Output 3: Strategies developed for managing diseases and pests in bean-based cropping systems

Activity 3.1 Characterizing and monitoring pathogen and insect diversity

Highlights:

- The causal agents of three new diseases of common bean that have recently emerged in Colombia have been identified.
- We showed a lack of host differential interaction in the common bacterial blight/ common bean pathosystem, and that the CBB pathogens might not have co-evolved with common bean gene pools.
- A molecular assay for specific detection and differentiation of CBB pathogens in bean seed, and the protocol for its application were developed.
- It was shown that the population structure of *C. lindemuthianum* is changing, as evidenced by the resistance of previously susceptible varieties.
- It was demonstrated that the varieties Widusa, Kaboon (Andean) and G 2333 (Mesoamerican) can be used to effectively manage all anthracnose races found in Colombia.
- The infection process of the angular leaf spot pathogen, *P. griseola*, was elucidated.
- The immune response of G 10474 to several pathotypes of *P. griseola* might be mediated through production of an antifungal compound.
- A protocol was developed for routine transformation of *P.griseola* and the fungus was transformed to express the GFP protein. Some transformants have lost their ability to infect bean (insertional mutagenisis).
- One hundred and thirty-four *Pythium* isolates from areas affected by bean root rots in Kenya and Rwanda were characterized by sequencing the ITS-1 region of ribosomal DNA and grouped into 22 species of which nine are new additions. Distribution maps for *Pythium* species were developed.
- Important changes in whitefly species composition in the target area were detected. Varying levels of resistance or susceptibility to some of the insecticides commonly used for whitefly control were observed.

3.1.1 Characterization of bean leaf crumple virus, a new whitefly-borne virus affecting common bean in Colombia

In 2003 Bean Virology described the emergence of a new viral disease of snap bean in the Cauca Valley of Colombia. The new virus severely affects the only snap bean cultivar ('Blue Lake') grown in this region, to the extent that snap bean production has been practically abandoned. In preliminary molecular characterization studies, we had reported an 85% homology between the new virus and *Bean golden yellow mosaic virus* (BGYMV) at the capsid protein level located in component DNA-A. The characterization of this virus was continued in 2004 in order to obtain partial sequence of DNA-B, the viral component that determines the pathogenicity of the virus to a large extent. This study showed that the B ss-DNA component of the snap bean begomovirus is most closely related to a group of begomoviruses present in the Americas, namely: *Cabbage leaf curl virus* (CaLcuV), *Squash leaf curl virus* (SLCV), *and Tomato yellow mosaic virus* (ToYMV).

Of these viruses, Bean Virology has detected begomoviruses in the Cauca Valley, closely related to CaLcuV (in soybean) and ToYMV (in tomato). It is thus apparent that this is a recombinant virus (Figures 40 A and B) capable of infecting common bean.

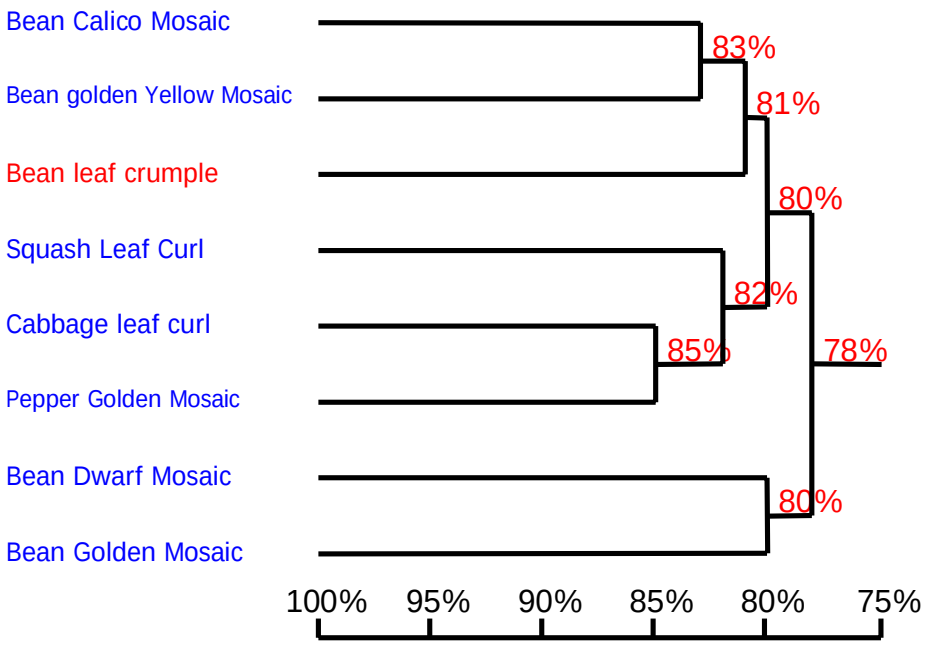

Figure 40 A. Phylogeny of Bean leaf crumple virus (DNA-A/capsid protein)

