3 population. Conversion of the recombination fraction into centiMorgans (cM) units was obtained with the Kosambi's mapping function. The final map was drawn using the software QGene V 3.04.

The diagnostic potential of the markers associated with the *Pi-1(t)* gene was also evaluated on DNA obtained from nineteen rice genotypes including seventeen elite cultivars grown in Latin America. For this purpose, the criteria followed for determining the presence or absence of the resistance gene was the amplification of the specific *Pi-1(t)* microsatellite allele in each rice genotype. Comparing with phenotypic evaluation obtained as indicated above, the veracity of the assay was corroborated.

Results and Discussion

Genetic analysis of the resistance was conducted using 283 F_3 near-isogenic lines of the cross CT 13432. Expected and observed segregation ratios for this population are shown in Table 1.5.1. The population analysis showed a good fit to the expected segregation ratio (1:2:1) for a single gene model confirming the hypothesis of a single dominant gene for *Pi-1(t)* locus.

Table 1.5.1. Segregation of F_3 near-isogenic lines of the genetic cross between C101LAC (*Pi-1(t)*)/C101A51 inoculated with the blast isolate Oryzica Yacu 9-19-1 of *Pyricularia grisea*.

Population	Expected ratio[1]	No. of lines expected			No. of lines observed		
		S	SG	R	S	SG	R
F_3 near-isogenic lines	1:2:1 ($\theta^2 = 1.0$, $p < 0.05$)	71	141	71	76	133	74

(1) According to a model based on a single dominant gene as indicated in materials and methods; (S): Susceptible, (SG): Segregant; (R): Resistant

From the reported position of *Pi-1(t)* on chromosome 11 relative to RZ536 RFLP marker, it was possible to estimate its approximate position on the Rice-Cornell microsatellite genetic map (Figure 1.5.1.A). Using this information, twenty-four microsatellite sequences were selected from this region of chromosome 11 from the Gramene database (www.gramene.org) as potential markers for *Pi-1(t)*. These markers were first tested for polymorphism in the susceptible and resistant parent and later for linkage to *Pi-1(t)* in pooled C101LAC/C101A51 samples. Of the twenty-four primer pairs tested six (corresponding to four microsatellite loci) were polymorphic in agarose gel electrophoresis, all of them showing positive results in bulked segregant analysis; five markers were not polymorphic, and thirteen principally repeats with TA sequences did not show consistent amplification with the different annealing temperatures assayed.

Linkage between these six markers and blast resistance was confirmed by screening 158 F_3 near-isogenic lines from the cross C101LAC/C101A51 segregating for *Pi-1(t)*. Chi-square test indicated that these markers were linked to *Pi-1(t)*. The genetic distance between the markers and the *Pi-1(t)* locus ranged from 0.0 (no recombination between the markers and the resistance factor) to 23.8 cM (Figure 1C). Among the six makers linked to *Pi-1(t)* gene, two (RM1233*I and RM224) mapped in the same position (0.0 cM) with the *Pi-1(t)* gene. Other three dominant markers corresponding to the same genetic locus (RM7654) were located at 18.5 cM above the *Pi-1(t)* gene, while marker RM6094 was identified at 23.8 cM below the gene.

To examine whether the markers identified would be of general utility on a wider range of rice germplasm used in applied breeding programs in Latin America, the presence of resistant bands for five markers were examined in elite rice cultivars and compared to the reported inheritance of *Pi-1(t)* (Table 1.5.2). For this purpose, we used known sources of blast resistance as positive controls and considered as predictive criteria of the resistance event the amplification in each variety of the resistant microsatellite band and therefore the presence of the resistant allele for the *Pi-1 (t)* gene. Comparing with phenotypic data on blast resistance our results showed that our known sources of resistance (C101LAC, Cica 8, Oryzica 2, BR IRGA 409, CR 1113, El Paso 144 and Panama 1048) carry the resistance *Pi-1(t)* allele; on the other hand, the susceptible cultivars (Colombia XXI, Epagri 108, Capirona and Oryzica 1 and CO-39) had not the resistant allele. In addition, other seven varieties (Jucarito-104, Fedearroz 2000, CR 1821, Primavera, Cimarrón, Bonanza and Fedearroz 50), which were resistant in the pathogenicity assay, did not show the allele characteristic of the *Pi-1(t)* gene.

This study demonstrates that approaches combining near isogenic progeny analysis and rice genome information available in a public database constitute a very useful tool for identifying molecular markers closely linked to blast resistance genes. The reported marker most closely linked to blast resistance gene *Pi-1(t)* was the cDNA Npb181, identified at 3.5 cM from the gene. Here, using a segregating population with identical genetic background (CO39) but with a higher number of segregant lines than the one used by these authors, we have identified two new microsatellite markers (RM1233*I and RM224) highly linked to gene *Pi-1(t)* (at 0 cM of the gene). From the reported position of the *Pi-1(t)* gene relative to the RZ536 marker, it was possible to estimate its putative position on the Rice-Cornell microsatellite genetic map flanked by the microsatellite RM254 and the RFLP RZ536 markers between the 110.0 and 125.6 cM at the end of chromosome 11. Here we have reported two microsatellite markers very closely linked to gene *Pi-1(t)*, which is in agreement with the information included in the Rice-Cornell microsatellite genetic map, positioning these markers between 112.9 and 120.1 cM at the end of chromosome 11. However, the two remaining microsatellite loci RM7654 and RM6094 were outside of the mentioned 7.2 cM chromosomal region, mapping to 18.5 and 23.8 cM from the gene *Pi-1(t)*, respectively.

Table 1.5.2. Analysis of the predictive capacity of six microsatellite markers for blast resistance gene *Pi-1(t)* in 19 commercial rice cultivars.

Variety	Origin	PA	Marker analyzed				
			RM1233*I	RM7654*A	RM7654*H	RM7654-2	RM224
CO-39 [1]	Philippines	S	-	-	-	-	-
C101LAC [2]	Philippines	R	+	+	+	+	+
Cica-8	Colombia	R	+	+	+	+	+
Fedearroz 2000	Colombia	R	-	-	-	-	-
Colombia XX1	Colombia	S	-	-	-	-	-
Oryzica 1	Colombia	S	-	-	-	-	-
Oryzica 2	Colombia	R	+	+	+	+	+
Fedearroz 50	Colombia	R	-	-	-	-	-
Epagri 108	Brazil (irrigated)	S	-	-	-	-	-
BRIRGA409	Brazil (irrigated)	R	+	+	+	+	+
Primavera	Brazil (upland)	R	-	-	-	-	-
Bonanza	Brazil (upland)	R	-	-	-	-	-
El Paso 144	Uruguay, Argentina	R	+	+	+	+	+
Cimarron	Venezuela	R	-	-	-	-	-
Capirona	Peru	S	-	-	-	-	-
Panamá 1048	Panama	R	+	+	+	-	+
CR 1113	Costa Rica	R	+	+	+	+	+
CR 1821	Costa Rica	R	-	-	-	-	-
Jucarito-104	Cuba	R	-	-	-	-	-

(1): Susceptible control; (2): Resistant control; PA: Results of the pathogenicity assay using blast isolate Yacu 9-19-1, R: resistant genotype, S: susceptible genotype, (+) presence of resistant allele, (-) absence of resistant allele.

We have shown that the known *Pi-1(t)* resistance sources such as C101LAC, Cica-8, Oryzica 2, BRIRGA409, El Paso 144, Panamá 1048 and CR1113 (Correa-Victoria et al., 2002. Fitopatología Colombiana 26: 47-54) exhibited microsatellite alleles associated with this gene of resistance, while susceptible varieties don't. Interestingly, six varieties (Fedearroz 2000, Fedearroz 50, Primavera, Bonanza, Cimarrón and Jucarito-104) that were resistant to the rice blast isolate Oryzica Yacu 9-19-1 did not show the resistant *Pi-1(t)* alleles. One possibility for this resistance reaction in these cultivars could be the presence of different resistance genes interacting with corresponding avirulence genes different from the avr *Pi-1(t)* in the pathogen.

This study is part of a molecular marker-assisted rice breeding program aiming at developing durable blast resistance in rice cultivars by pyramiding the resistance genes *Pi-1(t), Pi-2(t),* and *Pi-33(t)*, which are potentially useful for controlling blast pathogen populations in the Latin American region (Correa-Victoria et al., 2002. Fitopatología Colombiana 26: 47-54). Disease assays to evaluate resistance to rice blast are time-consuming and laborious procedures that also require specialized facilities. PCR analysis can greatly reduce the amount of labor needed for evaluating phenotypes by prescreening with MAS. Cost-effective microsatellite markers linked to the blast resistance *Pi-1(t)* gene and suitable for agarose gel electrophoresis facilitating the introgression and pyramiding of the gene into rice commercial cultivars, were developed here.

The microsatellites reported in this study seem to be suitable for assisting rice breeders in the introduction of the *Pi-1(t)* resistance gene in different rice cultivars, and serve as an indicator for the presence of others. Thus, the *Pi-1(t)* gene markers may serve as indicators for the presence of resistance gene clusters in the indicated chromosome region and for the selection of breeding parents for developing rice cultivars with a broader-resistance spectrum to blast. Additionally, these microsatellite markers could provide a starting point for efforts eventually aimed at cloning and isolating this gene.

Conclusions

The present work evidenced the usefulness of combining near-isogenic progeny analysis with rice genome information available in public databases to identify molecular markers highly linked to blast resistance genes in rice. Although a limited number of polymorphic markers can be expected when near-isogenic lines are used as progenitors, here we found six polymorphic markers in a region of only 13 cM surrounding the blast resistance gene *Pi-1(t)* (Figure 1.5.1). Additionally, two of these markers (RM1233*I and RM224) were closely linked to the gene. This finding supports the hypothesis that when polymorphisms are found in near-isogenic derived populations, differing only in the presence or absence of a gene, the probability that these markers be closely linked to the gene is very high. Besides, polymorphic markers linked to resistance genes in near-isogenic populations, can also be expected to detect polymorphism and presence of the linked genes in commercial rice varieties with certain level of inbreeding. Our results support the utility of these DNA markers in MAS and gene pyramiding rice breeding programs addressing the improvement of blast resistance in rice cultivars; and eventually to map based cloning of the gene. However, the use of these markers as a diagnostic tool for determining the presence of the resistance gene *Pi-1(t)* in a wider range of rice germplasm require additional studies for further confirmation of the results reported here. The speed, simplicity and reliability of PCR based approaches make microsatellite analysis on agarose gels an attractive tool for marker-assisted selection in rice breeding programs aiming at developing durable rice blast resistant cultivars.

Activity 1. 6. Characterization of strains of cassava frogskin virus.

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

Rationale

Cassava frogskin disease (CFSD) is a disorder of unknown etiology that affects cassava and was first reported in 1971 from southern Colombia, 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 primary 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. We report on the progress that has been made in characterizing the genomic segments, the identification of three strains of the virus and their association with cassava frogskin disease.

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 collected from different areas of Colombia between 1983 and 2003 and were designated Secundina 5, Secundina 80, FSD 29, CM-5460-10, SM 909-25, Regional Tolima, Amazonas 16, Catumare Jamundi and CMC-40. Recently additional collections were made from the Departments of Atlantico, Cauca, Meta, Tolima, and Valle de Cauca, Colombia.

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. Since Secundina develops mosaic leaf symptoms, it was used as a biological assay for the presence of CFSD. Control test plants were periodically grafted to Secundina to assure that they had not become affected with CFSD.

Double-stranded RNA extraction, cDNA synthesis and cloning. Double-stranded RNA (dsRNA) was isolated from young leaves, petioles or roots of CFSD affected plants. When the dsRNAs were purified from 2% low melting point agarose gels, the selected gel pieces were melted a 70C, 2X STE, 2% SDS and 0.1% bentonite were added, and the extraction procedure describe for the plant material was followed. The cDNAs were modified by adding a 3'A-overhangs and ligated in to the pCR 2.1 vector (TA cloning Kit, Invitrogen). The dsRNA were amplified using standard PCR protocols with primers designed from the CFSV S4 sequence. The PCR products were cloned into the TA plasmid (Invitrogen). Nucleotide sequences were determined using an ABI Prism 377 sequencer (Perkin-Elmer) 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

Molecular Characterization of CFSV. Approximately 7000 nucleotides of CSFV have been sequenced and this represents about 30% of the genome. Based upon the ten genomic segment of rice ragged stunt virus, we have cDNA clones of six of the genomic segments of CFSV. The predicted proteins from the cDNA clones and contigs were compared and found to have similarity with RRSV proteins. The percentage of similarity is reported in Table 1.6.1.

Several attempts were made to clone the 5'and 3'termini of selected genomic segments. The 5' terminus of RNA 4 of the isolates CMC-40 and Amazonia 16 and the 3'terminus of CMC-40 were successfully cloned and sequenced. These were compared with the termini from RRSV and they are distinct. Many of the other Reoviridae have conserved termini but these are general only six conserved nucleotides.

Table 1.6.1. The cDNA clones that represent approximately 30% of the genome of cassava frogskin virus.

Clone name and RNA segment	Viral Isolate	Size of cDNA Nucleotide	AA similarity with RRSV
CFSV RNA1 228	Amazonas 16	288	48%
CFSV RNA1 683	FSD 29	683	36%
CFSV RNA2 381	CMC-40	424	53%
CFSV RNA2 361	Contig		
CFSV RNA2 434	CMC-40	434	23%
CFSV RNA3 315	CMC-40	315	60%
CFSV RNA3 232	FSD 29	232	47%
CFSV RNA3 432	CMC 40	1476	30%
CFSV RNA3 716	contig		
CFSV RNA3 900			
CFSV RNA3 400			
CFSV RNA3 294 R			
CFSV RNA3 310	FSD 29	396	59%
CFSV RNA3 281	contig		
CFSV RNA4 743 R	Amazonia 16	743	35%
CFSV RNA4 743 R	CMC-40	1192	35%
CFSV RNA4 867	contig		
CFSV RNA4 580			
CFSV RNA4 652	CMC-40	652	62%
CFSV RNA4 284	FSD 29	284	60%
CFSV RNA5 351	5460-10	351	41%
CFSV RNA10 640	Secondia 5	640	27%

[1]. The comparison for amino acid similarity is to rice ragged stunt virus (RRSV). The assignment of the RNA genomic segment for CFSV is based on the similarity with RRSV.

Using PCR, hybridization probes were developed for the six genomic segments. These were used to detect the double-stranded RNA genomic segments of the isolate CMC-40. The genomic segments were the size that we have predicted previously using RNA markers and visualization of these dsRNAs. The only genomic segment that is slightly larger than predicted is the CFSV segment 5, but these polyacrylamide gels did not contain a denaturing agent and more precise gels are need to make a decision on the size of the genomic segments. This confirms that the cDNA clones recognize the genomic segments that were predicted by their size and with their similarity to RRSV.Conserved motifs among the RNA-dependent polymerase encoding elements are often used to study the relationships of viruses. There are four common motifs that are conserved in the sequences of the polymerases showing RNA template specificity (1989, EMBO, v8:p3887-3874). We have cloned that region of the polymerase encoded by the RNA S4 of CFSV and have compared it to other members of the family Reoviridae. A comparative analysis demonstrates that CFSV is most closely related to RRSV and then to the Cypo- viruses (Table 1.6.2). This analysis clearly identifies CFSV as a plant reovirus that is most closely related to RRSV. Further analysis is needed to determine if CFSV should be placed into a unique genus of the Reoviridae.

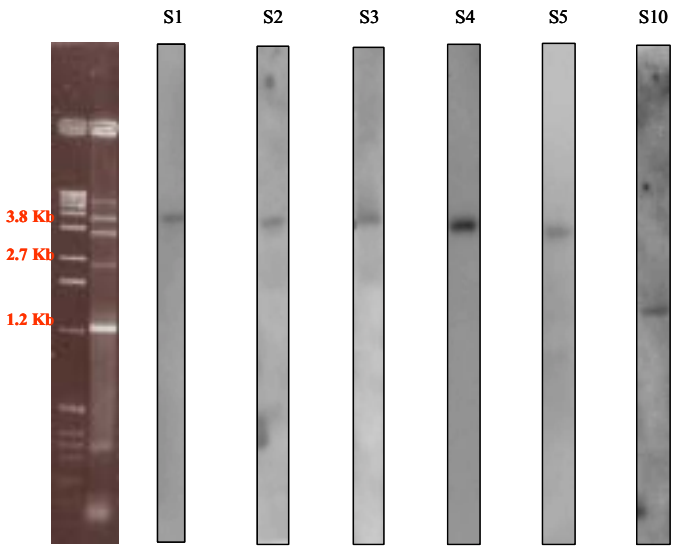


Figure 1.6.1. Hybridization of the genomic segments (S1-S6) of CFSV using probes generated from cDNA clones of this virus.

Table 1.6.2. A comparison of sequences surrounding the conserved RdRp motifs of members of the virus family Reoviridae. Under host vertebrates (V), plant (P), and insects (I).

Genus	Type species	Abbreviations	RdRp	Host
Orthoreovirus	*Avian orthoreovirus*NC98	**ARV**	RNA1	(V)
Aquareovirus	*Golden shiner virus*	**GSRV**	RNA2	(V, I)
Coltivirus	Colorado tick fever virus	**CTFV**-F1	RNA1	(V, I)
Fijivirus	*Fiji disease virus*	**FDV**	RNA1	(P, I)
Oryzavirus	*Rice ragget stunt virus*	**RRSV**	RNA4	(P, I)
Unclassified	*Cassava frogskin virus*	**CFSV**	RNA4	(P, ?)
Cypovirus	*Bómbix mori*	**BmCPV-1**	RNA2	(I)
Orbivirus	Bluetongue virus	**BTV**	RNA1	(V, I)
Phytoreovirus	*Rice dwarf virus*	**RDV**	RNA1	(P, I)
Rotavirus	Simian Rotavirus A	**SiRV-A**	RNA1	(V)
Seadornavirus	*Banna virus*	**BAV-Ch**	RNA1	(V, I)

Motifs	I	II	III	I
ARV	VQRRPRSI	**DISACDAS**	SGSTATSTEHTANNST	YVCQGDDGLM.IIDG
GSRV	VQRRARSI	**YAAFLAPI**	SGSTATSTEHTANNGA	IVKDMNIQNNYVCQ.
CTFV	..RRPRVI	**DSSTKPNT**	SGLLNTADQHT FLGV	GSVLGDDQVAGAFCQ
FDV	IDRRARVI	**DMKGMDAH**	SGFFATSAQHTLFLSL	HSVMGDDVFEVIVN.
RRSV	IGRRQRAI	**DASVQASV**	SGQPFTTVHHTFTLSN	LTVQGDDTRT.INYG
CFSV	IGRRQRAV	**DASVQAAV**	SGLPFTNVHHTFILTS	LTIQGDDIRM.AN--
BmCPV-1	SDRRQRAI	**DASVTTNT**	SGRADTSTHHTVLLQG	KIL GDDIME IFQG
BTV	PIKATRTI	**DYSEYDTH**	SGENSTLIANSMHNMA	EQYVGDDTLFYTKLD
RDV	AWRPVRPI	**DCTSWDQT**	SGRLDTFFMNSVQNLI	FQVAGDDAIM.VYDG
SiRV-A	PGRRTRII	**DVSQWDSS**	SGEKQTKAANSIANLA	IRVDGDDNYAVLQFN
BAV-Ch	VSDLDVVV	**MAPQLAVT**	EKPLKYKMNGLVCESA	TVALDDYNNRAYRLN

Isolate variation and the association of CFSV with CFS disease. During the development of an rt-PCR diagnostic method, it was determined that there existed substantial variation some isolates of CFSV. Using the information that was developed for the study of diversity, primers that generate a sequenced characterized amplified region (SCAR) were developed and they identify all known variants of the cassava frogskin virus. These amplify a 958 nucleotide region in the 5'region of CFSV S4 which is the polymerase gene. New isolates were collected from the Departments of Meta, Cauca, and Sucre. Also included in the analysis were older isolates from the Departments of Magdalena, Valle del Cauca, Amazonas and Tolima. A total of 39 independent cDNA clones were produced and sequenced representing 16 independent isolates. The majority of these isolates were in one group (Figure 1.6.3). It is remarkable, that the two isolates were collected in the Department Magdalena in 1980 and the seven isolates that were collected in 2005 from the Departments of Sucre, Meta and Cauca had 99% identity. The isolate Amazonia 16 collected from the Tarapoto Amazonia in 1990 and the isolate FSD 29 collected in Quilcacé, Cauca in 1983 were distinct from the other isolates an had only 89% identity with them. These isolated had 93% identity with each other. This is evidence that there are at least three distinct strains of CFSV. It is also evident from the finding of the same virus in plants affected with cassava frogskin disease from seven Departments of Colombia over a period of 25 years that there is a strong association of the virus with the disease.