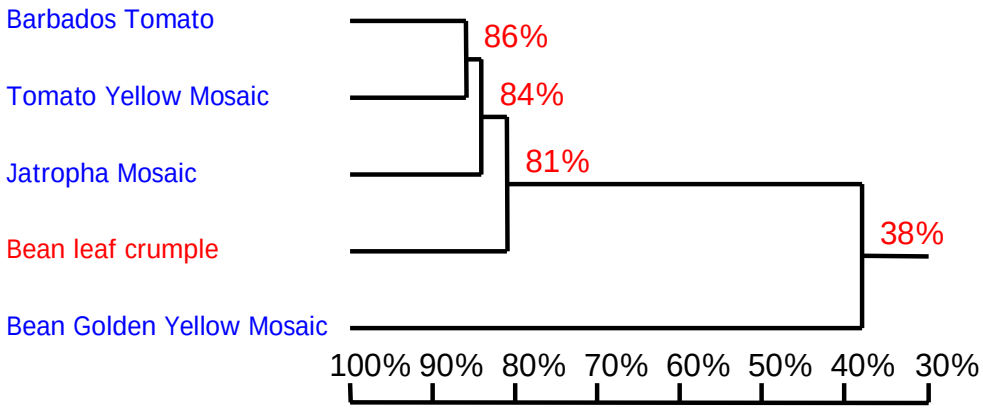

Figure 40 B. Phylogeny of Bean leaf crumple (DNA-B/Movement protein)

3.1.2 Characterization of a virus inducing systemic necrosis in common bean genotypes planted in the experiment station of Santander de Quilichao, Cauca, Colombia

Samples taken from common bean plants showing partial systemic necrosis under field conditions, were brought to CIAT for electron microscopy, partial molecular characterization by PCR, and pathogenicity tests for strain identification. The electron microscopy examination revealed the presence of flexuous filamentous virus-like particles approximately 750 nm in length and 15 nm in diameter. The PCR test was performed using primers specifically designed for potyviruses. A band of the expected molecular size was amplified, cloned and sequenced at CIAT. The comparison of the sequence obtained with that of other plant viruses filed in the GeneBank, indicated that the potyvirus was an isolate of the necrosis-inducing strain of *Bean common mosaic necrosis virus* (BCMNV). Whereas this strain is ubiquitous in temperate regions of South America and in Eastern Africa, it had not caused any problems in this part of the world in the past. However, the pathogenicity tests carried out at CIAT, using the differential common bean genotypes used for BCMV and BCMNV strain characterization, showed that the new virus does not behave exactly as the BCMNV-NL3 strain in these genotypes (Table 66).

Table 66. Reaction of the differential common bean genotypes used for BCMV/BCMNV strain characterization to a new necrosis-inducing potyvirus.

Genotype	SQ Virus*	BCMNV-NL3
Genotypes possessing recessive (I^+ I^+) resistance		
Dubbele Witte	+	+
Stringless Green Refugee	-	+
Redlands Greenleaf C	-	+v
Puregold Wax	-	+v
Imuna	-	+v
Redlands Greenleaf B	-	+
Great Northern 123	-	+
Sanilac	+	+
Michelite 62	+	+
Red Mexican 34	+	+
Pinto 114	-	+
Monroe	-	
Great Northern 31	-	
Red Mexican 35	-	
Genotypes possessing dominant (*II*) resistance		
Widusa	+	+
Black Turtle Soup	+	+
Jubila	-	+
Topcrop	-	+
Improved Tendergreen	-	+
Amanda		

* Virus from Santander de Quilichao, Cauca, Colombia; BCMNV=*Bean common mosaic necrosis virus*.

In fact, the pathogenicity of the potyvirus isolated in Santander de Quilichao, is quite different from the one expected for BCMNV-NL3, and it is practically identical (with the exception of the negative reaction of Stringless Green Refugee) to the spectrum of pathogenicity described for BCMNV-NL8, a different strain of the same species. The results obtained for the PCR test and partial molecular characterization of the SQ virus as BCMNV-NL3, are probably related to the close molecular organization of the NL3 and NL 8 strains of BCMNV.

3.1.3 Monitoring of Peanut stripe virus at CIAT-Headquarters

In 2002, Bean Virology reported on the detection of Peanut stripe virus affecting plots of the common bean cultivar 'Quimbaya' at CIAT Headquarters, Palmira, Colombia. This virus had been previously detected at CIAT infecting the forage legume species, *Arachis pintoi*. This forage species has also been utilized at CIAT as a cover crop in fruit orchards, which has been acting as a permanent virus reservoir for migrant aphids that acquire the virus from *A. pintoi* and transmit it to susceptible common bean genotypes on campus. Besides ICA-Quimbaya, commercial plots of cultivars ICA-Cafetero and Diacol-Calima have been affected by this virus at CIAT. Peanut stripe virus is currently considered as a strain of *Bean common mosaic virus*, but its biological behavior is different from that of most BCMV strains, being able to infect four of the six groups of bean genotypes possessing recessive resistance to BCMV. Peanut stripe virus does not induce systemic necrosis in common bean genotypes possessing the dominant *II* gene. The fact is that Peanut stripe virus has become an endemic pathogen of common bean in CIAT-HQ fields, but only in those old genotypes that do not have resistance to BCMV. This virus should not pose a problem for any of the advanced breeding lines developed by CIAT with resistance to BCMV.