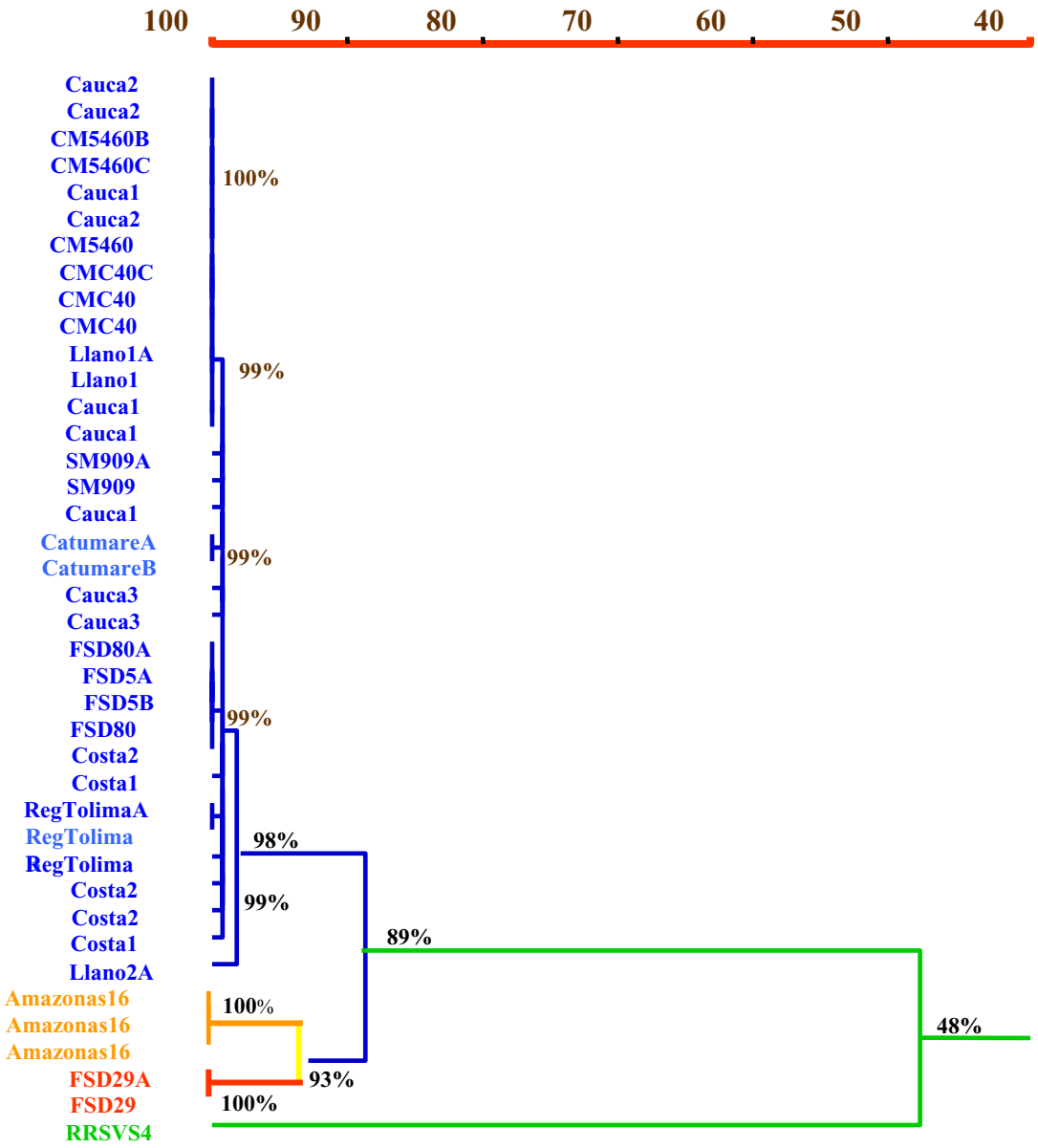


Figure 1.6.3. A comparison of a 958 nucleotide region of the RNA 4 of CFSV that were collected in 16 different sites over a period of 25 years. The percentages represent the identity between the different isolates.

Activity 1.7. Monitoring of whitefly populations in the Andean zone.

Contributors: J. M. Bueno, I. Rodríguez, C. Cardona and F. Morales.

Highlight:

∉ Detected important changes in whitefly species composition in the target area

Rationale

Continuous monitoring of changes in whitefly populations and species composition in target areas is one of the most important objectives of the DFID-funded project on Sustainable Management of Whiteflies. This is needed to develop appropriate management systems and, if necessary, to modify existing systems so as to be able to cope with new situations.

Materials and Methods

In 2006 we processed a total of 17 whitefly samples (adults and pupae) collected in 11 locations of the Cundinamarca Department of Colombia, at altitudes ranging between 1300 and 2100 meters above sea level (MSL). Samples were taken from beans, snap beans, tomatoes and several other annual crops. When possible, identification was initially based on morphological characteristics of pupae. To differentiate between biotypes (which are impossible to differentiate by morphology) RAPD techniques (primer OPA-04) were used. RAPD patterns of pupae and adults brought from the field were compared with those of existing mass rearings of different whiteflies maintained at CIAT (Figure 1.7.1).

Results and Discussion

In order to know about the distribution of the whitefly, other areas in Colombia are continued to be monitored, where it is considered to be an important pest. Four sites of the Cundinamarca Department (Fómeque, Cáqueza, Fusagasugá and Arbeláez) located in the Central Zone of Colombia, with altitudes ranging between 1300 and 2100 MSL, were sampled. We found that 64.7% of the collected samples were of *T. vaporariorum* and the other 35.3%, which were found under 1700 MSL, correspond to a complex of *T. vaporariorum* and *B. tabaci* biotype B. Presence of biotype B of *B. tabaci* was evidenced in the field by proofs of physiological disorders (irregular ripening) in tomatoes that was also associated with the presence of begomovirus and by morphological differentiations with *T. vaporariorum*. These identifications were confirmed by means of RAPD molecular tests, as seen in Figure 1.7.1.

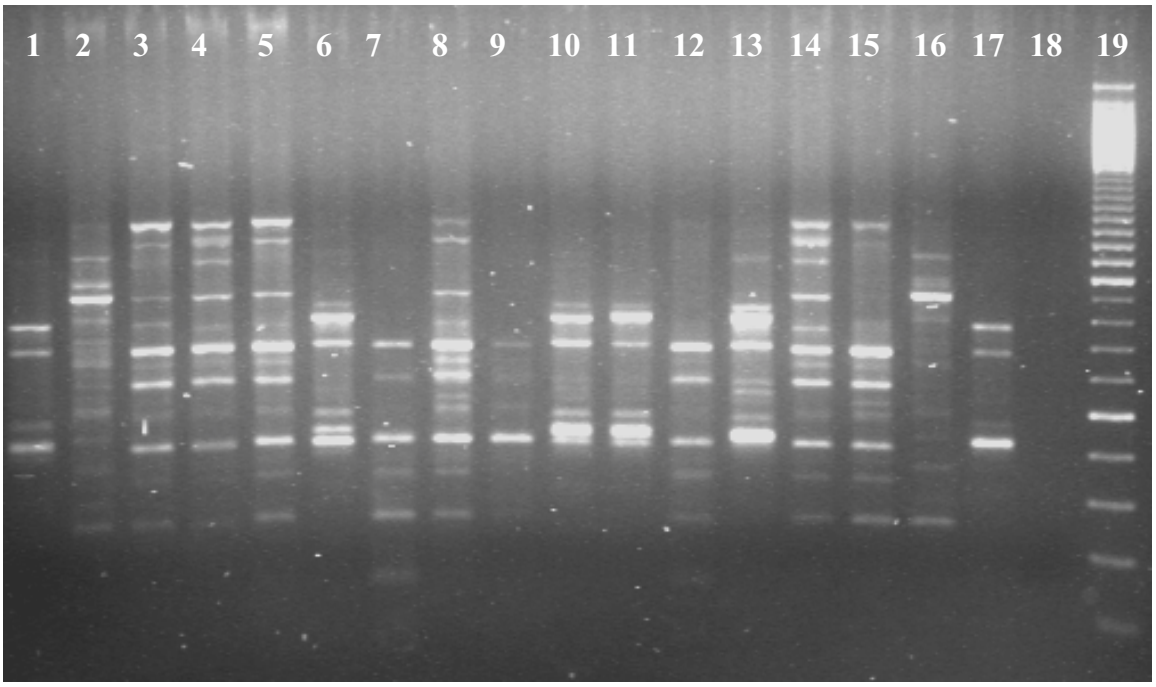


Figure 1.7.1. RAPD's of whiteflies collected in the Cundinamarca Department. Amplification of the primer OPA-04: 1, *T. vaporariorum* (CIAT breeding adults); 2, *B. tabaci* biotype A (CIAT breeding adults); 3, *B. tabaci* biotype B (CIAT breeding adults), 4-5, adults of *B. tabaci* biotype B collected in Fusagasugá (1491 MSL): 6-7, adults of *T. vaporariorum* and pupae of *B. tabaci* biotype B, collected in Fusagasugá (1719 MSL); 8-9, adults of *B. tabaci* biotype B collected in Fusagasugá (1312 MSL); 10, adults of *T. vaporariorum* collected in Fusagasugá (1376 MSL); 11-12, adults of *T. vaporariorum* and pupae of *B. tabaci* biotype B, collected in Arbeláez (1430 MSL); 13-14, adults of *T. vaporariorum* and pupae of *B. tabaci* biotype B, collected in Arbeláez (1478 MSL; 15, *B. tabaci* biotype B (CIAT breeding adults); 16, *B. tabaci* biotype A (CIAT breeding adults); 17, *T. vaporariorum* (CIAT breeding adults); 18, reaction target; 19, marker (100 pb).

As shown in Figure 1.7.2, species composition in the Cundinamarca Department has changed drastically in the past 9 years and the trend continues: since the introduction to Colombia of the biotype B, which is occupying niches previously reserved to *T. vaporariorum* even in areas located above 1300 MSL. The biotype B is an aggressive form of whitefly that is causing all the serious problems described in our 2003 and 2004 Reports. These include physiological disorders in several different crops (silver-leaf in squash, uneven ripening of tomatoes, pod chlorosis in snap beans), and the ability to transmit a geminivirus that has devastated snap beans in areas below 1200 MSL.

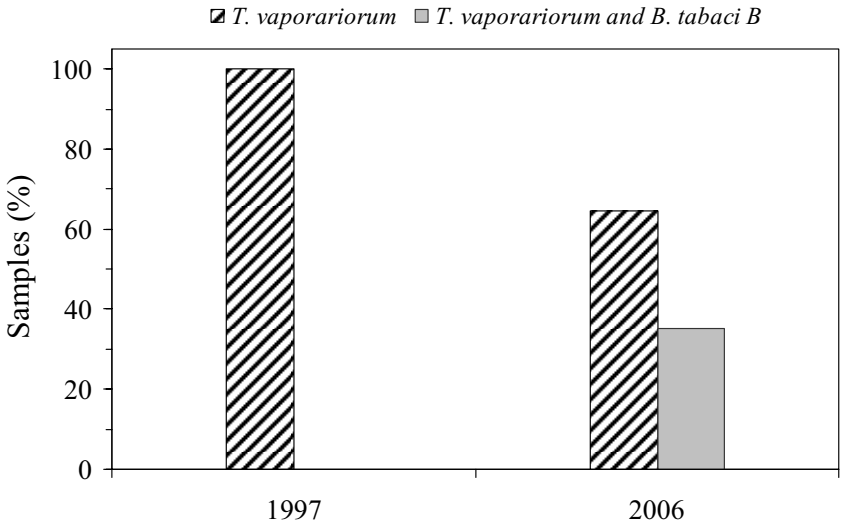


Figure 1.7.2. Changes in whitefly species composition in the Cundinamarca Department (Fómeque, Cáqueza, Fusagasugá and Arbeláez) located in the Central highlands of Colombia, with altitudes ranging between 1300 and 2100 MSL (1997-2006).

Activity 1.8. Mortality levels of new pesticides for the control of whitefly populations

Contributors: J.M. Bueno, H. Morales and C. Cardona.

Highlight :

- Evaluation of the efficiency of pesticide that could help to control whiteflies in the target region

Rationale

Besides monitoring resistance to pesticides, which is one of the major targets of the DFID-funded project on Management of Whiteflies in the Tropics, it is necessary to try new active ingredients and generic products that can help control this pest, without drastically affecting the economy of the farmer and harming the environment.

Materials and Methods

In order to evaluate the efficiency of pesticides on first instar nymphs (N1), we proceeded to obtain populations of nymphs in the same development stage of a susceptible strain by confining adults in clip cages attached to leaves of common bean seedlings. The adults (10 per clip cage) were allowed to oviposite during 24 hours and then were taken out of the cage. After the eclosion of the eggs, the area of the infested leaves with first instar nymphs was marked and the amount of individuals obtained was registered. Infested seedlings were further dipped during 5 seconds in 1000 mL of the pre-established concentration of each tested pesticide. Formulated material

was used in all the tests and the pre-established concentrations were prepared by dilution with distilled water. Controls were dipped in distilled water. Plants were left inside the cages for the counting of adult emergence (26 days after treatment). The difference between the initial N1 and the amount of adult emergence is a surviving method.

For the evaluation of adults, leaves were immersed in the pre-established dosage for each treatment. The leaves were dried at room temperature, and circles of about 5cm in diameter were cut placing them on noble agar in Petri cases. Adults were left inside each Petri case. After 48 hours, the amount of living and deceased individuals was registered. We used a randomized complete block design: 10 repetitions per treatment and 20 individuals per repetition. Everything was performed under controlled conditions of temperature and humidity. Mortality of the controls was not accepted over 10%. Mortality percentages were calculated, having Abbott making the corrections. The treatments used are as follow:

Orgocontrol 1 = one part of Orgocontrol and 32 parts of water
Orgocontrol 2 = one part of Orgocontrol and 64 parts of water
Orgocontrol 3 = one part of Orgocontrol and 128 parts of water
Imidor 1 = 600cc/ha (generic Imidacloprid)
Imidor 2 = 300cc/ha
Confidor = 600cc/ha (chemical control)
Control (Test) = water control

Results and Discussion

The toxicological reaction of the *T. vaporariorum* N1's to the six treatments are presented in Figure 1.8.1. It is observed that treatments with Orgocontrol 1 and 2, and Imidor 1 present mortality higher than 80%, which is a similar result obtained with the chemical control with Confidor. This indicates that these treatments can be used as a solution for the control of immature insects. For the control of adults, the pre-established dosages of Orgocontrol only reached a 60% of mortality, while the dosages of Imidor 1 and Imidor 2 were similar in results obtained with Confidor. The treatment with Imidor can be used for the control of both immature and adult stages of *T. vaporariorum* (Figure 1.8.2).

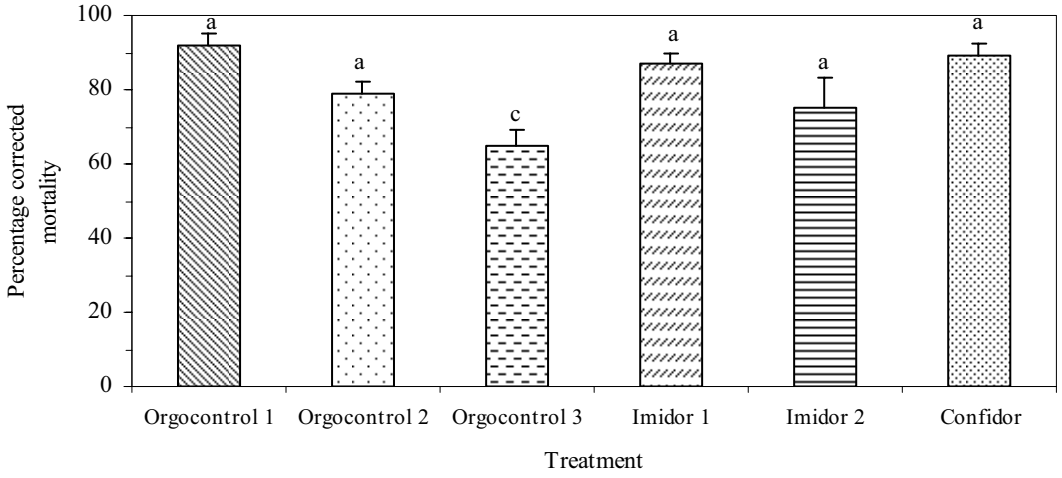


Figure 1.8.1. Response (corrected percentage mortality) of *Trialeurodes vaporariorum* first instarnymph of three insecticides with diferent doses. Dosages were as follows: Orgocontrol 1= one part Orgocontrol to 32 parts water; Orgocontrol 2= one part Orgocontrol to 64 parts water; Orgocontrol 3= one part Orgocontrol to 128 parts water; Imidor 1= 600cc/ha; Imidor 2= 300cc/ha and Confidor = 600cc/ha.

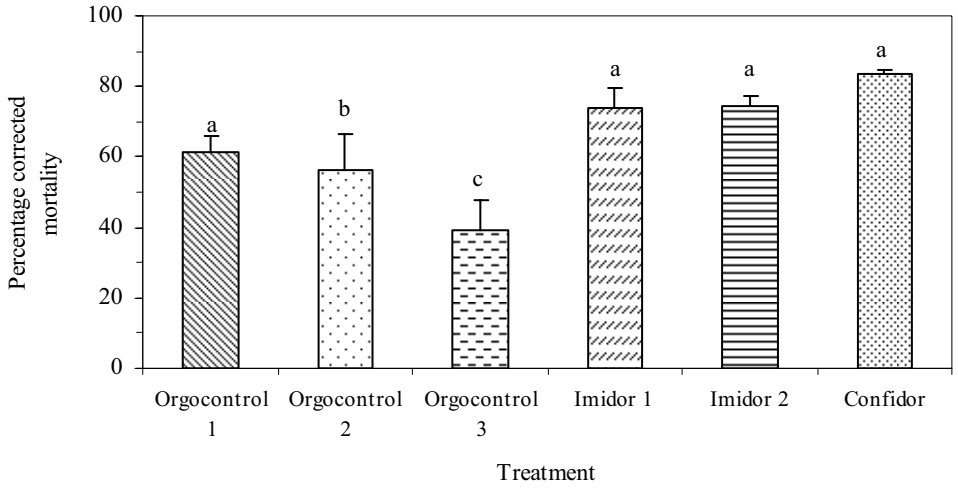


Figure 1.8.2. Response (corrected percentage mortality) of *Trialeurodes vaporariorum* Adultsof three insecticides with diferent doses. Dosages were as follows: Orgocontrol 1= one part Orgocontrol to 32 parts water; Orgocontrol 2= one part Orgocontrol to 64 parts water; Orgocontrol 3= one part Orgocontrol to 128 parts water; Imidor 1= 600cc/ha; Imidor 2= 300cc/ha and Confidor = 600cc/ha.

Figure 1.8.3 shows the response of N1 nymphs of *Bemisia tabaci* biotype B to different treatments, evidencing high susceptibility to the different pre-established dosages of each pesticide. For adults, the pre-established dosages of Orgocontrol only eliminated a 30%, while the dosages of Imidor produced a mortality of 80%, similar to Confidor results (Figure 1.8.4).

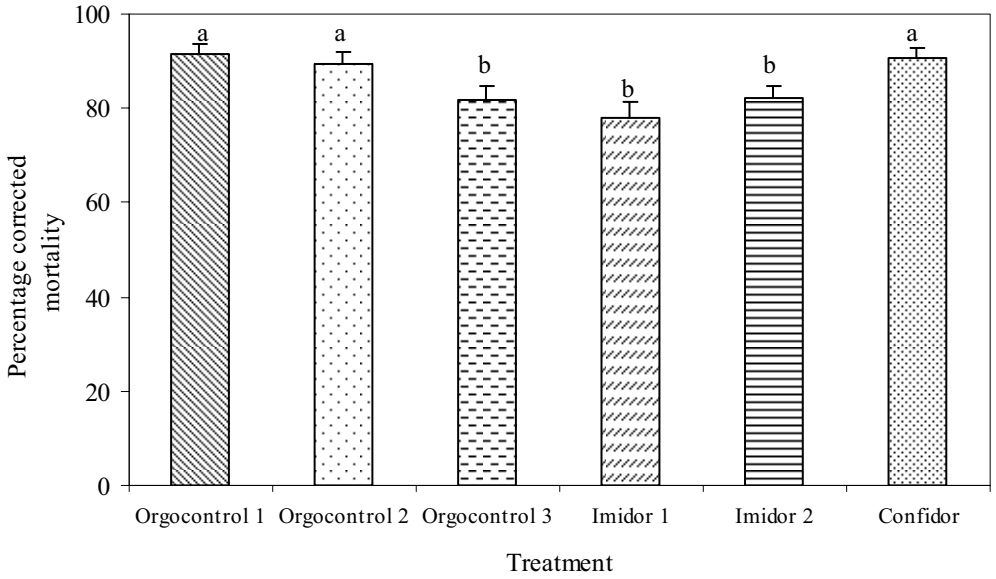


Figure 1.8.3. Response (corrected percentage mortality) of Bemisia tabaci B first instar nymph of three insecticides with diferent doses. Dosages were as follows: Orgocontrol 1= one part Orgocontrol to 32 parts water; Orgocontrol 2= one part Orgocontrol to 64 parts water; Orgocontrol 3= one part Orgocontrol to 128 parts water; Imidor 1= 600cc/ha; Imidor 2= 300cc/ha and Confidor = 600cc/ha.

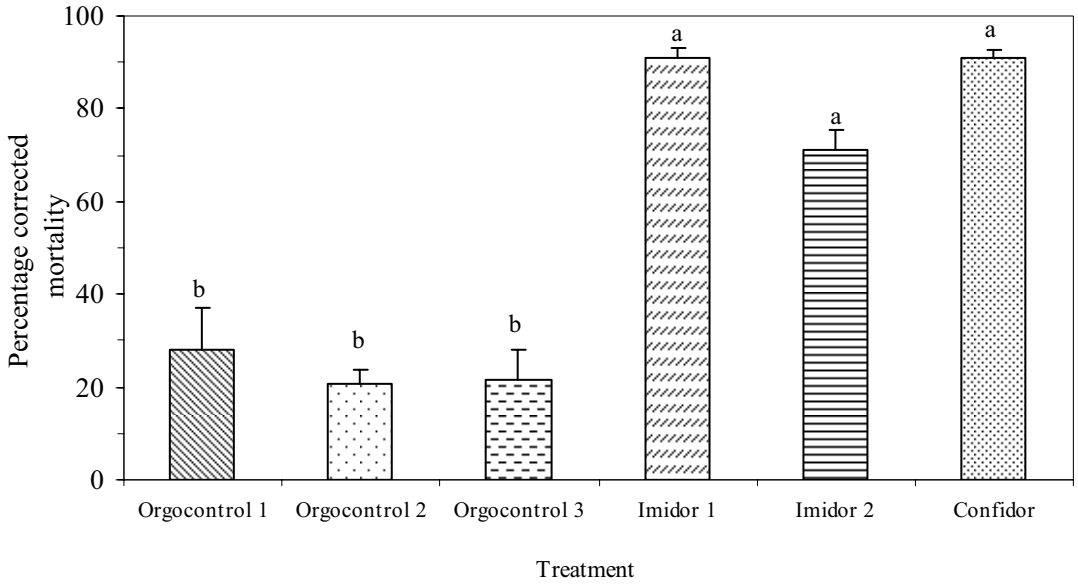


Figure 1.8.4. Response (corrected percentage mortality) of Bemisia tabaci B adults of three insecticides with diferent doses. Dosages were as follows: Orgocontrol 1= one part Orgocontrol to 32 parts water; Orgocontrol 2= one part Orgocontrol to 64 parts water; Orgocontrol 3= one part Orgocontrol to 128 parts water; Imidor 1= 600cc/ha; Imidor 2= 300cc/ha and Confidor = 600cc/ha.

Activity 1.9. 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 and Valle 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 have analyzed the internal transcribed spacer (ITS) regions of the ribosomal DNA of the *Colletotrichum* isolates using the polymerase chain reaction (PCR) method. Furthermore, we used RAPD (random amplified polymorphic DNAs) and AFLP (amplified fragment length polymorphism) to characterize the isolates. We report here the results of this work.

Materials and Methods

Fungal isolates: Ninety-one monoconidial isolates of *Colletotrichum* spp. that are maintained at the Integrated Disease and Pest Management Program of CORPOICA were used (Table 1.9.1). 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 20 ng/σl, and stored at –80 ∀C for further analysis.

Table 1.9.1. Isolates of *Colletotrichum* spp infecting citrus fruits and tree tomato used in this study.

Isolate code	Zone/Municipality	Farm	Host	Tissue	*Colletotrichum* spp.	Extracted DNA concentration ng/σl
5	Caicedonia	Danubio	Limón Tahití	Flor	*C. acutatum*	120
6	Manizales	La Bejuca	Limón Tahití	Botón	*C. acutatum*	65
14	Caicedonia	Danubio	Limón Tahití	Botón	*C. acutatum*	98
55	Pereira	Catalina (FEDECAFË)	Limón Tahití	Botón	*C. acutatum*	81
83	Pereira	Catalina (FEDECAFË)	Limón Tahití	Botón	*C. acutatum*	100
100	Caicedonia	Maracaibo	Limón Tahití	Flor	*C. acutatum*	101
107	Pereira	Catalina (FEDECAFË)	Limón Tahití	Botón	*C. acutatum*	132
212	Caicedonia	Maracaibo	Limón Tahití	Flor	*C. acutatum*	95
269	Pereira	Yarima	Limón Tahití	Botón	*C. acutatum*	110
275	Pereira	Yarima	Limón Tahití	Botón	*C. acutatum*	264
589	Villavicencio	El Refugio	Limón Tahití	Flor	*C. acutatum*	107
590	Villavicencio	El Refugio	Limón Tahití	Flor	*C. acutatum*	116
592	Cumaral	Las Brisas	Limón Tahití	Flor	*C. acutatum*	277
593.a.	Cumaral	Las Brisas	Limón Tahití	Flor	*C. acutatum*	189
593.b.	Cumaral	Las Brisas	Limón Tahití	Botón	*C. acutatum*	83
594	Cumaral	Las Brisas	Limón Tahití	Botón	*C. acutatum*	143
595	Cumaral	Las Brisas	Limón Tahití	Botón	*C. acutatum*	127
596	Cumaral	Las Brisas	Limón Tahití	Botón	*C. acutatum*	113
597	Cumaral	Las Brisas	Limón Tahití	Flor	*C. acutatum*	195
599	Cumaral	Las Brisas	Limón Tahití	Botón	*C. acutatum*	114
600	Villavicencio	El Refugio	Limón Tahití	Flor	*C. acutatum*	113
611	Villavicencio	El Refugio	Limón Tahití	Flor	*C. acutatum*	209
619	Villavicencio	El Refugio	Limón Tahití	Flor	*C. acutatum*	119
644	Zona Bananera	La Inmaculada	Limón común	Flor	*C. acutatum*	125
651	Ciénaga	Las Margaritas	Limón común	Botón	*C. acutatum*	126
656	Ciénaga	Las Margaritas	Limón común	Flor	*C. gloeosporioides*	308
663	Ciénaga	Las Margaritas	Limón común	Flor	*C. acutatum*	126
677	Zona Bananera	La Inmaculada	Naranja Tangüelo	Hoja	*C. gloeosporioides*	374
679	Zona Bananera	La Inmaculada	Limón común	Botón	*C. acutatum*	339
687	Zona Bananera	La Inmaculada	Limón común	Botón	*C. acutatum*	130
699	Montenegro	Estancia	Naranja Valencia	Flor	*C. gloeosporioides*	125
754	Meta	El Naranjal	Limón tahití		*C. acutatum*	220
755	Meta	El Naranjal	Limón tahití		*C. acutatum*	350
756	Meta	El Naranjal	Limón tahití		*C. acutatum*	249
757	Meta	El Naranjal	Limón tahití		*C. acutatum*	149
758	Meta	El Palmar	Limón tahití		*C. acutatum*	196

Table 1.9.1 continued

Isolate code	Zone/Municipality	Farm	Host	Tissue	*Colletotrichum* spp.	Extracted DNA concentration ng/σl
759	Meta	El Palmar	Limón tahití		*C. acutatum*	149
761	Meta	Los Ramos	Limón tahití		*C. acutatum*	241
762	Meta	El Elefante	Limón tahití		*C. acutatum*	195
763	Meta	El Elefante	Limón tahití		*C. acutatum*	325
764	Meta	El Elefante	Limón tahití		*C. gloeosporioides*	178
765	Meta	Las Brisas	Limón tahití		*C. acutatum*	251
766	Meta		Limón tahití		*C. acutatum*	217
768	Santander	Casaloma	Limón tahití		*C. acutatum*	232
769	Santander	Casaloma	Limón tahití		*C. acutatum*	273
771	Santander	Casaloma	Limón tahití		*C. acutatum*	219
774	Santander	Lorenzo	Limón tahití		*C. acutatum*	478
775	Santander	San Antonio	Limón tahití		*C. acutatum*	396
776	Santander	San Antonio	Limón tahití		*C. acutatum*	295
777	Santander	San Antonio	Limón tahití		*C. acutatum*	284
778	Santander	La Esmeralda	Limón tahití		*C. acutatum*	445
779	Santander	La Esmeralda	Limón tahití		*C. acutatum*	446
780	Santander	La Esmeralda	Limón tahití		*C. acutatum*	422
781	Santander	La Esmeralda	Limón tahití		*C. acutatum*	233
782	Santander	La Esmeralda	Limón tahití		*C. acutatum*	386
783	Santander	Alto de la Sabana	Limón tahití		*C. acutatum*	415
784	Santander	Alto de la Sabana	Limón tahití		*C. acutatum*	246
785	Santander	Los Mangos	Limón tahití		*C. acutatum*	258
786	Santander	Los Mangos	Limón tahití		*C. acutatum*	424
787	Santander	Asomadita	Limón tahití		*C. acutatum*	587
788	Santander	Asomadita	Limón tahití		*C. acutatum*	381
789	Santander	El Mirador	Limón tahití		*C. acutatum*	396
790	Santander	Chimita	Limón tahití		*C. acutatum*	410
791	Santander	El Pangil	Limón tahití		*C. acutatum*	291
792	Santander	Villa Diana	Limón tahití		*C. acutatum*	204
793			Tomate de árbol		*C. acutatum*	291
794			Tomate de árbol		*C. acutatum*	230
796			Tomate de árbol		*C. acutatum*	244
797			Tomate de árbol		*C. acutatum*	196
798			Tomate de árbol		*C. acutatum*	278
801			Tomate de árbol		*C. acutatum*	356
802			Tomate de árbol		*C. acutatum*	243
805			Tomate de árbol		*C. acutatum*	311
806			Tomate de árbol		*C. acutatum*	247
808			Tomate de árbol		*C. acutatum*	300
809			Tomate de árbol		*C. acutatum*	364
810			Tomate de árbol		*C. acutatum*	381
812			Tomate de árbol		*C. acutatum*	210
813			Tomate de árbol		*C. acutatum*	478
814			Tomate de árbol		*C. acutatum*	262
816			Tomate de árbol		*C. acutatum*	377
820			Tomate de árbol		*C. acutatum*	353
821			Tomate de árbol		*C. acutatum*	242
822			Tomate de árbol		*C. acutatum*	378
824			Tomate de árbol		*C. acutatum*	199
826			Tomate de árbol		*C. acutatum*	327
827			Tomate de árbol		*C. acutatum*	262
828			Tomate de árbol		*C. acutatum*	218
830			Tomate de árbol		*C. acutatum*	478
831			Tomate de árbol		*C. acutatum*	416
832			Tomate de árbol		*C. acutatum*	338

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.

RAPD markers: Forty-five arbitrary primers of 10 bases purchased from Operon Technologies⊇ were evaluated, of which 13 were selected for generating polymorphisms among the fungal isolates tested. Table 1.9.2.shows the list of RAPD primers that were selected for evaluation of all ninety-one isolates (65 isolated from citrus and 26 from tree tomato)

AFLP (Amplified Fragment Length Polymorphism) *analysis:* The Kit AFLP⊇ *Analysis System for Microorganisms* (Invitrogen, 2004) was used largely according to the manufacturer's instructions except that the reaction volume was reduced by 75% while still maintaining the recommended concentrations.

Table 1.9 2. RAPD primers selected in this study.

Primer code	Sequence (5`⇓ 3`)
A-04	AATCGGGCTG
AJ-05	CAGCGTTGCC
AJ-08	GTGCTCCCTC
AJ-09	ACGGCACGCA
AJ-11	GAACGCTGCC
AJ-15	GAATCCGGCA
AJ-20	ACACGTGGTC
AK-04	AGGGTCGGTC
AK-09	AGGTCGGCGT
AN-03	AGCCAGGCTG
B-01	GTTTCGCTCC
B-04	GGACTGGAGT
C-01	TTCGAGCCAG

The genomic DNA of each isolate was digested with restriction enzymes *Eco*RI and *Mse*I. Specific adopters were then ligated to the ends of the digested DNA fragments. Subsequently, PCR amplifications were conducted using primers that are specifically designed that recognize the sequences of the adopters.

For AFLP analysis, only 57 isolates (31 from citrus and 26 from tree tomato) were used to date. Various combinations of primers, E-AC/M-C, E-AC/M-A, and E-AA/M-C, were used in order to generate information that reflects genetic diversity among the isolates. The amplified products were separated on denaturing 6% polyacrylamide gels with 5M urea and run in electrophoretic vertical equipment Sequi-Gen⊇ GT BIO-RAD. OncThe gels were stained with silver nitrate according to protocols provided by Promega (1998. Technical manual. Silver sequence ^{TM} DNA sequencing system. Promega Corporation). to visualize the results. Inorder to identify the fragment sizes, a molecular size marker (molecular DNA ladder 10 bp Invitrogen⊇) was used that has size markers in the range of 30-330 bp.

Analysis of RAPD and AFLP data: comparisons of each banding profile for each primer were conducted on the basis of presence or absence (1/0) of amplified products of the same size. Band of the same size were scored as identical. An analysis of similarity was conducted with data collected for the two types of makers. The similarity matrix was constructed using NTSYS version 2.1 (Rohlf, 2000, Exeter Publ). The coefficients of similarity were introduced in the subprogram SAHN in order to construct dendrograms. Furthermore, a multiple correspondence analysis (MCA) was conducted using SAS (SAS Institute, 1989).

Results and Discussion

PCR amplifications: The DNA extraction protocol used generated high quality DNA. Many of the arbitrary primers tested so far resulted in limited polymorphisms). 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, 764 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, and 764 as well as the control isolates generated a DNA product of 450 bp indicating that they all belong to the species *Colletotrichum gloeosporioides* (Figure 1.9.1). The results of the molecular identification of all the isolates tested are presented in Table 1.9.1.

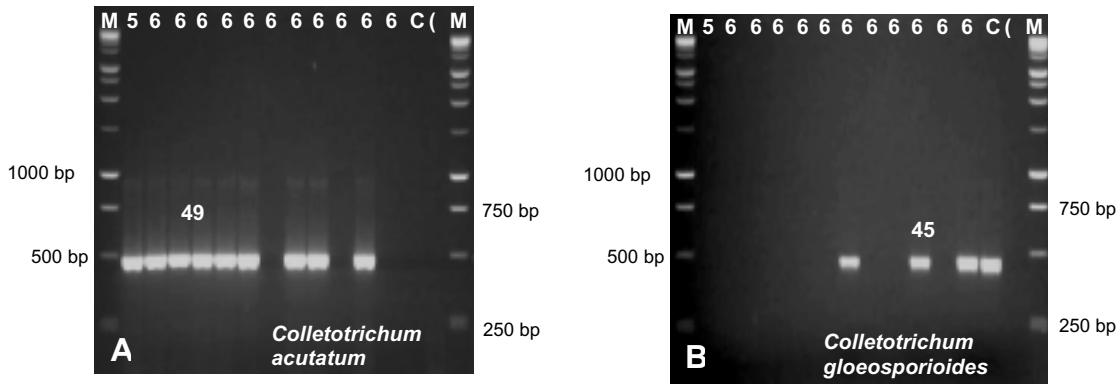


Figure 1.9.1. Taxonomic identification of isolates of *Colletotrichum* spp. using PCR analysis **A,** *Colletotrichum acutatum* (using primers CaInt2 and ITS4); **B,** *Colletotrichum gloeosporioides* (with primers CgInt and ITS4). The numbers at each lane are isolate numbers described in Table 1.9.1. Lanes (∝), negative control without sample DNA; M, size marker; **Cg**, positive control *C. gloeosporioides*.