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3.1.4 Determination of pathogenic variation within the common bacterial blight pathogens (*Xanthomonas campestris* pv *phaseoli* (Xcp) and *Xanthomonas campestris* pv *phaseoli* var *fuscans* (Xcpf))

Rationale: The question of physiological races within common bacterial blight has been the subject of much discussion. Some studies have reported the presence of physiological races in the CBB pathogens while others have found the contrary. The presence of physiological races would suggest a gene for gene interaction, which would have profound influence as to how breeding for CBB resistance is undertaken. To date, all information shows that CBB resistance in *Phaseolus vulgaris* is quantitative in nature. To test the hypothesis of the presence of physiological races in the CBB pathogen, we collected all bean genotypes that have been reported in the past to show differential response when challenged with the CBB pathogen, and inoculated these under greenhouse conditions, using isolates of a diverse origin. The objective was to provide insights into the co-evolution of Xcp and Xcpf with Andean and Mesoamerican gene pools, and corroborate the results of Gilbertson et al. (2004). In addition, this would lead to the formulation of a differential series, that can be used to rapidly characterize the CBB pathogen, identify the most effective resistance genes to use in breeding programs, as well as formulate ways to effectively deploy CBB management strategies.

Materials and Methods: Bacterial isolates: Bacterial isolates were selected to represent different geographical areas where bean is grown, and for which we had isolates in stock. A total of 29 isolates were selected, 15 Xcp and 14 Xcpf.

Bean germplasm: Fifty bean genotypes were used in this study, 26 belonging to the Andean gene pool and 24 to the Mesoamerican gene pool (Table 67). The majority of these genotypes have previously been reported to show a differential reaction to the CBB pathogen and as having the capability to distinguish between isolates. In addition, six lines specifically developed for resistance to the CBB pathogen (VAX 1 to VAX 6) through interspecific hybridization of *P. vulgaris* and *P. acutifolius* and embryo rescue techniques were included.

Table 67. Reaction of *Phaseolus* genotypes to inoculation with 14 isolates of *Xanthomonas campestris* pv. *phaseoli* var fuscans (Xcpf) and 15 isolates of *Xanthomonas campestris* pv. *phaseoli* (Xcp).

		Xcp		Xcpf	
Genotype	**Gene Pool[a]**	**Incompatible[b]**	**Compatible[c]**	**Incompatible**	**Compatible**
Nuña maní Roja	A	2	13	7	7
A 196	A	1	14	3	11
Bola 60 días	A	1	14	1	13
Burros Argentinos	A	1	14	1	13
G 76	A	0	15	0	14
Taylor	A	0	15	0	14
ICA CERINZA	A	0	15	0	14
A 475	A	0	15	0	14
A 36	A	0	15	0	14
G 5686	A	0	15	0	14
Jatu Rong	A	0	15	0	14
Ecuador 1056	A	0	15	0	14
Jalo EEP 558	A	0	15	0	14
Alubia Cerrillos	A	0	15	0	14
Mortiño	A	0	15	0	14
Bolón Bayo	A	0	15	0	14
Ecuador 299	A	0	15	0	14
G 11867	A	0	15	0	14
MCD 4011	A	0	15	0	14
MCD 4012	A	0	15	0	14
Radical San Gil	A	0	15	0	14
Frutilla Corriente	A	0	15	0	14
Coscorrón Corriente	A	0	15	0	14
Tórtolas Corriente	A	0	15	0	14
Caballero	A	0	15	0	14
Bolón Rojo	A	0	15	0	14
VAX 3	M	15	0	14	0
VAX 4	M	15	0	14	0
VAX 6	M	15	0	14	0
VAX 5	M	14	1	14	0
VAX 1	M	12	3	13	1
VAX 2	M	12	3	11	3
XAN 266	M	7	8	8	6
Guanajuato 31	M	3	12	5	9
SEA 14	M	1	14	3	11
SEA 13	M	1	14	2	12

Table 67. cont'd

Genotype	Gene Pool[a]	Xcp		Xcpf	
		Incompatible[b]	Compatible[c]	Incompatible	Compatible
Durango 222	M	1	14	2	12
Cejita	M	1	14	2	12
San Cristóbal	M	0	15	1	13
APN 114	M	0	15	1	13
MAM 28	M	0	15	1	13
DICTA 17	M	0	15	1	13
Flor de Mayo Bajio	M	0	15	1	13
Carioca	M	0	15	1	13
Porrillo Sintético	M	0	15	0	14
Orgulloso	M	0	15	0	14
Rió Tibagi	M	0	15	0	14
Zacatecano	M	0	15	0	14
Ojo de Cabra	M	0	15	0	14
Frijola	M	0	15	0	14
Garbancillo Zarco	M	0	15	0	14
Flor de mayo IV	M	0	15	0	14
Amarillo 154	M	0	15	0	14
México 235	M	0	15	0	14
México 309	M	0	15	0	14
BAT 41	M	0	15	0	14

[a] *Phaseolus* gene pool; A = Andean; M = Mesoamerican.
[b] Number of isolates that had a resistant response.
[c] Number of isolates that had a susceptible response.

Plant inoculation: Each bacterial isolate was inoculated onto the first trifoliate leaf of six plants for each genotype using the multiple needle method (CIAT, 2003), at a concentration of 5×10^7 CFU. Disease severity and progression was recorded starting 10, 13 and 17 days after inoculation using the CIAT 1-9 scale.

Results and Discussion: Considering the Andean genotypes only, 98.7% had a susceptible response to inoculation with Xcp isolates and 96.7% were susceptible when inoculated with Xcpf isolates, revealing that Andean genotypes where equally susceptible to both Xcp and Xcpf isolates of CBB (Table 67)**.** A similar result was evident for Mesoamerican genotypes when inoculated with Xcp (96.1% susceptible) and Xcpf (91.6% susceptible). These results show no significant differences in the reaction of Andean and Mesoamerican gene pools to Xcp and Xcpf isolates, revealing a lack of co-evolution of Xcp or Xcpf with bean gene pools as has been reported (Mkandawire et al., 2004). Principal component analysis showed no differences in the reaction of Andean and Mesoamerican genotypes to Xcp and Xcpf. (data not shown). Contrary to reports of differential interaction between bean and CBB isolates, no such interaction was apparent in this study (Table 67). The VAX lines showed high levels of resistance to all isolates, in particular VAX 3, VAX 4, and VAX 6.

Conclusion: The results obtained in this study do not permit us to establish host differential varieties for the CBB pathogen, as no differential interaction was observed in the interaction of bean and the CBB pathogens from different geographical areas. In addition, these results reveal a lack of co-evolution between Xcp or Xcpf with gene pools established for the common bean host. However, such a conclusion can only be made following evaluation of wild beans with the same spectrum of CBB isolates, as the genotypes that have been used in this study are improved, therefore the original host diversity might have been lost during bean improvement. We are in the process of evaluating Andean and Mesoamerican wild bean accessions.

References:

CIAT 2003. Annual Report, Bean Program 2003. CIAT, Cali, Colombia annual.

Mkandawire, A.B.C., Mabagala, R.B., Guzmán, P., Gepts, P., and Gilbertson, R.L. 2004. Genetic diversity and pathogenic variation of common blight bacteria (*Xanthomonas campestris* pv. *phaseoli* and *X. campestris* pv. *phaseoli* var. *fuscans*) suggests pathogen coevolution with the common bean. Phytopathology 94: 593-603.

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

3.1.5 A specific molecular assay for detecting and differentiating *Xanthomonas campestrsi* pv. *phaseoli* and *Xanthomonas campestri* pv. *phaseoli* var. *fuscans*

Rationale: Common bacterial blight and fuscous blight, caused by *Xanthomonas campestris* pv. *phaseoli* (xcp) and *Xanthomonas campestris* pv. *phaseoli* var. fuscans (xcpf) respectively, are major diseases of common bean world wide. Yield losses range from 0 to 40% on susceptible cultivars. The two pathogens are seed borne, this being the principal source of inoculum and means of dissemination to new areas. One way to minimize the impact of the CBB pathogens is to ensure the distribution of disease-free seed. Current assays to identify and quantity *X. c. phaseoli* in bean tissues include plating on selective media, phage typing, immunoassay, and host inoculation. Although valuable, they are labor intensive and not sufficiently precise for routine use. We describe the development of a rapid, sensitive and specific assay for detecting the CBB pathogens in seed, and for differentiating between the two causal agents of the disease.

Material and Methods: Previous studies reported the presence of an Xcpf diagnostic 820 bp fragment following amplification with the RAPD primer OPG11 (Birtch et al., 1997). Amplification of representative Xcp and Xcpf isolates using this primer resulted in two fragments, a 900 bp fragment that was present in all Xcp isolates and an 820 pb present in all Xcpf isolates (Figure 41). The fragments were excised from gels, cloned, DIG labeled and used as probes in Southern hybridization analysis of total genomic DNA either digested with EcoRI or not digested and PCR amplified products, to confirm specificity of these fragments to the two bacteria. Once confirmed, the fragments were sequenced and specific primers designed. The specificity of these primers for Xcp or Xcpf was tested using DNA from different bacteria

(*Xanthomonas campestris* pv. *phaseoli*, *Xanthomonas campestris* pv. *phaseoli* var. fuscans, *Xanthomonas campestri* pv. *manihotis*, *Xanthomonas campestri* 36062 and *Xanthomonas campestri* 1622/16 isolated from Bracharia, *Xanthomonas campestri* pv.o*ryzae, Agrobacterium tumefaciens, Pseudomonas fuscovaginae)*, *Phaseolus vulgaris*, and from several fungi that infect beans (*Pythium ultimum, Colletotrichum lindemuthianum, Phaeoisariopsis griseola* and *Macrophomina phaseolina).* DNA amplification was performed in an MJ Research Thermal Cycler with one cycle at 94^{o}C for 5 min, 65^{o}C for 40 s and 72^{o}C for 2 min, followed by 35 cycles at 94 ^{o}C for 1 min, 65^{o}C for 40 s and 72^{o}C for 2 min and a final cycle at 72^{o}C for 10 min. Reactions were carried out in 12.5 µl reaction volumes containing 5ng of genomic DNA, 0.5 unit Taq polymerase (Promega), 0.16 µM of each primer, 200 µM of each dNTP, 1x PCR reaction buffer, and 1.5 mM $MgCl_{2.}$