RAPD data analysis: Amplification products of the 91 isolates using the selected random primers showed very limited polymorphisms (see Figure 1.9. 2). The isolates that were identified as C. gloeosporioides (656, 677, 699 and 764), generated patterns very different from the rest of the isolates of C. acutatum. The C. acutatum isolates with the exception of numbers 596, 611, and 792 generated very similar band patterns (Figure 1.9. 2). Fungal isolates from citrus produced more applification bands than those from tree tomato (Figure 1.9. 2C)

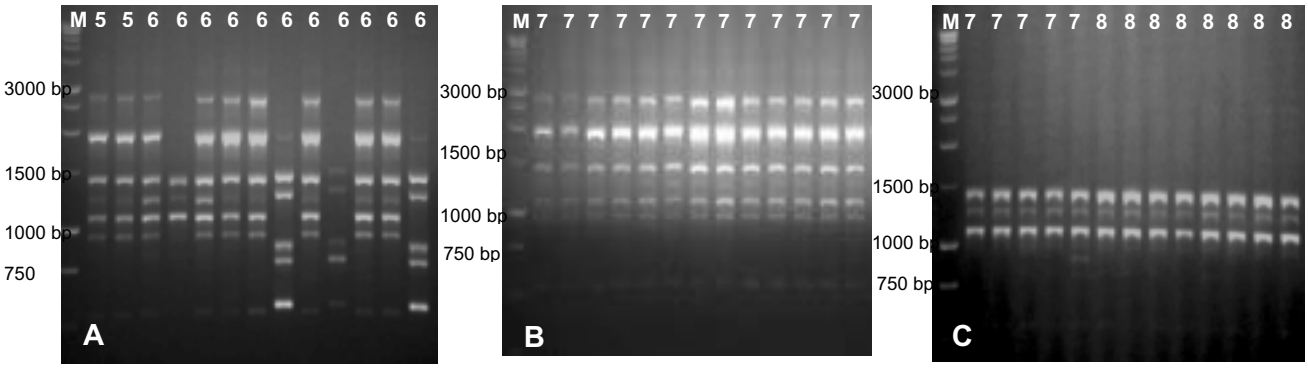


Figure 1. 9. 2. RAPD amplification products of DNA from isolates of *Colletotrichum* spp**.** isolated from anthracnose lesions amplified using random primer B-01 (see sequence in Table 1. 9. 2). **A and B**, citrus; **C**, tree tomato. The numbers indicated in each lane correspond to the isolate codes listed in Table 1.9. 1. Lane **M** corresponds to the 1 kb molecular size marker Promega⊇.

Analysis of the collected data corroborates these observations. Figure 1. 9.3 demonstrates a similarity dendrogram using coefficient of Nei-Li (1979, Proc. Natl. Acad. Sci. USA. 79:5269-5273), where a clear separation of C. *acutatum* and C. *gloeosporioides* is observed at the level of 23% similarity. Interestingly, the isolates of *C. acutatum* were separated along the line of their original hosts: citrus and tree tomato, with a 61% similarity. However, the isolates 596 and 611, although isolated from citrus, the dendogram grouped them within the group formed by isolates from tree tomato at a level of 74% similarity (Figure 1.9.3). Within each group, there is a high level of similarity that ranged between 88-100% indicating a very limited level of diversity.

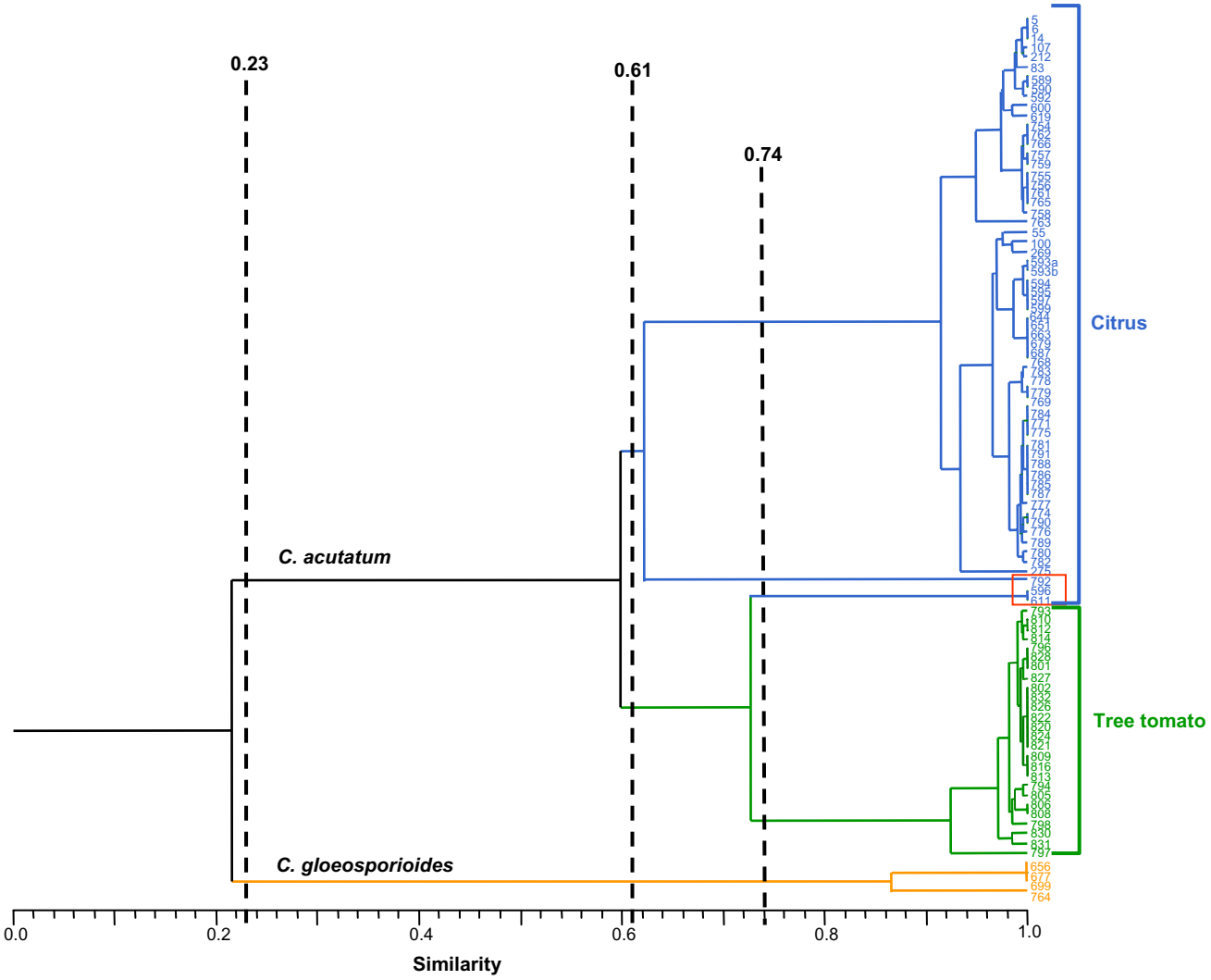


Figure 1.9.3. Similarity dendrogram (UPGMA) of 91 isolates of species of *Colletotrichum* infecting citrus and tree tomato based on RAPD data using 13 random primers listed in Table 1.9. 2.

Multiple correspondence analysis (MCA) further corroborates the results of the similarity analysis. Figure 1.9.3 presents a three dimensional visualization of the variation among the fungal isolates. In the first dimension, there is a clear differentiation of *C. acutatum* and *C.*

gloeosporioides. In the second dimension, a clear separation among isolates that infect citrus and tree tomato is observed within the species *C. acutatum*. The third dimension demonstrates a narrow separation forming two related groups (similarity analysis of 92%).

These results using the selected random primers and the 91 isolates indicate that there is a narrow genetic base with high level of similarity among these isolates. The geographic origin and the exact source of the isolates 596, 611 and 792 remain unclear.

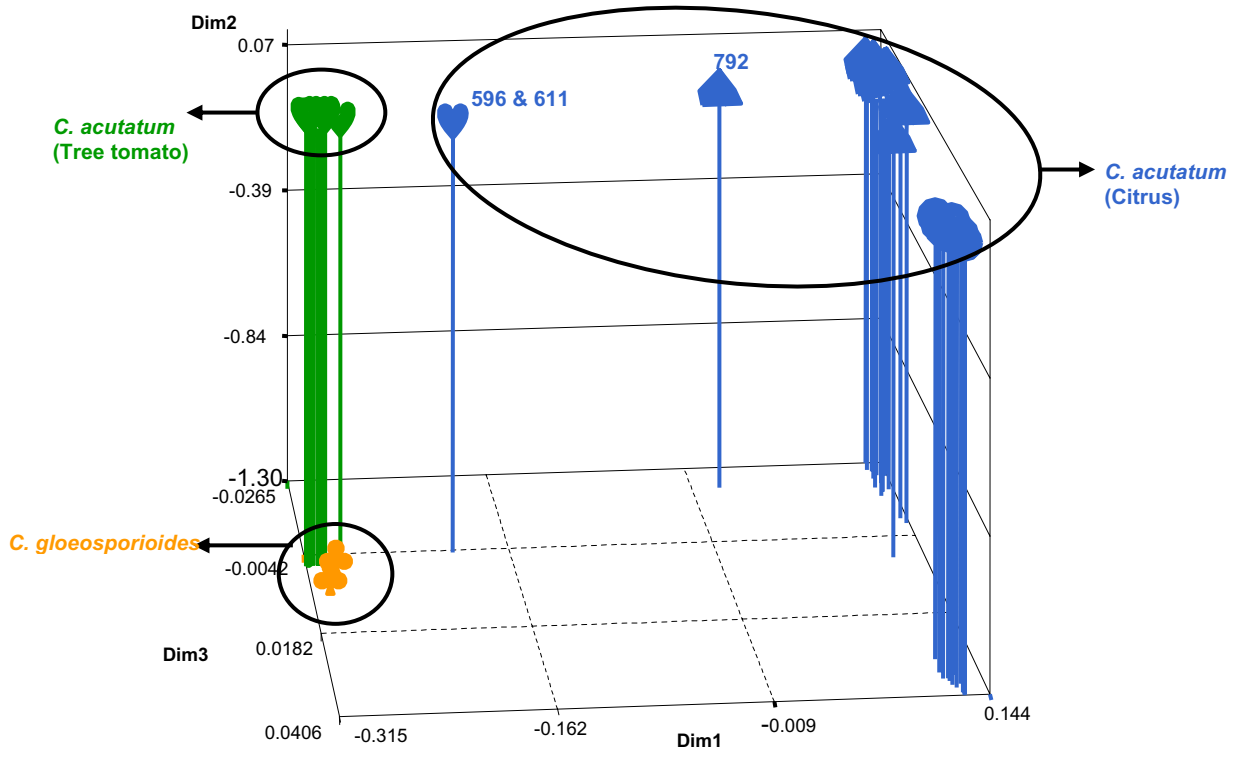


Figure 1.9.4. Three-dimensional graph derived from multiple correspondence analysis (MCA) of 91 isolates of species of *Colletotrichum* infecting citrus and tree tomato based on RAPD data with 13 arbitrary primers. *AFLP analysis:* Of the 91 isolates of the anthracnose pathogen used in this study and evaluated with RAPD analysis, 57 were examined with three combinations of AFLP primers, E-AC/M-C, E-AC/M-A, and E-AA/M-C. Figure 1.9.4 shows an example of AFLP gel pattern of some isolates. A large number of bands were generated with AFLP analysis which allow the evaluation of greater number of loci and thus a wide coverage of the genome. The isolates that showed a pattern of polymorphism were 656, 677, and 699 that were identified as *C. gloeosporioides*;, and isolates 596 and 611 that belonged to *C. acutatum* (Figure 1.9.4), exactly corresponding to the results obtained with RAPD analysis (Figure 1. 9.2). Among the isolates originating from the two plant hosts, there was some differentiation, although they share a number of bands in common. The isolates that originated from citrus generated more bands than those from tree tomato (Figure 1.9.5B).

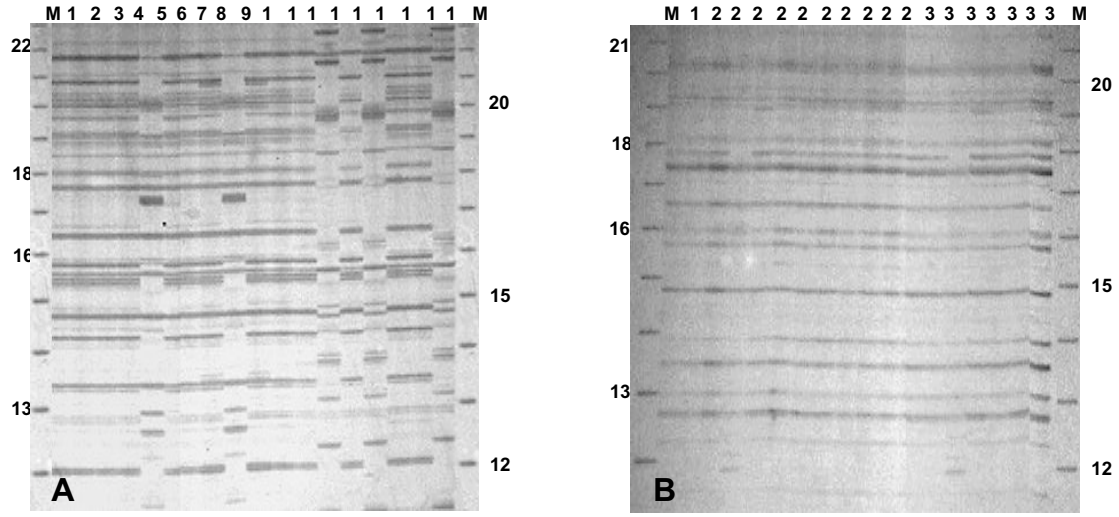


Figure 1.9.5. AFLP amplification profiles of DNA from isolates of *Colletotrichum spp*. infecting citrus (A) and tree tomato (B), amplified with a combination of primers E-AA/M-C. Lane **M** is size marker 30-330 bp AFLP Invitrogen⊇. Lanes (**1**) isolate 593a, (**2**) 593b, **(3**) 594, (**4**) 595, (**5**) 596, (**6**) 597, (**7**) 599, (**8**) 600, (**9**) 611, (**10**) 619, (**11**) 644, (**12**) 651, (**13**) 656, (**14**) 663, (**15**) 677, (**16**) 679, (**17**) 687, (**18**) 699, (**19**) 808, (**20**) 809, (**21**) 810, (**22**) 812, (**23**) 813, (**24**) 814, (**25**) 816, (**26**) 820, (**27**) 821, (**28**) 822, (**29**) 824, **(30**) 826, (**31**) 827, (**32**) 828, (**33**) 830, (**34**) 831, (**35**) 832, (**36**) 797.

Within the isolates of *C. acutatum*, those infecting citrus were separated from those that infect tree tomato with a 60% similarity. However, isolates 596 and 611, although isolated from citrus, the dendrogram grouped them within the tree tomato subgroup with a 66% similarity (Figure 1.9.6). Isolate 792 was not included in this AFLP analysis and thus, it is not shown in the AFLP dendrogram. Within each group and excluding the unique isolates described, a high similarity of 86-100% was observed indicating a low level of diversity.

Multiple correspondence analysis further confirmed the results obtained using the similarity index analysis. Figure 1.9.7 presents a tri-dimensional figure demonstrating the variation among isolates. In the first dimension, the clear separation of *C. acutatum* and *C. gloeosporioides* is shown. In the second dimension, a distinct differentiation within isolates of *C. acutatum* is evident based on their respective hosts. In the third dimension, no separation was evident within the isolates that originated from the same host plant. There is an 87% similarity among isolates from tree tomato and a 91% similarity within those that originated from citrus (Figure 1.9.6).

The statistical analysis conducted using three combinations of AFLP primers and with the 57 isolates tested to date, indicate that a limited genetic diversity exist. These conclusions corroborate those obtained using RAPD analysis.

In the next several months, we plan to increase the number of isolates, including isolates from mango, collected from more locations and analyze them using methods described in this study.

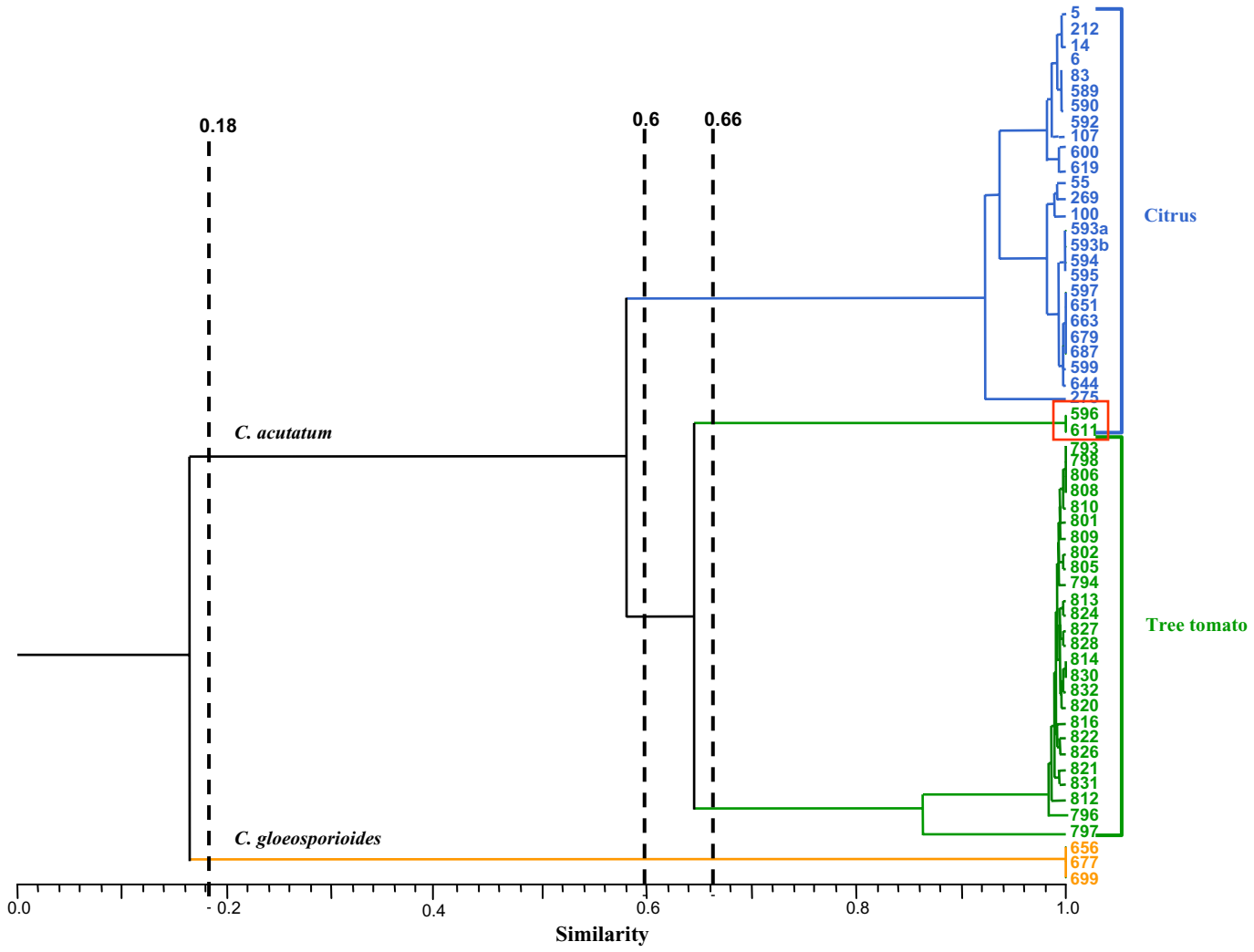


Figure 1.9.6. Similarity dendrogram (UPGMA) of 57 isolates of species of *Colletotrichum* infecting citrus and tree tomato based on AFLP data.