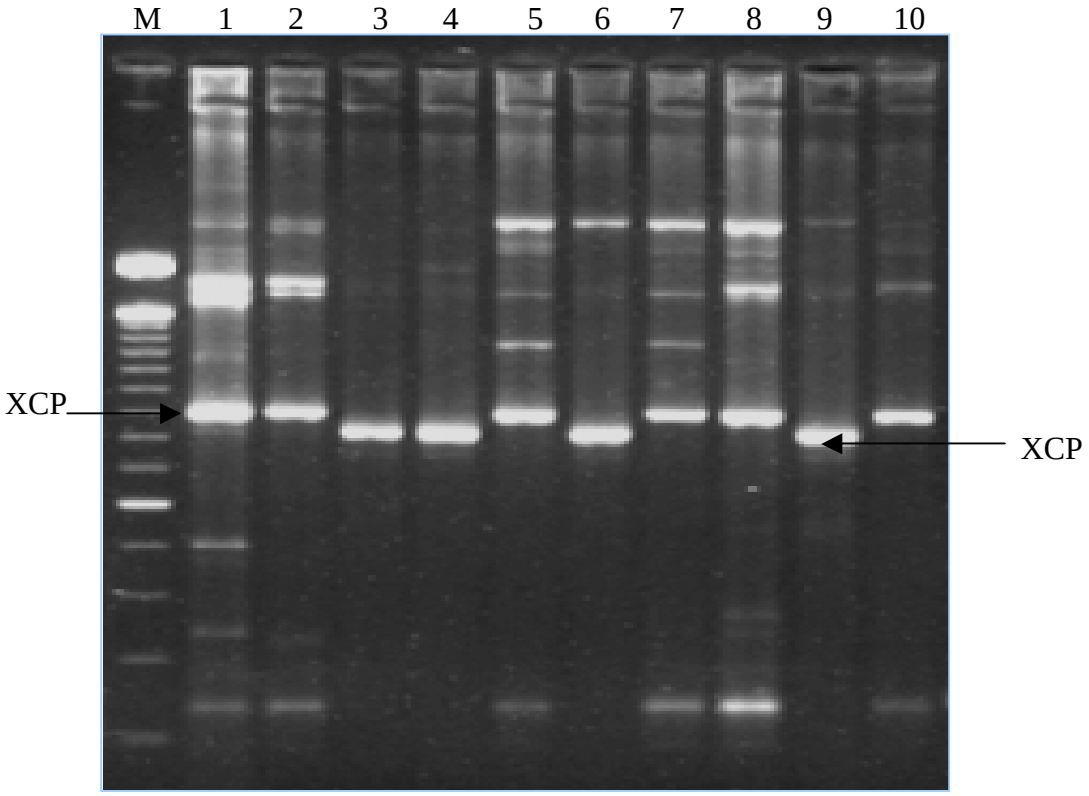

Figure 41. Amplification of *Xanthomonas campestris* pv. *phaseoli (*Xcp*)*, *Xanthomonas campestris* pv. *phaseoli* var. fuscans (Xcpf) using RAPD primer OPG11. Lanes 1, 2, 5, 7, 8, and 10 are Xcp isolates; lanes 3, 4, 6, and 9 are Xcpf isolates. Lane M is the 100 bp molecular size marker.

Utility of developed probes

The utility of the designed probes for detecting the CBB pathogens was validated by amplification of DNA from seeds collected from known infected pods and leaf tissues. In addition, bacterial DNA extracted using different methods (Mahuku, 2004) were used to test the sensitivity of this method to impurities in the PCR assay.

DNA extraction from seed

Ten seeds were washed with sterile distilled water, placed in a plastic bag and 4 ml of a salt solution (8.5 g NaCl in 1 liter of sterile distilled water) added. Alternatively, ten seeds in a plastic bag were macerated in NaCl solution. The bags were put on a shaker (~100 rpm) at room temperature for ~18 hrs, the contents transferred to a 15 ml falcon tube, centrifuged (4000 rpm) for 1 hr at 4°C. The pellet was resuspended in 100 µl of sterile double-distilled water and a serial dilution of up to 1:500 done. One µl from each dilution was used in a 12.5 µl PCR reaction volume.

Extraction of bacterial DNA from single seeds

A single bean seed from infected pods was thoroughly washed with sterile distilled water, macerated in a plastic bag and the contents washed into a 2 ml eppendorf tube using 100 µl of NaCl solution. A plastic pestle that tightly fits the eppendorf tube was used to further macerate and homogenize the solution, the mixture was left standing for ten minutes at room temperature. The supernatant was transferred to a new tube, and centrifuged at 800 rpm for 5 minutes at 4°C. The supernatant was removed, the pellet resuspended in 100 µl of sterile distilled water and a 1 µl of a 1:100 dilution used in a 12.5 µl PCR reaction volume.

Detection level (specificity)

To determine the detection level, 1 µl of the pellet was added to 100 µl of sterile distilled water and plated on YCGA medium and incubated at 28 °C. After 18-24 hrs, the number of CFUs was counted, incubated for 48 hrs, to distinguish between Xcpf and Xcp.

Results and Discussion:

Amplification with OPG11 resulted in two diagnostic fragments, a 900 bp for Xcp and an 820 bp fragment for Xcpf (Figure 41). Southern blot analysis revealed that these fragments were unique to Xcp an Xcpf respectively (Figures 42A and 42B). A set of three primer combinations was developed; one set (xcpG11-L1/xcpG11-R1) was specific to the CBB pathogens (Xcp and Xcpf), amplifying an 800 bp (Figure 43A). This primer pair did not amplify DNA from other pathogens or bean DNA (Figure 43). The primer pair xcpfG11-L1/xcpfG11-R1 was specific for Xcpf (Figure 43B), while the primer pair xcpG11-L2/xcpG11-R1 was specific to Xcp (Figure 43C). When tested on seed from infected pods of plants that had been inoculated with Xcpf under field conditions, only Xcpf was detected (Figure 44C). Amplification with the CBB general primers (Figure 44A) revealed the presence of the bacteria, while amplification with Xcp specific primers revealed the absence of Xcp in seed (Figure 44B). Cultures of the same seeds after seed

washings revealed that they were infected with Xcpf, and all seeds that were negative in the PCR assay where also negative using the culturing method, showing that this assay can potentially be used as a faster method of detecting the CBB pathogens in seed. This PCR assay could detect a minimum of 5 CFU of the bacterium.

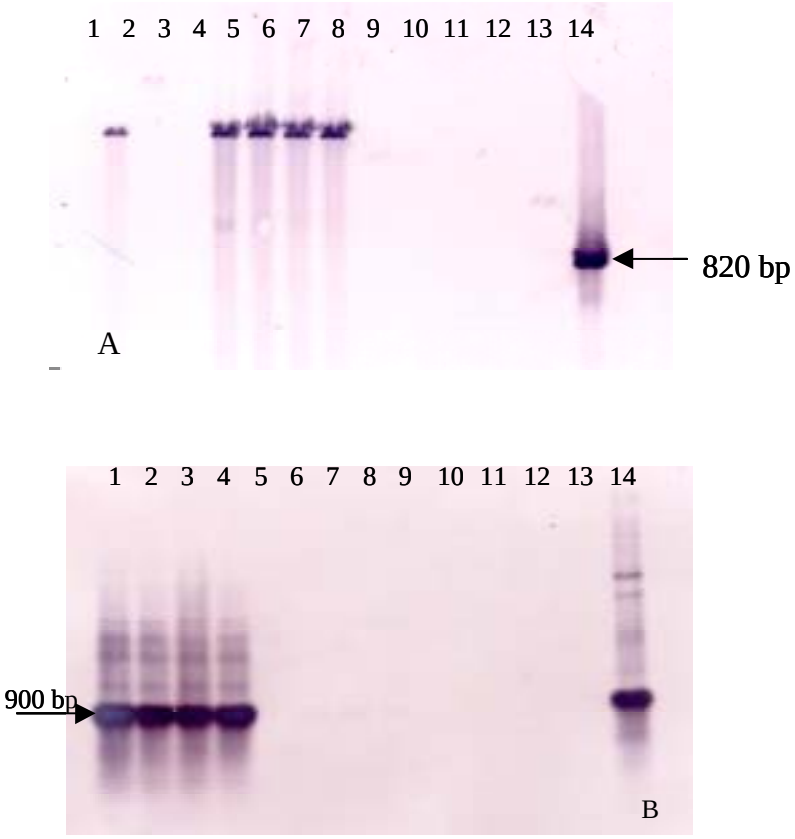

Figure 42. Specificity of the CBB probes for *Xanthomonas campestris* pv. *phaseoli* (Xcp) and *X. campestris* pv. *phaseoli* var fuscans (Xcpf). (A): Genomic DNA was digested with EcoRI and hybridized using the DIG-labeled Xcpf specific fragment; Lane 1, 3 4 correspond to Xcp, lanes 2, 5, 6, 7, 8 are Xcpf isolates; Lane 9 is *X. campestris* pv. *manihotis*,; lane 10 is *X. campestris* pv. *oryzae*; lane 11 is *Phaeoisariopsis griseola*, Lane 12 is *Colletotrichum lindemuthianum*; lane 13 is bean and lane 14 is the plasmid containing the 820 bp fragment specific to Xcpf. (B) PCR amplified DNA using RAPD primer OPG11 and hybridized using the DIG-labeled Xcp specific fragment. of Xcp, Xcpf. Lanes 1-4 is Xcp isolates, lanes 5-8 is Xcpf; Lane 9 is *X. campestris* pv. *manihotis*; lane 10 is *X. campestris* pv. *oryzae*; lane 11 is *Phaeoisariopsis griseola*, Lane 12 is *Colletotrichum lindemuthianum*; lane 13 is bean and lane 14 is the plasmid containing the 820 bp fragment specific to Xcp