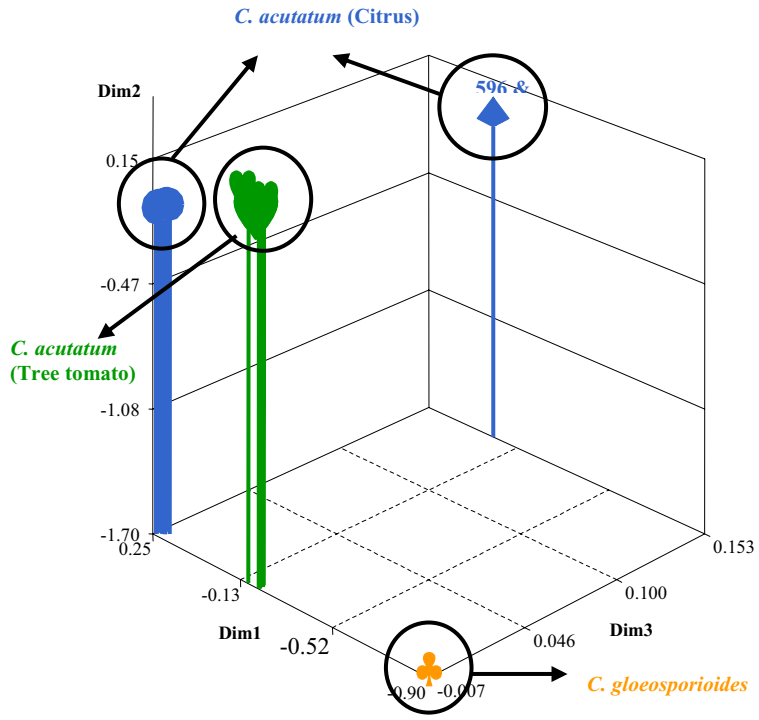


Figure 1.9.7. Three-dimensional graph derived from multiple correspondence analysis (MCA) of 57 isolates of species of *Colletotrichum* infecting citrus and tree tomato based on AFLP data.

Activity 1.10. Identifying strategies for managing anthracnose (*Glomerella cingulata* (*Anamorph Colletotrichum gloeosporioides*) of soursop (*Annona muricata* L.), emphasizing varietal resistance

Contributors*:* A. Rojas Triviño, E.Álvarez, and D. Sánchez

Rationale

Anthracnose is the disease that most limits soursop production in Colombia and other countries. Incidence and losses can be 100% (Oliveros 2000, Recopilación bibliográfica de seis especies de frutales tropicales: lulo, mora, uchuva, pitaya, guanábana y tomate de árbol. CIAT, Cali, Colombia.), depending on agroecological conditions, crop management, and planting material. The disease is favored by inadequate cultural management practices. To successfully manage the disease, we must identify genetic resistance in soursop and understand the disease's epidemiology. Activities towards these ends would include determining markers for pathogenicity and the pathogen's genetic variability, and genetically characterizing different soursop clones selected for their desirable agronomic traits and good performance in production zones.

Materials and Methods

Collecting plant materials: Sampling of plant materials infected with anthracnose was carried out in the Departments of Valle del Cauca, Cauca, Huila, Tolima, Meta, Santander del Sur, Norte de Santander, Quindío, Caldas, Córdoba, and Sucre. Samples were taken from established crops and individual trees in which symptoms appeared in leaves, branches, stems, flowers, and/or fruits. Each sample was identified according to a format for noting information on origin, conditions, and observations.

*Isolating, identifying, and storing the fungus***:** *Colletotrichum* isolates were obtained according to the direct method for isolating plant pathogenic microorganisms (Castaño-Zapata 1997,Manual para el diagnóstico de hongos, bacterias, virus y nemátodos fitopatógenos. Universidad de Caldas, Colombia), but with some modifications. Observations were then made, using a compound light microscope and examining for presumed fungal growth such as types of colony growth and the presence of acervuli, cirri, and spore types. The fungus was then purified on PDA agar, and monosporic cultures later prepared on 2% water agar. The monosporic cultures thus obtained were stored at 4º and -20ºC on Whatman No. 1 filter paper colonized by the fungus (Aricapa and Correa, 1994. ASCOLFI Inf 20(3): 29–30.).

Morphological characterization of Colletotrichum isolates: One objective of morphological characterization is to determine the macroscopic variability of the fungus such as color and type of colony growth, cirrus color, length of conidia, presence of microsclerotia, and other parameters such as speed of growth, presence of the teleomorph stage, and length of asci and ascospores.

Molecular characterization of Colletotrichum isolates: To extract the maximum amount of DNA from each *Colletotrichum* isolate, we followed the methodology reported by Mahuku (Mahuku, 2004. Plant Molecular Biology Reporter 22: 71-81). We made some modifications such as adding 1.5 σL of proteinase K of 10 mg/mL, adding an equal volume of cold isopropanol to the supernatant obtained by adding chloroform and isoamyl at a ratio of 24:1, and washing twice with ethanol at 70%.

Amplifying the ITS region. To identify the species of each isolate, we used the following primers to amplify the internal transcribed spacer region (ITS) of rDNA: those specific to *Colletotrichum gloeosporioides* (*Cg*Int) and *C. acutatum* (*Ca*Int2) (Brown et al. 1996); and *Col*1 for the related *C. graminicola* and *C. dematium* that had not amplified with the first two primers (Afanador-Kafuri et al. 2003.Phytopathology 93:579 – 587). These primers were coupled with the primer ITS4 (White et al. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA; Gelfand DH; Sninsky JJ, eds. PCR protocols: a guide to methods and applications. Academic Press, San Diego, CA, USA. pp 315–322.). Table 1.10.1 presents the primers used and their corresponding sequences.

Table 1.10.1. Primers used in PCR analysis for amplifying specific fungal taxa.

Primer	Sequence 5N3Nj
*Ca*Int2[a]	GGGGAAGCCTCTCGCGG
*Cg*Int[a]	GGCCTCCCGCCTCCGGGCGG
*Col*1[b]	GCCGTCCCCTGAAAAG
ITS4[c]	TCCTCCGCTTATTGATATGC

a. Brown et al. (1996).

b. Afanador-Kafuri et al. (2003).

c. White et al. (1990).

To visualize the amplified products for their later analysis, they were separated on agarose gel with ethidium bromide at 1.0 mg/mL and adding buffer 10X TBE to a final concentration of 0.5X. A marker with a molecular weight of 100 bp was included, together with positive controls (*C. gloeosporioides* and *C. acutatum*) and a negative control composed of a PCR cocktail (Álvarez et al., 2005, Fitopatología Colombiana 28: 1-8.).

Amplifying RAM microsatellites at random: To determine the genetic variability existing among the isolates belonging to the same *Colletotrichum* species (previously determined by amplification of the ITS region), we used the technique of random amplification of microsatellites (RAMs), based on the polymerase chain reaction (PCR) (Hantula et al., 1996. Eur J Forest Pathol 26:159–166.).

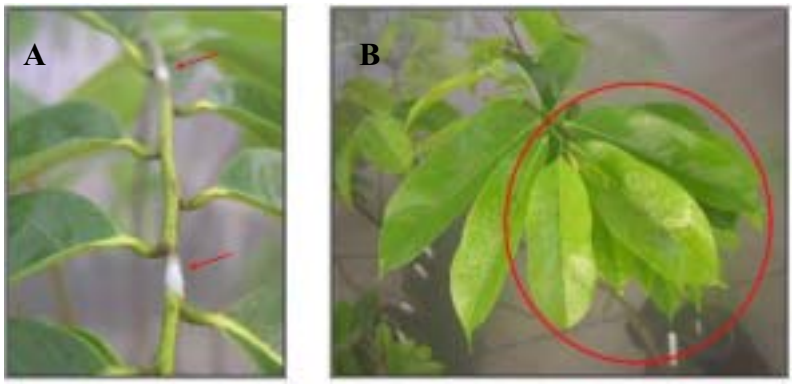


Figure 1.10.1. Inoculating soursop trees of cv. Elita. (A) Wounding method. (B) Spraying method.

Pathogenic characterization of Colletotrichum *isolates:* To evaluate the pathogenicity of *Colletotrichum* isolates, we are currently conducting tests in the greenhouse, artificially inoculating plants of cv. Elita to select the most pathogenic isolates belonging to different RAM groups.

Preparing Colletotrichum *inoculum:* Each isolate conserved on filter paper was planted on AA+E basal medium (Silveira et al. 2004. Sci Agric Piracicaba Brazil 61:542–544) and incubated in an inverted box at 28°C for 15 days under 24 h of light. Alternatively, isolates of *Colletotrichum* spp. were propagated on Marthur's agar medium (0.1% yeast extract, 0.1% Bacto™ Peptone, 1% sucrose, 0.25% $MgSO_4$ 1 $7H_2O$, 0.27% KH_2PO_4, 1.2% agar supplemented with 25 mg ampicillin in 1 L sterilized distilled water). With this medium, the fungus produced considerable sporulation (Freeman et al., 1996. Appl Environ Microbiol 62(3): 101–1020.).

After incubation, a suspension of spores was prepared, directly adding 10 mL of sterilized distilled water over the growing organisms in the Petri dish. This initial suspension was collected in 50-mL sterilized BD Falcon™ tubes, filtering with sterilized gauze to eliminate mycelia and fragments of medium. The suspension was then adjusted to a concentration of 1×10^7 spores/mL, using a hemacytometer (Reichert, Buffalo, NY, USA). Finally, Inex-A⊇ (COSMOAGRO S.A., Colombia) was added to disperse spores at a final concentration of 0.5%.

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

Inoculating the plants: On completing the treatment mentioned above, each isolate was inoculated on three plants, each constituting a replication, as follows (Figure 1.10.1):

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

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

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

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

Designing the severity scale and diagrams to evaluate anthracnose on leaves and branches of artificially inoculated soursop: The scale was designed according to percent values corresponding to 1, 5, 10, 25, and 50% (represented by grades 1, 3, 5, 7, and 9). Once the severity scale was established, and to conduct the evaluation in the greenhouse, we prepared severity diagrams corresponding to each established value.

First, we determined the types of lesions most frequently observed on leaves and branches in the field, and then we examined symptom development in the same tissues inoculated in vitro.

Once we ascertained the typical symptoms of anthracnose on leaves and branches of soursop we designed the diagrams to show the typical symptoms at different levels of severity. Each diagram was digitalized in an Epson Expression 1680 scanner, on a scale of grays, with a resolution of 300 dpi and stored in the TIFF format. The stored diagrams were interpreted with the WinRHIZO™ image analysis system (Regent Instruments, Inc., Quebec, Canada). With the values obtained on the infected area as interpreted by the system (black area = healthy tissue; white area = infected tissue), we selected diagrams that adjusted to the scale, ending up with three patterns of symptom development on leaves and one pattern for branches.

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

Evaluating germplasm in the greenhouse: Characterizing the pathogenicity of the *Colletotrichum* isolates and the RAM analysis, which permitted the formation of genetic groups, allowed us to select, at least one pathogenic isolate from each group and at least one isolate that was minimally pathogenic to cv. Elita. Thus, we could evaluate 20 accessions of soursop with participation of farmers (Table 1.10.2). The evaluations of disease's progress were conducted at equal intervals of time, using the same scale designed to characterize the pathogenicity of the *Colletotrichum* isolates.

Table 1.10.2. Soursop (*Annona muricata* L.) accessions evaluated for anthracnose.

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

Molecular evaluation of the germplasm

To conduct a molecular characterization of the 20 accessions, we extracted DNA from each accession, using the random amplification of microsatellites or RAM technique, and electrophoresis of single-stranded conformational polymorphisms (SSCPs). The primers (synthesized by Technologies, Inc.) used to amplify the DNA extracted from soursop (Table 1.10.3) had been reported as polymorphic in assessments of plant and animal diversity

(Piedrahita et al. 2005. Biotecnol Sect Agropecu Agroind 3:16–26.; Oslinger 2003. Caracterización molecular de cerdos criollos colombianos mediante la técnica molecular RAMs. BSc thesis in Zootechny. Universidad Nacional de Colombia, Palmira, Colombia; Alvarez et al., 2005. Fitopatología Colombiana Vol. 28 (1): 1-8.; Morillo et al. 2005. Acta Agronómica 54 (2): 15-24; Espinosa et al. 2005. Colección y caracterización molecular con marcadores tipo RAMs (microsatélites aleatorios de heliconias y especies relacionadas). *In* Proc. IX Congress of the Asociación Colombiana de Fitomejoramiento y Producción de Cultivos, held in Palmira, 11–13 May 2005. CORPOICA, Palmira, Colombia. p. 118. ; Arcos et al., 2005. Colección, caracterización fenotípica y molecular de poblaciones de uchuva Physalis peruviana. In Proc. IX Congress of the Asociación Colombiana de Fitomejoramiento y Producción de Cultivos, held in Palmira, 11–13 May 2005. CORPOICA, Palmira, Colombia. p. 103; Sanabria et al. 2006. Acta Agron 55 (1): 23-30).

Table 1.10.3. RAM primers and nucleotide sequences.

RAM primer	Condensed sequence (5N to 3N)	Sequence (5N to 3N)
TG	HVH $(TG)_7T$	5Nj HVH TGT GTG TGT GTG TGT 3Nj
CGA	DHB $(CGA)_5$	5Nj DHB CGA CGA CGA CGA CGA 3Nj
CT	DYD $(CT)_7C$	5Nj DYD CTC TCT CTC TCT CTC 3Nj
CA	DBDA $(CA)_7$	5Nj DBD ACA CAC ACA CAC ACA 3Nj
GT	VHV $(GT)_7G$	5Nj VHV GTG TGT GTG TGT GTG 3Nj
AG	HBH $(AG)_7A$	5Nj HBH AGA GAG AGA GAG AGA 3Nj
CCA	DDB $(CCA)_5$	5Nj DDB CCA CCA CCA CCA CCA 3Nj
ACA	BDB $(ACA)_5$	5Nj BDB ACA ACA ACA ACA ACA 3Nj

Source: Hantula et al., 1996. Eur J Forest Pathol 26:159–166.

Results and Discussion

*Collecting plant materials***.** In forming the bank of *Colletotrichum* strains, we collected and processed 93 samples of soursop from Valle del Cauca, Cauca, Huila, Tolima, Meta, Santander del Sur, Norte de Santander, Quindío, Caldas, Córdoba, and Sucre. We obtained 80 isolates from trees infected with the disease and conserved them for use in the trials (Table 1.10. 4).

Table 1.10.4. Sites (departments and municipalities) and number of *Colletotrichum* isolates collected from soursop with symptoms of anthracnose.

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

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

Molecular characterization of Colletotrichum isolates, amplifying the ITS region: With these amplifications, we sought to determine the presence of one unique genus by observing the typical electrophoretic patterns of the *Colletotrichum* genus (Figure 1.10.2).

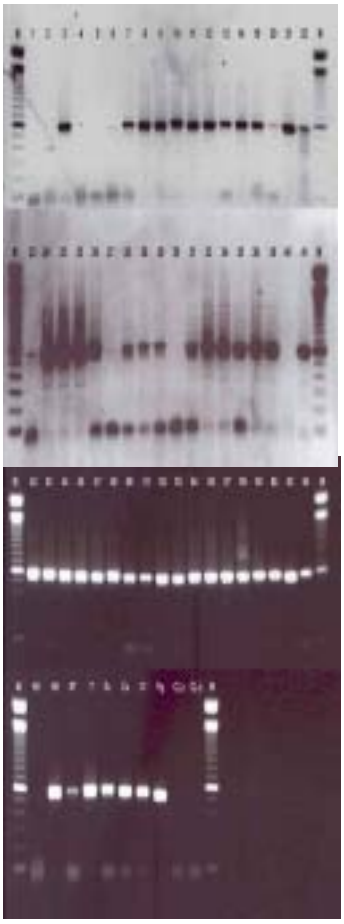


Figure 1.10.2. Electrophoretic profiles of DNA from 55 isolates of *Colletotrichum* spp., obtained from amplifying the ITS region, using primers ITS1 and ITS4. Lane M = marker with a molecular weight of 100 bp; positive control = *C. lindemuthianum*; negative control = PCR cocktail; check = *Phaeoisariopsis griseola*. The lanes identified as T, Gr, and Ca constitute *Colletotrichum* isolates from tea, granadilla, and cacao, respectively.

We partly determined the hybridization temperatures at which the primers in the PCR reaction would amplify the ITS region, coupling ITS4 primers with the specific primers *Ca*Int2, *Cg*Int, and *Col*1 to identify the species. With primers *Cg*Int and *Col*1, we obtained the best results for hybridization at 60ºC, even though hybridization was more specific at this temperature for primer *Col*1 (Figure 1.10.3) and at 62ºC for *Cg*Int (Figure 1.10.4).

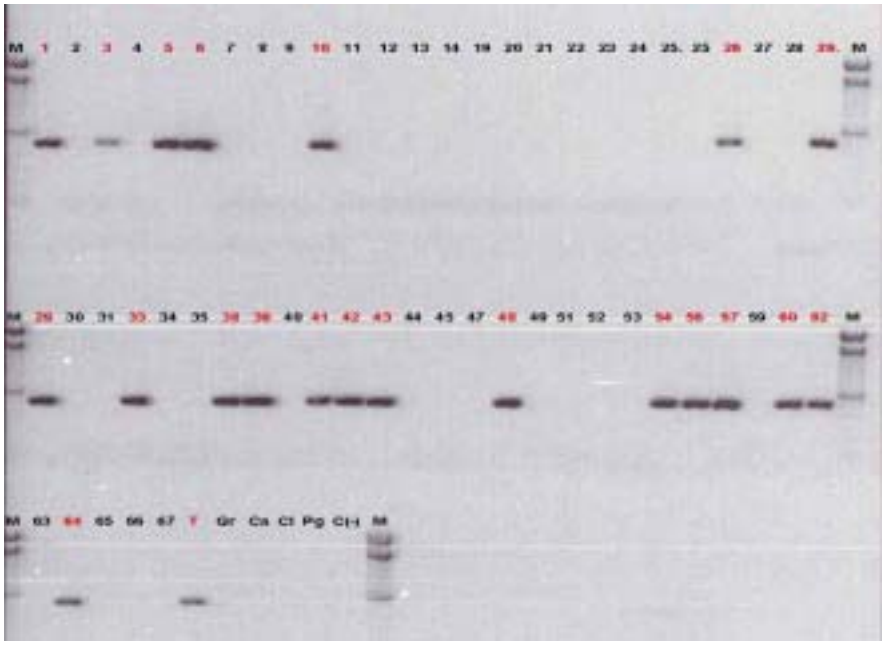


Figure 1.10.3. Electrophoretic profiles of DNA from 55 isolates of *Colletotrichum* spp., obtained by amplifying the ITS4 region + *Col*1 at a hybridization temperature of 60ºC. Lane M = marker with a molecular weight of 100 bp; positive control = *C. lindemuthianum*; negative control = PCR cocktail; check = *Phaeoisariopsis griseola*. The lanes identified as T, Gr, and Ca constitute isolates of *Colletotrichum* spp. from tea, granadilla, and cacao, respectively.

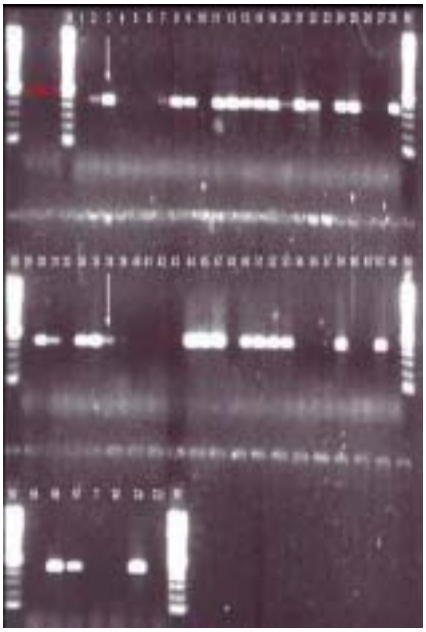


Figure 1.10.4. Electrophoretic profiles of DNA from 55 isolates of *Colletotrichum* spp., obtained by amplifying the ITS4 region + *Cg*Int at a hybridization temperature of 62ºC. Lane M = marker with molecular weight of 100 bp; positive control = *C. lindemuthianum*; negative control = PCR cocktail; check = *Phaeoisariopsis griseola*. The lanes identified as T, Gr, and Ca constitute isolates of *Colletotrichum* spp. from tea, granadilla, and cacao, respectively.