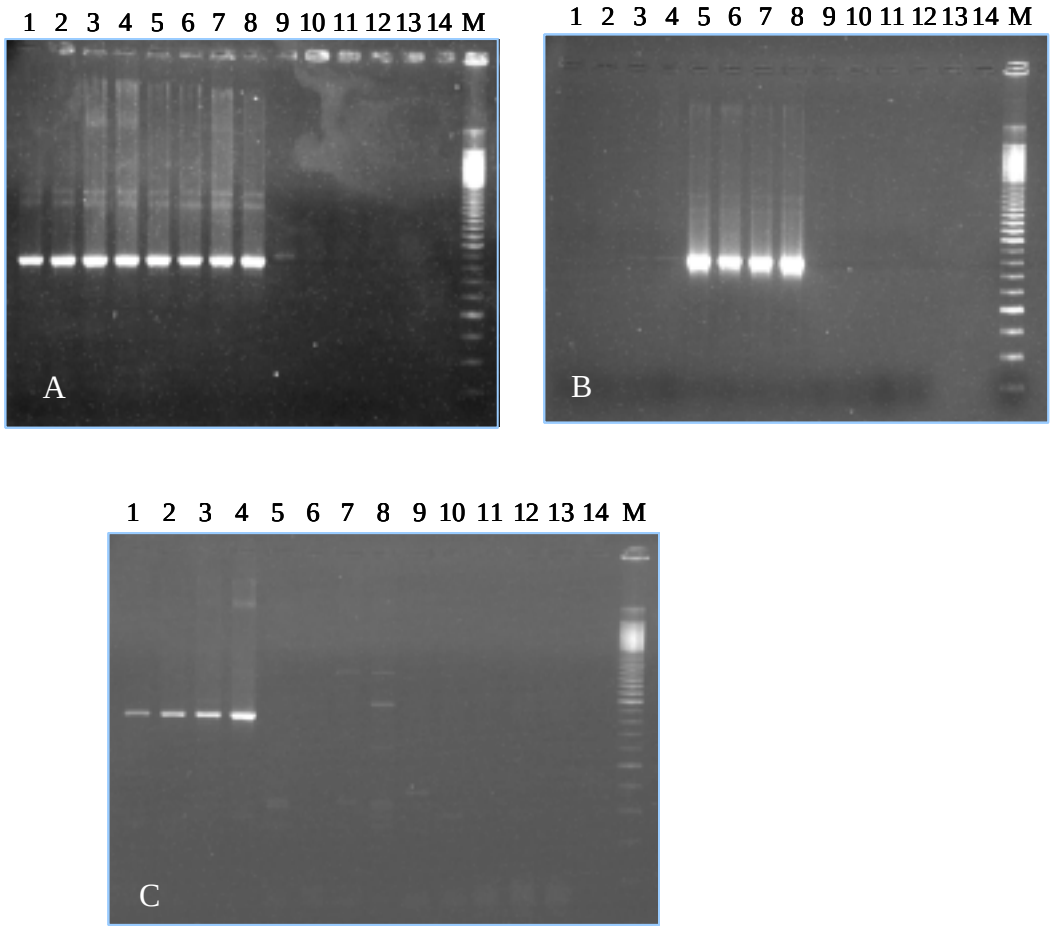

Figure 43. Specific detection of the common bacterial blight pathogens. Lane 1-4; *Xanthomonas campestris* pv. p*haseoli* , lane 5-8 *Xanthomonas campestris* pv. p*haseoli* var, fuscans; lane 9; *Xanthomonas campestri* pv. m*anihotis*, lane 10; *Xanthomonas campestri* pv.o*ryzae*, lane 11; *Phaeoisariopsis griseola*, lane 12; *Colletotrichum lindemuthianum*, lane 13; *Phaseolus vulgaris*, lane 14; negative control, lane 15; 100 pb molecular ladder. (A) Specific detection of the CBB pathogens (Xcp and Xcpf); (B) specific detection of Xcpf; and (C) specific detection of Xcp.