The partial results obtained with primer *Ca*Int2 are presumed to indicate the absence of *C. acutatum* as none of the isolates so far evaluated had amplified with the primer specific to this species (Figure 1.10.5).

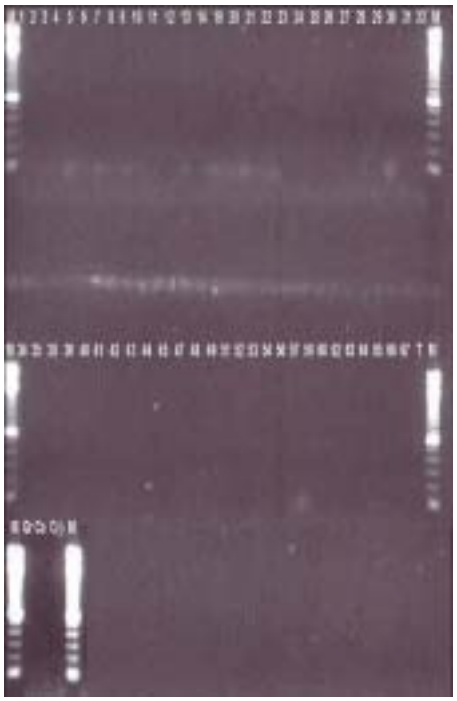


Figure 1.10.5. Electrophoretic profiles of DNA from 55 isolates of *Colletotrichum* spp., obtained by amplifying the ITS4 region + *Ca*Int2 at a hybridization temperature of 60ºC. Lane M = marker with molecular weight of 100 bp; positivecontrol = *C. lindemuthianum*; negative control = PCR cocktail; check = *Phaeoisariopsis griseola*. Lanes identified as T, Gr, and Ca constitute isolates of *Colletotrichum* spp. from tea, granadilla, and cacao, respectively.

The results so far obtained with amplifications, using three primers specific to *Colletotrichum* spp. are summarized in Table 1.10.5. The species so far established are *C. gloeosporioides* and *Colletotrichum* sp., as no isolate was observed to amplify for *C. acutatum*.

Table 1.10.5. Prior identification of *Colletotrichum* species.

Consecutive number	Sample code	Department of origin	Primer reaction		
			*Ca*Int2/ITS4	*Cg*Int/ITS4	*Col*1 /ITS4
1	GM01-L02	Huila	(-)	(+d)[a]	(+)
2	GM03	Huila	(-)	(+)	(-)
3	GM04-L01	Huila	(-)	(+)	(+d)
4	GM04-L02	Huila	(-)	(-)	(-)
5	GM05	Huila	(-)	(-)	(+)
6	GM06	Huila	(-)	(+d)	(+)
7	GM25-L01	Valle del Cauca	(-)	(+)	(-)
8	GM25-L02a	Valle del Cauca	(-)	(+)	(-)
9	GM25-L02b	Valle del Cauca	(-)	(+)	(-)
10	GM26	Valle del Cauca	(-)	(+d)	(+)
11	GM27	Valle del Cauca	(-)	(+)	(-)
12	GM28	Valle del Cauca	(-)	(+)	(-)
13	GM29	Valle del Cauca	(-)	(+)	(-)
14	GM30-L01	Valle del Cauca	(-)	(+)	(-)
19	GM35-L01	Valle del Cauca	(-)	(+)	(-)
20	GM35-L02	Valle del Cauca	(-)	(+)	(-)
21	GM36-L02	Valle del Cauca	(-)	(+)	(-)
22	GM37	Valle del Cauca	(-)	(+)	(-)
23	GM38a	Valle del Cauca	(-)	(+d)	(-)
24	GM38b	Valle del Cauca	(-)	(+)	(-)
25	GM39-L02	Valle del Cauca	(-)	(+)	(-)
26	GM40	Valle del Cauca	(-)	(+d)	(+)
27	GM41	Valle del Cauca	(-)	(+)	(-)
28	GM42	Valle del Cauca	(-)	(+)	(-)
29	GM44-L01	Quindío	(-)	(+d)	(+)
30	GM49-L01	Valle del Cauca	(-)	(+)	(-)
31	GM49-L02	Valle del Cauca	(-)	(+)	(-)
33	GM52- L01	Valle del Cauca	(-)	(-)	(+)
34	GM52- L02	Valle del Cauca	(-)	(+)	(-)
35	GM52- L03	Valle del Cauca	(-)	(+)	(-)
38	GM57	Valle del Cauca	(-)	(+)	(+)
39	GM58-L02	Valle del Cauca	(-)	(+d)	(+)
40	GM59a	Norte de Santander	(-)	(-)	(-)
41	GM59b	Norte de Santander	(-)	(+d)	(+)
42	GM60-L01	Valle del Cauca	(-)	(+d)	(+)
43	GM61-L01	Norte de Santander	(-)	(+d)	(+)
44	GM61-L02	Norte de Santander	(-)	(+)	(-)
45	GM62-L01	Valle del Cauca	(-)	(+)	(-)
47	GM62-L03	Valle del Cauca	(-)	(+)	(-)
48	GM63	Cauca	(-)	(-)	(+)
49	GM64-L02	Valle del Cauca	(-)	(+)	(-)
51	GM66-L01	Valle del Cauca	(-)	(+)	(-)
52	GM66-L02	Valle del Cauca	(-)	(+)	(-)
53	GM67-L01	Valle del Cauca	(-)	(+)	(-)
54	GM67-L02	Valle del Cauca	(-)	(+d)	(+)
56	GM68	Valle del Cauca	(-)	(+d)	(+)
57	GM69-L01	Valle del Cauca	(-)	(+d)	(+)
59	GM70	Valle del Cauca	(-)	(+)	(-)
60	GM71	Tolima	(-)	(+d)	(+)
62	GM73	Tolima	(-)	(+d)	(+)
63	GM74	Tolima	(-)	(+)	(-)
64	GM75	Santander del Sur	(-)	(+)	(+)
65	GM77	Quindío	(-)	(-)	(-)
66	GM78	Quindío	(-)	(+)	(-)
67	GM79	Quindío	(-)	(+)	(-)
T	Tea	Valle del Cauca	(-)	(-)	(+)
Gr	Granadilla	Huila	(-)	(-)	(-)
Ca	Cacao	Huila	(-)	(+)	(-)

a. (+d) indicates weak positive reaction

Pathogenic characterization of Colletotrichum isolates: Initially, an evaluation scale was designed for soursop plants inoculated artificially by the methods described above.

Designing the severity scale and diagrams to evaluate anthracnose on artificially inoculated leaves and branches of soursop: With the scale we previously designed in grades and percentages (Table 1.10.6), we prepared severity diagrams corresponding to each value, using the previous information on the types of symptoms that develop on branches, stems, and leaves.

Table 1.10.6. Severity scale to evaluate anthracnose on leaves and branches of soursop.

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

Finally, we determined three patterns of symptom development on leaves and one pattern for stems and branches (Figure 1.10.6).

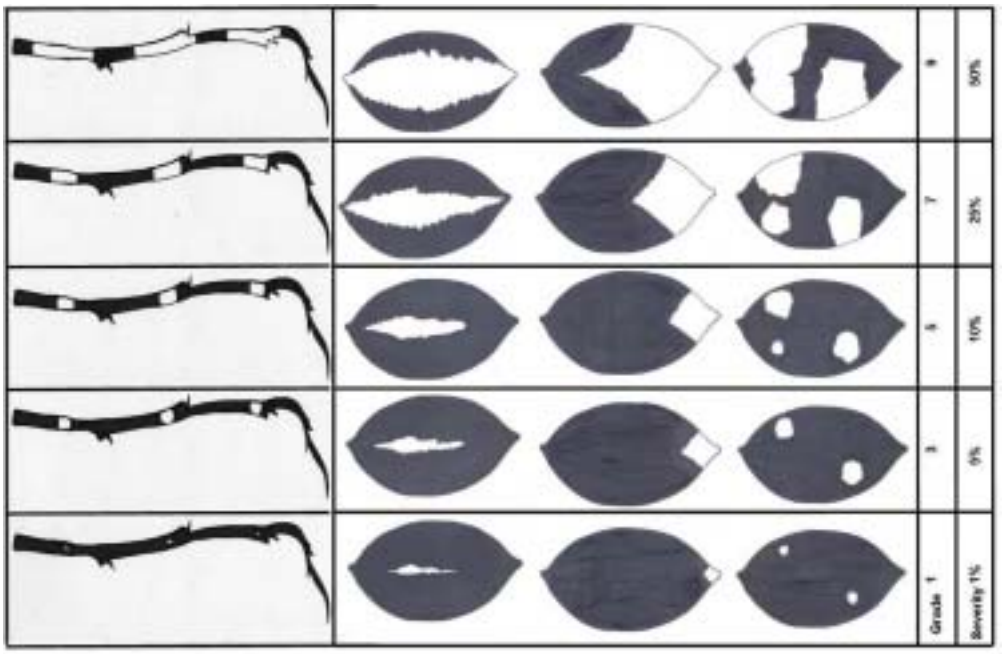


Figure 1.10.6. Severity scale (at right) and diagrams for evaluating anthracnose on leaves and branches of soursop. White areas indicate infected areas

Evaluating the disease on plants of cv. Elita. We conducted the respective evaluations with the isolates inoculated on soursop cv. Elita. With the values obtained, we calculated the rates of development (r) for each treatment (Table 1.10.4) and conducted curves of disease development. We observed differences in disease progress occasioned by each isolate and by the rate at which each progressed over time.

Table 1.10.4. Rates of development of anthracnose in soursop according to *Colletotrichum* isolate.

Isolate	Origin	r (units per day)
GM01-L02	Huila	0.12
GM03	Huila	0.12
GM04-L01	Huila	0.01
GM04-L02	Huila	0.06
GM05	Huila	0.04
GM59a	Norte de Santander	0.04
GM59b	Norte de Santander	0.06
GM63	Cauca	0.04
GM68	Valle del Cauca	0.15
GM89-L01	Sucre	0.02
GM89-L02	Sucre	0.13
GM90-L01	Sucre	0.10
GM90-L02	Sucre	0.09
GM91-L01a	Sucre	0.05
GM91-L01b	Sucre	0.15
GM91-L02	Sucre	0.21
GM92a	Sucre	0.25
GM92b	Sucre	0.08
GM93	Sucre	0.08
GM94	Córdoba	0.06

Isolates GM91-L02 and GM92a presented the highest rates of development (0.21 and 0.25 units per day, respectively). That is, these two isolates showed more progress than the others evaluated, which fluctuated between 0.01 and 0.15 units per day (Figures 1.10.7 and 1.10.8 and 1.10.9). These latter isolates were therefore less virulent.

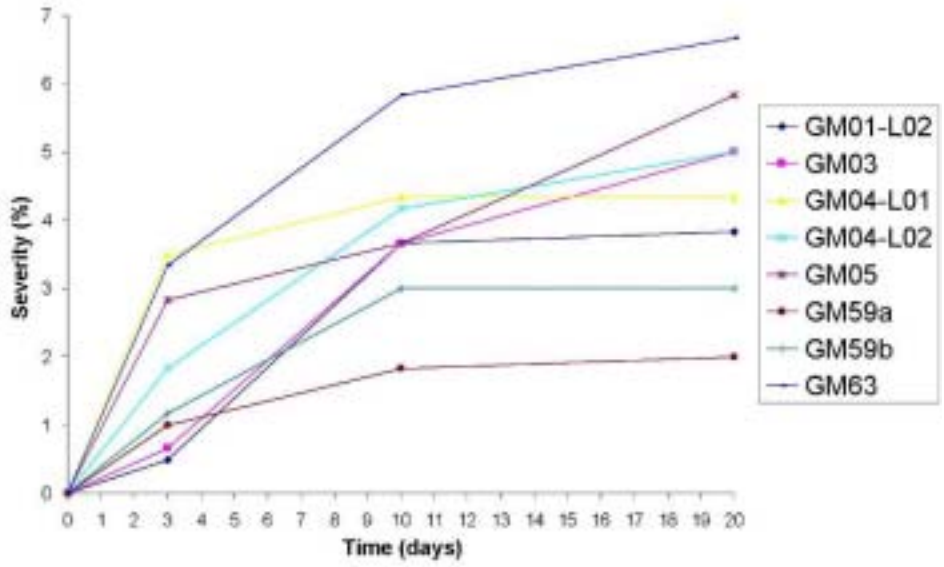


Figure 1.10.7. Progress curves of soursop anthracnose in cv. Elita inoculated with **eight** different isolates of *Colletotrichum* spp.

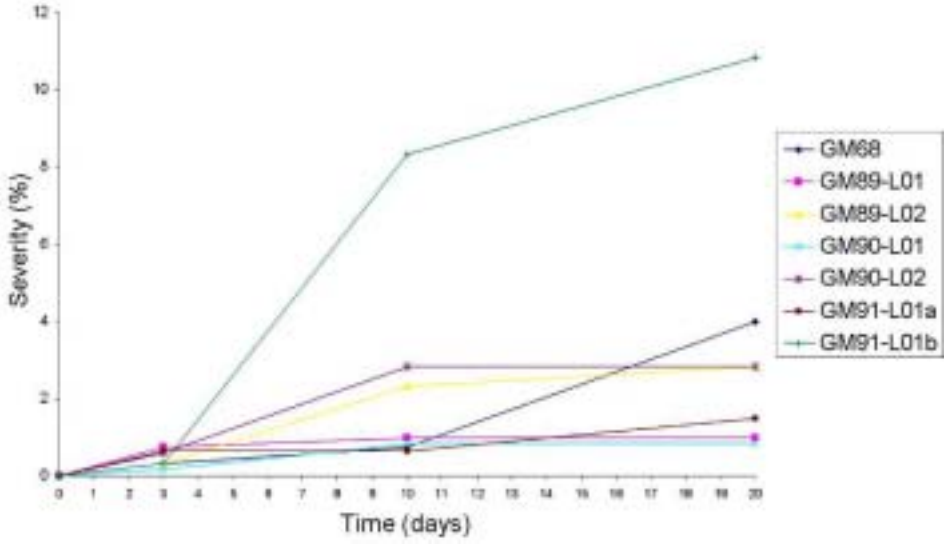


Figure 1.10.8. Progress curves of soursop anthracnose in cv. Elita inoculated with **seven** different isolates of *Colletotrichum* spp.

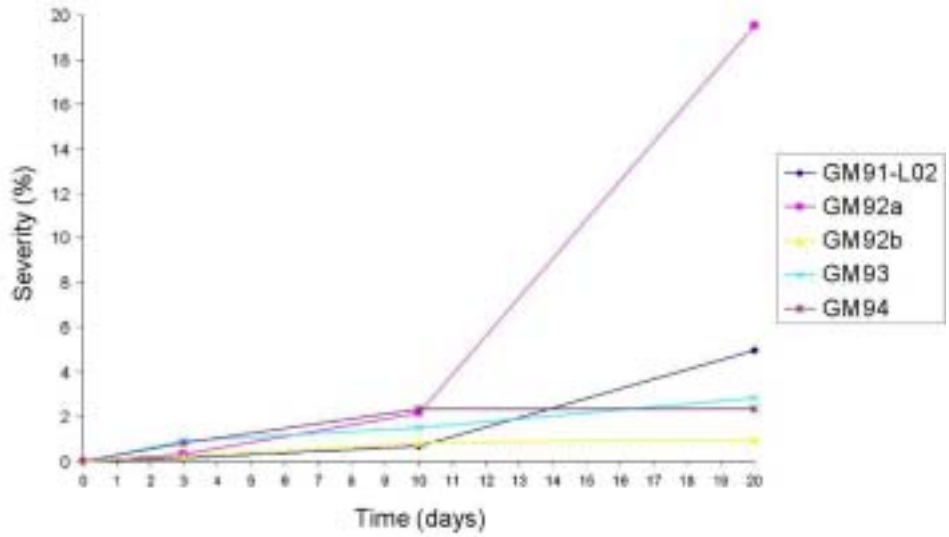


Figure 1.10.9. Progress curves of soursop anthracnose in cv. Elita inoculated with **five** different isolates of *Colletotrichum* spp.

Acknowledgment to Myriam Sanchez, Corporación BIOTEC.

Activity 1.11. Molecular and pathogenic characterization of isolates of *Colletotrichum* spp. associated with anthracnose of Andean blackberry on accessions from Valle del Cauca

Contributors: E. Álvarez, A. Arenas, and J. F. Mejía

Highlight:

Anthracnose of Andean blackberry crops evaluated in the Department of Valle del Cauca, Colombia, is caused by *Colletotrichum.*

Rationale

Anthracnose is an economically important disease that affects stems of 50% to 70% of Andean blackberry crops grown in Colombia (Tamayo, 2003, Boletín Técnico 20. CORPOICA–Regional 4, Rionegro, Department of Antioquia, Colombia. 40 pp). Incidence may even be as high as 100% in some crops (UNISARC and SENA 2006). Moreover, control of the disease is inefficient, despite the use of chemical products and cultural practices (Saldarriaga 2005. MSc Thesis. Faculty of Agricultural Sciences, Universidad de Caldas, Manizales, Colombia. 192 pp). Tools are needed to generate technological alternatives that will contribute to the integrated management of the disease ((Saldarriaga 2005. MSc Thesis. Faculty of Agricultural Sciences, Universidad de Caldas, Manizales, Colombia. 192 pp) This study assesses molecular characterization as a means of identifying pathogen species, and relating their variability and population composition to aspects of pathogenicity.

Materials and Methods

Sampling sites: Field sampling was conducted in 15 village districts and 29 farms of 10 municipalities of the Department of Valle del Cauca: Buga, Tuluá, Ginebra, Palmira, Cerrito, Bolívar, Guacarí, Trujillo, Jamundí, and Dagua (Figure 1.11.1). For most of the municipalities at least two farms were visited.

Figure 1.11.1 Location of sampling sites in Valle del Cauca, Colombia. Samples of Andean blackberry (*Rubus glaucus*), infected by *Colletotrichum* spp., were obtained according to coordinated geographic planes, using the program DivA-GIS (a geographic information system). This is a preliminary analysis of the location of points, as the exact locations of some sampled populations are yet to be refined.

Sampling: We collected 143 samples of tissues of Andean blackberry (Table 1.11.1), principally from young semi-woody stems, but also from fruit and petioles. Although most tissues showed symptoms of anthracnose, healthy ones were also sampled. We used pruning secateurs that were previously disinfected with hypochlorite at 2.5%. For each plant, 3 to 5 stakes (15 cm long) were placed inside a paper bag that was duly labeled. In the field, we also collected crop data on, or example, management, incidence, other phytosanitary problems, and geo-referencing (GPS). The samples were conserved in a cold room at 4°C until processing.

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

Table 1.11.1. Samples of Andean blackberry collected mostly from stems, Department of Valle del Cauca, Colombia.

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

DNA extraction: DNA was extracted from monosporic isolates, following Mahuku's protocol (2004), with modifications by Álvarez (2005). The method involved inactivating proteins, using SDS/proteinase K, and precipitating polysaccharides in the presence of a high concentration of salts (Mahuku, 2004. Plant Molecular Biology Reporter 22: 71-81.) The quality of DNA was determined in agarose gel at 0.8% and quantified through fluorometry (Hoefer DyNA Quant™ 200 Fluorometer).

Amplifying the ITS region: To identify species from the *Colletotrichum* genus, we used the ITS4 universal primer in combination with primers specific to *C. acutatum* (CaInt2), *C. gloeosporioides* (CgInt), and *Colletotrichum* spp. (Col1) (Afanador et al. 2003 Phytopathology 93(5):579–587).

For each PCR reaction, we used 10X *Taq* buffer at a concentration of 1X M/µL (100 mM Tris-HCl, pH 8; 2.5 mM $MgCl_2$; and 500 mM KCl), 0.2 mM of each of the dNTPs, 0.5 µM of each primer, 1.5 mM $MgCl_2$, 2 ng/µL of DNA, HPLC water (0.22 µm), and 0.1 U/µL of *Taq* polymerase (Bioloine). The amplification protocol for the DNA was carried out in a PTC–100 thermal cycler (MJ Research, Inc., Watertown, MA, USA). Initial denaturation was at 95°C for 5 min; one denaturation per cycle at 95°C for 30 s; annealing at 55ºC for 30 s for Col1; and extension at 72°C for 90 s, with a total of 40 cycles of amplification from step 2. The final extension was at 72°C for 4 min, finishing at 4°C for 30 min.

Because bands were not specific at the temperature suggested in Álvarez's PCR protocol (2005), temperatures for hybridization were tested at 55ºC, 60ºC, 62ºC, 64ºC, 65ºC, and 68ºC for primers CaInt2 and CgInt. Visualization of the bands was carried out on an agarose gel at 1.2%, with an electrophoretic current of 90 volts.

The 5.8S-ITS region of the rDNA was amplified, using the universal primers ITS1 and ITS4 (Álvarez 2005 Fitopatol Colomb 28:1–8.). The cocktail for the PCR was prepared with concentrations and quantities equal to those for the specific primers. Amplification was

programmed with an initial denaturation at 94°C for 2 min, one denaturation per cycle at 94°C for 30 s, hybridization at 55°C for 30 s, and extension at 72°C for 120 s, making a total of 40 cycles of amplification. The final extension was at 72°C for 240 s, finishing at 4°C for 30 min.

SSCP and electrophoresis in polyacrylamide gel: To find single-stranded conformational polymorphisms (SSCP) that would permit rapid identification of *Colletotrichum* species, we mixed 2 µL of individual PCR product with 8 µL denaturing buffer (formamide at 95%, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol blue). The mixture was then centrifuged at a low pulse, heated in the thermal cycler at 96ºC for 10 min, and finally conserved on ice for 15 min. The mixture was placed in a Mini-PROTEAN 3-Cell chamber (Bio-Rad Laboratories, Hercules, CA, USA) with 1X TBE buffer (89 mM Tris-borate and 2 mM EDTA, pH 8.0), and on a non-denaturing mini-gel with proportions of acrylamide to bis-acrylamide of 29:1 at 8%. Of the mixture, 10 µL were taken and the samples were run for 6 h at 15 mA (150–200 V). We included 3 µL of a marker with a molecular weight of 1 kb (Invitrogen, CA, USA) in the extreme right and left lanes of the gel to facilitate comparisons with SSCP patterns (Kong et al. 2004. Appl Microbiol 38:433–439). Later, the bands were visualized with ethidium bromide (1 mg/mL of final concentration) for 5 min and any excess washed off in a tray of water for 10 min.

Results and Discussion

From the field samplings and cultures, we obtained 83 monosporic isolates of *Colletotrichum* spp. stored on filter paper. Two species in particular were found to associate with stem tissue; these were *C. acutatum* and *C. gloeosporioides*. When these were further analyzed with the specific primers CaInt2 and CgInt, *C. acutatum* appeared to be the more frequent (62%) species than *C. gloeosporioides* (38%).

By standardizing the PCR protocol to amplify the ITS region, we determined that the hybridization temperatures adequate for the specificity of bands were 64ºC and 65ºC for primers CaInt2 and CgInt, respectively. This study attempted to genotype 50 isolates, of which 40 were evaluated with the specific primers CaInt2, CgInt, and Col1 (Figure 1.11.2). We found that 21 isolates amplified for *C. acutatum*, 13 for *C. gloeosporioides,* none for Col1, and 6 did not amplify for any primer. Because these last isolates did not amplify, we analyzed them with primer Col1, varying the hybridization temperature.

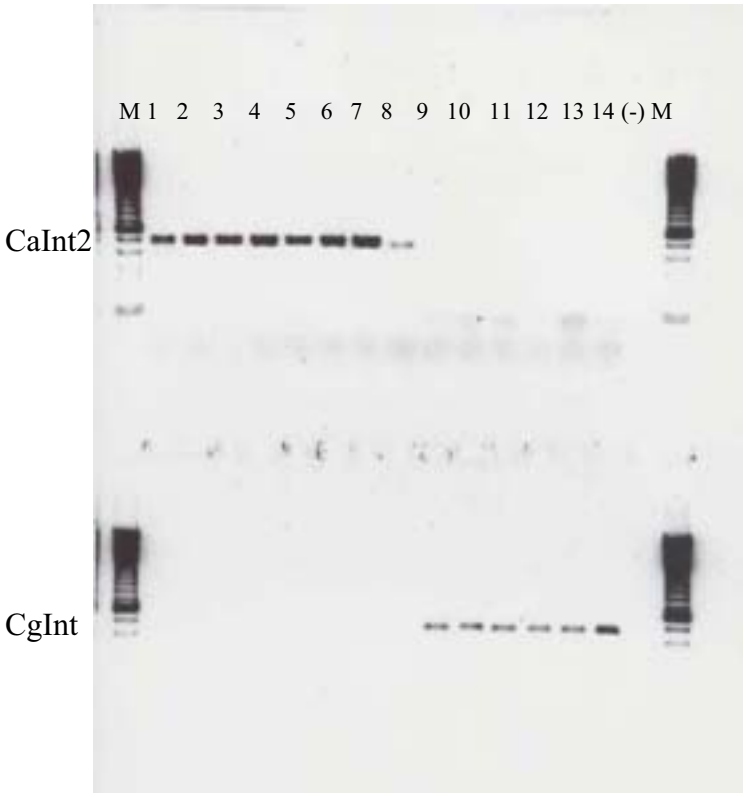


Figure 1.11.2. Isolates of *Colletotrichum* species were amplified with the specific primers CaInt2 and CgInt, and visualized on agarose gel at 1.2%. Lane M = marker with a molecular weight of 100 bp; lane 1 = 20t V; lane 2 = 23f V; lane 3 = 23t V; lane 4 = 26 V; lane 5 = 28 V; lane 6 = 66 V; lane 7 = 81 V; lane 8 = 89 V; lane 9 = 35 V; lane 10 = 46C1 V; lane 11 = 46C2 V; lane 12 = 50 V; lane 13 = 51 V; lane 14 = 59V; lane 15 = negative control.

Currently, an analysis of single-stranded conformational polymorphisms (SSCP; Figure 1.11.3) is being carried out and the samples evaluated have presented an apparent correlation with the amplification of the specific primers. Figure 1 shows that the pattern for *C. lindemuthianum* is different to the others, presenting a double band and below it, the fastest migration, thus indicating a different conformation. *Colletotrichum acutatum* presents a double band and a one band above that of *C. lindemuthianum* and one band below that of *C. gloeosporioides*. This last fungus presented only one band, and the band with the slowest migration. Accordingly, we determined that the six samples analyzed had the same pattern as *C. gloeosporioides*—a result that is coherent with the analysis on the specific primers.

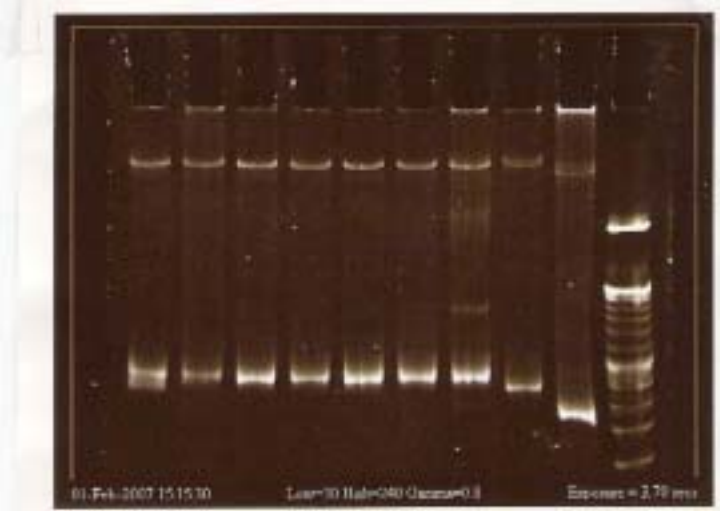


Figure 1.11.3. Electrophoresis of SSCP from *Colletotrichum* spp. on polyacrylamide gel. Lanes 1–6 = six isolates that amplified for *C. gloeosporioides* with the specific primers; lane 7 = *C. gloeosporioides*; lane 8 = *C. acutatum*; lane 9 = *C. lindemuthianum*; lane M = ladder with a molecular weight of 1 kb.

Conclusions

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

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

Activity 1.12. Anthracnose of Andean blackberry (*Rubus glaucus* Benth.): variability in species and races of the causal agent and identification of sources of resistance to the disease

Contributors: L. Afanador Kafuri, E. Álvarez, and A. González

Rationale

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

understanding constitutes the basis on which to develop genetic improvement programs for this species.

Materials and Methods

Collecting germplasm of Andean blackberry and Colletotrichum strains: The collection of *Colletotrichum* strains and germplasm of Andean blackberry was carried out in 29 municipalities of the Departments of Antioquia, Caldas, Cundinamarca, Huila, Quindío, Risaralda, Santander, and Valle del Cauca (Table 1.12.1). In each municipality, four farms were visited and, from each farm, four samples were taken of stems, branches, and/or fruits with typical symptoms of anthracnose.

Multiplying germplasm: The collected germplasm was propagated through germinated microstakes as described by Molina (Molina,1998. Microestacas pregerminadas, una nueva alternative de propagación de mora. Paper presented at the 2nd seminar on Frutales de Clima Frío Moderado,held at the Centro de Desarrollo Tecnológico de Frutales, Manizales, Colombia, August 12–14). This method consisted of first treating with carbendazim (5 g/L) or benomyl (0.5 g/L) for 5 min, and then treating with Hormonagro® in powder, ensuring the tissues were in contact with the product. The treated stakes were planted at an angle in trays containing sterilized sand. With this methodology, the stakes germinated in 25 to 30 days. We also evaluated in vitro propagation from cauline buds, using 4E culture medium (Roca et al., 1984. Procedures for recovering cassava clones distributed in vitro. CIAT, Cali, Colombia. 8 pp).

Table 1. 12.1. Germplasm of *Rubus* spp. collected from study areas in Colombia.

Source	Ecotype	Entries (no.)	Greenhouse	In vitro
Antioquia (CORPOICA)	Germplasm bank	38	2	10
Caldas	Castilla	2	2	0
Cundinamarca	Castilla (sexual seed)	4	3	0
Huila	Castilla	2	1	0
Quindío	Sin Tuna, Castilla	3	1	2
Santander	Castilla, wild blackberry, wild raspberry	8	5	0
Valle del Cauca	Ecuatoriana, Castilla, Bejucuda, Ranchona, Sin Tuna	20	14	2
Nariño	Castilla	2	2	
	Total	**79**	**30**	**14**

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

Evaluating inoculation methods: We first evaluated a method of inoculating leaves, fruits, and stems removed from Andean blackberry. To inoculate the stems, we followed the methodology described by Stewart et al. (Stewart et al., 2003. Horticultural Studies 2003, AAES Research

Series 520: 32-34), which consisted of cutting portions of stems 20 cm long and 1 cm wide. The two extremes were sealed with paraffin and then the surfaces disinfected. An incision was then made in the central part of the stem, removing the external layer of tissue. A block of agar with mycelia from the fungus was placed on the incision and then sealed to prevent dehydration.

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

To inoculate leaves, we applied, on each side of the main leaf vein, 20-εL drops from an aqueous suspension of fungal spores, adjusted to a concentration of 1×10^6 spores/mL. Fruits were inoculated by adding to the center of each fruit one drop of the same suspension. Incubation was carried out under the same conditions as for the stems.

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

Results and Discussion

Collecting blackberry germplasm and Colletotrichum *strains:* The fungus and Andean blackberry germplasm were collected in 10 departments, obtaining a total of 315 samples of tissues infected by anthracnose and 79 accessions of *Rubus* spp. Of these, 30 (38%) were established in the greenhouse, and 14 (18%) under in vitro conditions (Tables 1.12.1 and 1.12.2).

Table 1.12.2. Departments and municipalities in which sampling for anthracnose in Andean blackberry was conducted, together with a collection of *Rubus* germplasm.

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

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

We isolated 232 strains of *Colletotrichum* spp., as well as other types of fungi such as *Botrytis cinerea*, *Alternaria* sp., *Phomopsis* sp., *Mycosphaerella* sp., *Rosellinia* sp., *Kuehneola loeseneriana* (rust), and two types of viruses (a potyvirus and CMV (Table 1.12.3).

Table 1.12.3. Monosporic isolates of *Colletotrichum* spp. collected in the Departments of Antioquia, Caldas, Cauca, Cundinamarca, Huila,Nariño, Quindío, Risaralda, and Santander, Colombia.

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

Each isolate was given a preliminary morphological characterization, based on colony characteristics, morphological variants, and the presence or absence of the fungus's sexual phase Figure 1.12.1).

Figure 1.12.1. Monosporic cultures of *Colletotrichum* spp. in which morphological variability occurs within strains (left), and the sexual phase is present (center and right).

Evaluating inoculation methods: In stems, the first symptoms began on Day 4 after inoculation as dark coffee-brown lesions around the site of inoculation. By Day 12, these lesions had expanded to completely cover the stem. Most present abundant sporulation of the fungus. In fruits, the first symptoms appeared on Day 5 after inoculation as depressed lesions that were dark coffee-brown in color. By day 10, the fruit was completely necrotic, and covered with mycelia of the fungus with abundant sporulation. In leaves, the first symptoms appeared on Day 9 after inoculation as necrosis of the central vein. It then expanded over most of the foliar blade (Figure 1.12.2).

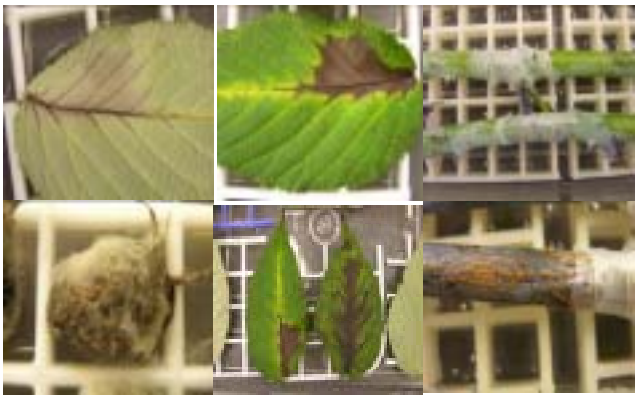


Figure 1.12.2. Symptoms of anthracnose in extracted stems, leaves, and fruits of Andean blackberry. At left are leaves and fruits inoculated with aqueous suspensions of spores from *Colletotrichum* strain 7(1) from Valle del Cauca; at right, inoculated stems and controls.

Activity 1.13. Characterization and identification of phylotypes and sequevars of isolates of *Ralstonia solanacearum* obtained from plantain, banana, and *Heliconia* sp. in Colombia

Contributors: E. Álvarez and J. F. Mejía

Rationale

Characterization and knowledge of the genetic structure of pathogen populations have direct applications in disease management. This study therefore aimed to obtain information on the genetic diversity of a population of *Ralstonia solanacearum* race 2 from Colombia, causal agent of bacterial wilt, a major disease affecting crops of plantain, banana, and *Heliconia* sp.

Traditionally, *Ralstonia solanacearum* has been classified into five races according to differences in the range of hosts and into six biovars according to biochemical properties. Cook and Sequeira, 1994, Bacterial wilt: the disease and its causative agent, *Pseudomonas solanacearum*, 77-94), using restriction fragment length polymorphism (RFLP) analysis, showed that *R. solanacearum* could be classified into two divisions: 1, including biovars 3, 4, and 5, with isolates principally from Asia; and division 2, including biovars 1, 2, and N2, with isolates principally from the Americas. Other authors such as Taghavi (Taghavi *et al.*, 1996, Int. J. Syst. Bacteriol. 46:10-15), using sequence analysis of the 16S rDNA region, also confirmed the existence of these two divisions. The sequencing of the ITS region (16S-23S rRNA gene intergenic spacer region), the polygalacturonase gene, and the endoglucanase gene also

corroborated the existence of these two divisions, but indicated the existence of another group of isolates originating from Indonesia (Fegan *et al.*, 1998, Bacterial Wilt Disease, 19-33).

Poussier (Poussier *et al.*, 2000, Syst. Appl. Microbiol. 23:479-486) conducted a phylogenetic analysis of the *hrp* gene region, using PCR-RFLP and complementing it with amplified fragment length polymorphism (AFLP) and sequencing of the 16S rRNA gene. They observed a new cluster of isolates from Africa—biovar 1. Phylogenetic analysis of the endoglucanase and *hrpB* genes confirmed the presence of this group in strains originating from Africa. Under this classification system, members of the *R. solanacearum* species complex can be subdivided into four phylotypes, corresponding to the four genetic groups identified via sequence analysis (phylotypes I, II, III, and IV).

The phylotype to which a strain belongs can be rapidly identified through multiplex PCR, based on sequence information from the ITS region. This PCR employs four forward primers: one specific one for each phylotype and a single reverse primer that is specific for the species. It also includes a primer pair described by Opina (Opina *et al.*, 1997, Asia Pacific J. Mol. Biol. Biotech. 5:19-30). All were *R. solanacearum*. Blood disease bacterium (BDB; *Pseudomonas syzygii*) strains generate the 280-bp fragment that is specific to the *R. solanacearum* species complex (Allen *et al.*, 2005, Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. 1-510).

Isolates of *R. solanacearum* infecting *Musa* spp. (also known as race 2 strains) are a major menace for crops such as plantain, banana, and heliconias all over the world, including Colombia. French and Sequeira (1970, Phytopathology 70:506-512) defined five groups or ecotypes with strains of *R. solanacearum* race 2 that cause bacterial wilt of banana, plantain, and *Heliconia* sp. in Central and South America. The groups differ in virulence, where some are pathogenic to both plantain and banana (A, SFR, B, and D types) and others are pathogenic only to plantain (H). The groups also differ in transmission and aggressiveness.

Cook and Sequeira (1994, Bacterial Wilt: the disease and its causative, *Pseudomonas solanacearum* by molecular genetic methods, p. 77-94) then discovered, through RFLP analysis, that all strains of *R. solanacearum* race 2 are found in three multi-locus genotypes (MLGs), designated as MLGs 24, 25, and 28. Moreover, from this description, Allen (Allen *et al.*, 2005, Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. 1-510) conducted a classification based on the phylogenetic analysis of sequences of the ITS region (16S-23S) and the endoglucanase gene (*egl)*. Under this scheme, strains of *R. solanacearum* race 2 were classified into phylotype II, sequevars 3, 4, and 6. On the basis of these results and specific classification, genomic DNA fragments were used to develop a multiplex PCR-based molecular test for *R. solanacearum* race 2 (Allen *et al.*, 2005, Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society (APS), St. Paul, MN, USA. 510p.).

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

Materials and Methods

Strains were chosen, based on studies of: 1. pathogenicity on the plantain hybrid 'Africa 1', 2. genetic diversity through RAM primers, and 3. the amplification of the pathogen's 16S rRNA gene by Álvarez (Álvarez *et al.*, 2005, Fitopat. Colom. 28(2): 71-75). Of the 58 strains from the collection at CIAT, originated from infected crops of plantain (37), banana (5), and heliconias (3). The degrees of pathogenicity in the area under the disease progress curve (AUDPC) were between 10 and 70. The strains were collected from the Departments of Magdalena, Valle del Cauca, Quindío, Antioquia, Caquetá, and Meta (Colombia). The remaining 13 strains were controls and came from plantain (1), *Heliconia* sp. (1), tobacco (6), eggplant (1), tomato (1), potato (1), arrowroot (1), and capsicum (1) from Kenya, Japan, Asia, USA, and Colombia. Five nonpathogenic strains of *R. solanacearum* were isolated from soil for comparative purposes (Table 1.13.1) [(Álvarez *et al.*, 2005, Fitopat. Colom. 28: 71-75)].

Table 1. 13.1. Origin and pathogenicity of 58 strains of *Ralstonia solanacearum* race 2, causal agent of bacterial wilt, isolated from banana, plantain, and *Heliconia* sp.

No.	Strain	Region	Host	Tissue	AUDPC[a]	Sequevar	Phylotype			
							I	II	III	IV
1	1 S.A	Quindío	Plantain	Rachis	18	4		+		
2	3	Quindío	Plantain	Petioles	69.38	4		+		
3	5 (Sunisa 8)	Antioquia (Urabá)	Banana	Rhizome	38.75	4		+		
4	6	Antioquia (Urabá)	Banana	Fruit	31.83	4		+		
5	15	Quindío	Soil		37.63	4		+		
6	16b1	Quindío	Soil	*Mucuna*	0	–		+		
7	17	Valle (Jamundí)	Soil		69.5	4		+		
8	18	Valle	Plantain	Sucker	42.5	4		+		
9	32	Caquetá	Plantain	Pseudostem	33.88	4		+		
10	34	Caquetá	Plantain	Raceme rachis	27.63	4		+		
11	38	Quindío	Soil	Coffee pulp	59.5	4		+		
12	40	Quindío (Quimbaya)	Soil	Center of focus	15.75	4		+		
13	41	Quindío (Quimbaya)	Soil	Center of focus	56.25	4		+		
14	42	Meta	Plantain	Pseudostem	28	4		+		
15	43	Meta	Plantain	Pseudostem	20.75	4		+		
16	48	Quindío (Armenia)	Plantain	Fruit	37.13	4		+		
17	54a	Meta (Puente de Oro)	Plantain	Pseudostem	36.25	4		+		

Table 1.13.1. continued

No.	Strain	Region	Host	Tissue	AUDPC[a]	Sequevar	Phylotype			
							I	II	III	IV
18	58-1R	Meta (Puente de Oro)	Plantain	Petioles	56.63	4		+		
19	59	Meta (Puente de Oro)	Plantain	Pseudostem	0	4		+		
20	65	Meta (Granada)	Plantain	Pseudostem	47.63	4		+		
21	67	Meta (Puente de Oro)	Plantain	Pseudostem	41.63	4		+		
22	69-1	Meta (Granada)	Plantain	Pseudostem	27	4		+		
23	70	Meta (Granada)	Plantain	Pseudostem	5.75	4		+		
24	71aR	Antioquia (Urabá)	Plantain	Rhizome	21.25	4		+		
25	72b	Antioquia (Urabá)	Plantain	Pseudostem	10.75	4		+		
26	73a	Antioquia (Urabá)	Plantain	Pseudostem	10.75	4		+		
27	76	Quindío (Montenegro)	Plantain	Pseudostem	61.88	4		+		
28	78	Quindío (Montenegro)	Plantain	Rachis	73.38	4		+		
29	79	Quindío (Montenegro)	Plantain	Rhizome	66.88	4		+		
30	80	Quindío (Montenegro)	Plantain	Pseudostem	67.88	4		+		
31	81	Quindío (Montenegro)	Plantain	Fruit	10.88	4		+		
32	83	Quindío (Calarca)	Plantain	Fruit	55	4		+		
33	84	Quindío (Calarca)	Plantain	Pseudostem	61	4		+		
34	85	Quindío (Calarca)	Plantain	Sucker	68.38	4		+		
35	86	Quindío (Calarca)	Plantain	Rachis	59.75	4		+		
36	88	Quindío (La Tebaida)	Plantain	Rhizome	61.75	4		+		
37	89	Quindío (La Tebaida)	Plantain	Pseudostem	60.38	4		+		
38	97	Quindío (Quimbaya)	Plantain	Rhizome	28.63	4		+		
39	107	Quindío (Armenia)	Plantain	Fruit	68.25	4		+		
40	110	Magdalena	Banana	Pseudostem	63.25	6		+		
41	111	Magdalena	Banana	Rhizome	34.38	6		+		
42	112	Magdalena	Banana	Sucker	29.5	6		+		
43	113	Valle (Rozo)	Heliconia wameiana	Pseudostem	40.5	4		+		
44	114	Valle (Rozo)	Heliconia wameiana	Rhizome	40.38	4		+		

Table 1.13.1. continued

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

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

The selected strains were amplified by multiplex PCR, their classification being evaluated according to phylotype with primers Nmult 21:1F, Nmult 21:2F, Nmult 22:InF, Nmult 23:AF, Nmult 22:RR, 759, and 760 (Figure 1B); and to sequevars in Musas with primers Mus 20-F, Mus 20-R, Mus 35-F, Mus 35-R, Mus 06-F, Mus 06-R, Si28-F, and Si28-R. Amplification conditions were as according to the methodology described by Fegan and Prior (2005) and cited in Allen (Allen *et al.*, 2005, Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society (APS), St. Paul, MN, USA. 510p.).

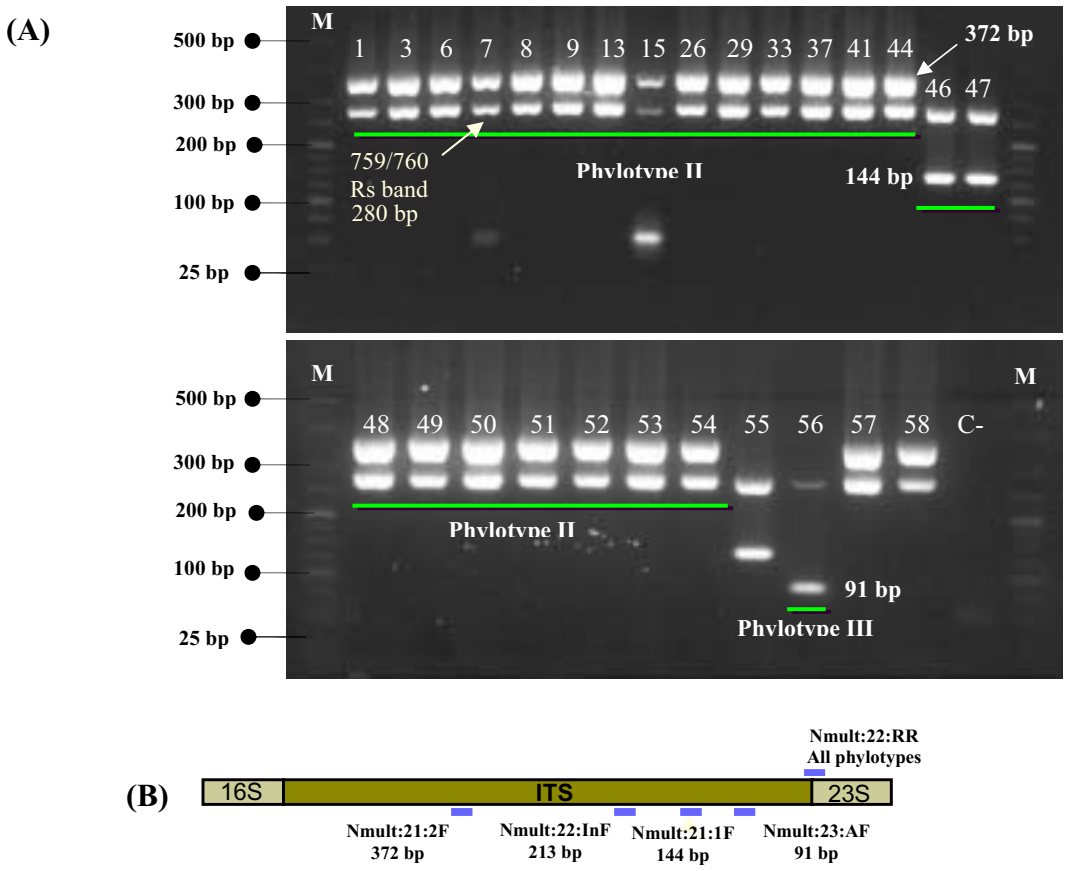


Figure 1.13.1. (A) Evaluation of phylotypes by multiplex PCR for 58 isolates obtained from banana, *Heliconia*, plantain, and controls from the collection held at CIAT. (B) Location of primers for multiplex PCR (phylotypes) in the ITS region. Lane M = marker with molecular weight according to HyperLadder V (100 lanes).

Results and Discussion

All the isolates obtained from Colombia, regardless of geographic region, host, tissue, or pathogenicity, belonged to phylotype II. In contrast, the control strains were characterized as belonging to phylotypes I, II, and III. Specifically, those from eggplant (Kenya), tobacco (Japan), and capsicum (Asia) belonged to phylotype I; those from plantain, arrowroot, tobacco (Colombia), tobacco (Quency, FL, and North Carolina), potato (Australia), and tomato (North Carolina), to phylotype II; and the sole *Heliconia* isolate (Costa Rica) to phylotype III (Figure 1.13.1A; Table 1.13.1).

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

- *Phylotype I* is equivalent to division I, as defined by Cook (Cook *et al.*, 1994, Bacterial Wilt, 77-94). The strains in this phylotype all belong to biovars 3, 4, and 5 and were isolated primarily from Asia.

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

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

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

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

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

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

- The remaining 8 isolates did not amplify for any sequevar (Figure 1.13.2).

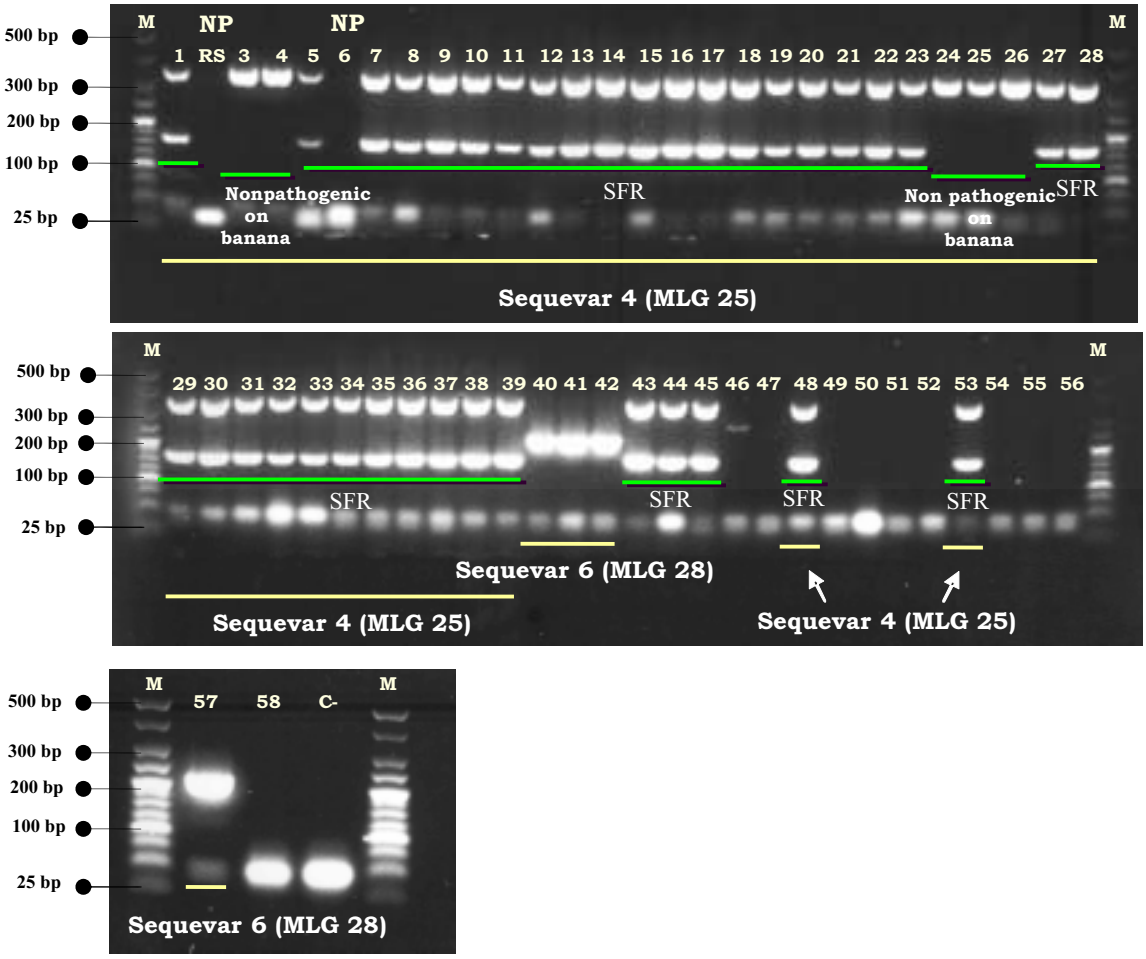


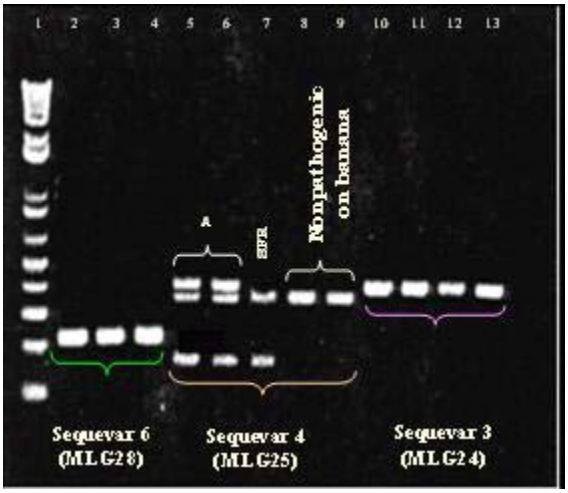
Figure 1.13.2. Evaluation of sequevars by multiplex PCR for 58 isolates obtained from banana, *Heliconia*, plantain, and controls from the collection held at CIAT. Lane M = marker with molecular weight according to HyperLadder V (100 lanes).

Based on Allen *et al.* classification (Allen *et al.*, 2005, Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society (APS), St. Paul, MN, USA. 510p.), strains identified as "SFR" (small, fluidal, round colony form, insect transmitted) are found in MLGs 25 and 28. Strains identified as "D" (causing leaf distortion and slow wilting of banana) belong to MLGs 24 and 25. In contrast, strains designated as "B" (large elliptical colony form, rapid wilt of banana, not commonly insect transmitted) belong to MLG 24 only, as do the strains classified as "H" (slightly pathogenic on plantain but not pathogenic on banana). The authors' phylogenetic work showed that isolates belonging to MLGs 24 and 25 are closely related to each other, but are slightly more distant to MLG 28. Given that strains classified as SFR are present in MLGs 25 and 28, it is conceivable that these strains may have differing properties, including their capacity to survive in soil and host range (Figure 1.13.3A).

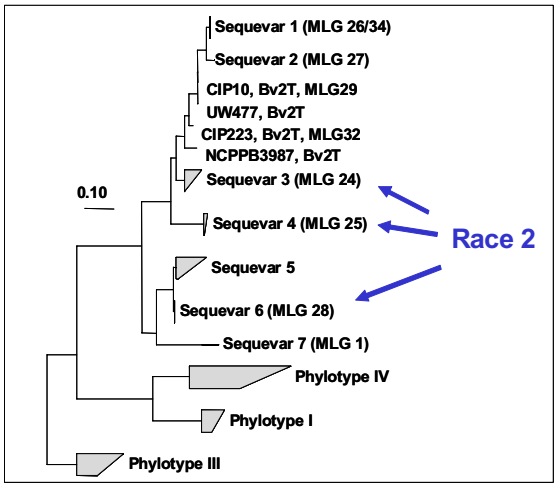
Comparing the band patterns for the different isolates from plantain, banana, and *Heliconia* spp.

with those of the race 2 strains described above, the isolates that characterized as SFR-type strains, sequevar 4 (MLG 25), were isolates 1, 5, 7–23, 27–39, 43–45, 48, 51, and 53; those that are SFR-type, nonpathogenic on banana, sequevar 4 (MLG 25) were 3, 4, and 24–26; and SFR-type, sequevar 6 (MLG 28), were 40–42 and 57 (Figure 1.13.2; Table 1.13.1).

The primers designed for sequevar 4 (MLG 25) were each developed separately, using strains isolated from Peru, Colombia, Costa Rica, Martinique, and Florida (USA). Their host plants were banana, plantain, *Heliconia* sp., pothos (*Epipremnum aureum*), and anthurium. Strains isolated from bacterial wilt-infected anthurium from Martinique in the French West Indies and from pothos in Florida were found to cluster with *R. solanacearum* strains belonging to sequevar 4. The strains from anthurium were nonpathogenic on banana. However, the strains from pothos caused wilt of banana. The pathogenic potential of these strains for banana needs to be confirmed (Allen *et al.*, 2005, Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society (APS), St. Paul, MN, USA. 510p).



(A) Fegan and Prior (2005)



(B) Fegan and Prior (2005)

Figure 1.13.3. (A) Musa-specific region from the subtracted sequences specific to phylotype II, sequevars 3, 4, and 6, and used in a multiplex PCR. (B) Phylogenetic tree of phylotype II based on partial endoglucanase gene sequences. The corresponding sequevars can be seen where strains of *R. solanacearum* race 2 are classified. (Taken from Fegan and Prior [2005]).

Strains belonging to sequevars 3 (MLG 24) and 4 (MLG 25) are closely related and form a branch, together with sequevars 1 and 2, which contain potato disease-causing strains belonging to race 3/biovar 2 (Figure 1.13..3B). All strains previously identified as belonging to MLG 28 fell in sequevar 6. Strains in this sequevar were isolated from host plants of banana, plantain, and *Heliconia* sp. in Honduras, Venezuela, Hawaii, and Australia (where the disease has been eradicated).

Sequevar 6 is phylogenetically distinct from strains of sequevar 3 (MLG 24) and 4 (MLG 25) in which other bacterial wilt-causing strains are found. Hence, the *R. solanacearum* race 2 strains are polyphylectic, which indicates a separate evolutionary origin for the two groups of strains. All strains of sequevar 6 were also found to belong to biotype 6 (Figure 1.13.3B) (Allen *et al.*, 2005, Bacterial Wilt Disease and the *Ralstonia solanacearum* Species Complex. American Phytopathological Society (APS), St. Paul, MN, USA. 510p)

This finding is of vital importance, considering that strains isolated from banana in Magdalena, Colombia (Table 1.13.1), belong to sequevar 6 and are either moderately or highly pathogenic on plantain. The possibility of strains being introduced from Honduras or Venezuela exists and, hence, the danger of re-invasion of zones where the disease has been eradicated is constant.

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

Conclusions

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

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

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