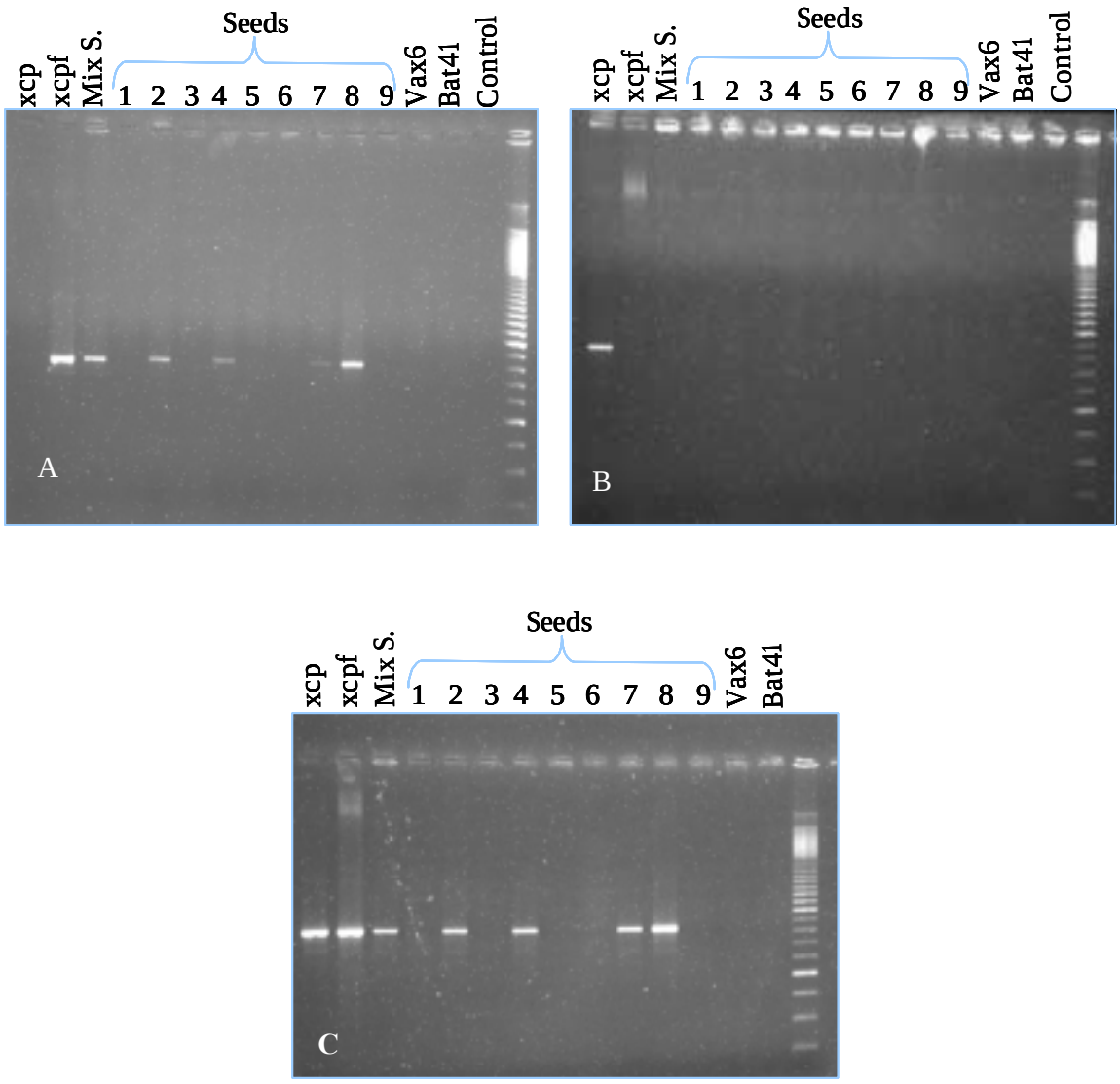

Figure 44. Detection of CBB pathogens in common bean seed, collected from infected pods of BAT 41 inoculated with . *Xanthomonas campestris* pv. p*haseoli* var, fuscans (Xcpf). Lane 1 is Xcp, lane 2 Xcpf, lane 3 is DNA extracted from mixed seeds; lanes 4 individual seeds, lane 12 is bacteria free seed from VAX 6, lane 13, bacteria free seed from BAT 41. (A) the CBB pathogen speific primers were used for PCR amplification in A; while (B) Xcp specific primers were used, and in (c), Xcpf specific primers were used.

Conclusions: A fast and accurate method for detecting the CBB pathogens in seed was developed. This method should significantly improve the accuracy and efficiency of diagnostic of bean common blight and can be easily implemented to certify seed lots.

References:

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Mahuku, G. 2004. A simple extraction method suitable por PCR-Based analysis of plant, fungal and bacterial DNA. Plant Molecular Biology Reporter 22: 71-81.

Contributors: M.A. Henríquez, M. Navia, G. Mahuku

3.1.6 Pathogenic characterization of *Colletotrichum lindemuthianum* isolates from different regions of Colombia

Rationale: Anthracnose of common bean, caused by *Colletotrichum lindemuthianum* continues to be one of the most important diseases of common bean in Colombia, especially in high areas with cool temperatures, high rainfall and relative humidity. Farmers in these areas produce climbing beans, mainly Cargamantos, Bola roja, Radicales, Mortiño. These varieties fetch high prices in the market. Since 2002, we have been monitoring the population structure so as to determine the most prevalent races and compare this with information collected in the eighties, in the hopes of determining whether there has a shift in the pathogen population structure, introduction of new pathogen races or both.

Materials and Methods: Forty-three samples were received from different departments of Colombia where anthracnose of common bean is a serious problem. A total of 43 single spore isolates were made, 16 from Antioquia, 21 from Cundinamarca, 4 from Santander and 2 from Darién in the Cauca valley (Table 68). Fungal characterization on a set of 12 differential varieties (Table 69) established for *C. lindemuthianum* was done in the greenhouse as described previously (Mahuku et al., 2003).

Results and Discussion: Sixteen pathotypes were identified among the 43 isolates and the most frequently characterized pathotype was 3 (Table 68). This pathotype (with 17 isolates) was present in all the departments from where samples were received. All pathotypes have been described before in Colombia. The most susceptible varieties were Michelite (infected by 93% of the isolates), MDRK (58.1%), PI 207262 (46.51%), Perry Marrow (32.6%), Cornell 49242 (25.2%), and TU (4.6%) (Figure 45). None of the isolates infected Widusa, Mexico 222, Kaboon, and G 2333, and these can potentially serve as source of anthracnose resistance. In addition, the resistance genes in Widusa and Kaboon have been well characterized and tagged, making the use of molecular markers in MAS in breeding programs involving these genotypes possible.

Table 68. Frequency distribution of *Colletotrichum lindemuthianum* races characterized from different departments of Colombia

Pathotype	Locality				
	Altiplano Norte	Oriente Antioqueño	Santander	Cundinamarca	Valle
1		1			
3		4	4	8	1
5	2				
7				1	
11				1	
129		1			
131				1	
132		1		2	
133		1		2	
135	1				
137		1		4	
139	1			1	
141	1	1			
143					1
515		1			
641				1	
Total isolates	5	11	4	21	2

Table 69. Common bean Anthracnose differential varieties and their respective identified resistance genes.

Code	Differential Variety	Gene Pool[a]	Resistance(s) gene[b]	Binary Value[c]
A	Michelite	M	?	1
B	MDRK	A	Co-1	2
C	Perry Marrow	A	Co-1^3	4
D	Cornell 49242	M	Co-2	8
E	Widusa	M	Co-1^5	16
F	Kaboon[d]	A	Co-1^2	32
G	Mexico 222	M	Co-3	64
H	PI 207262	M	Co-4^3, Co-9	128
I	TO	M	Co-4	256
J	TU	M	Co-5	512
K	AB 136	M	Co-6, Co-8	1024
L	G 2333[d]	M	Co-4^2, Co-5, Co-7	2048

a= *Phaseolus* gene pool; A = Andean; M = Mesoamerican.
b = identified resistance genes.
c=Binary value assigned for each differential variety and used for race designation.
d= differential varieties that are resistant to all isolates that have been characterized from Antioquia and Santander.

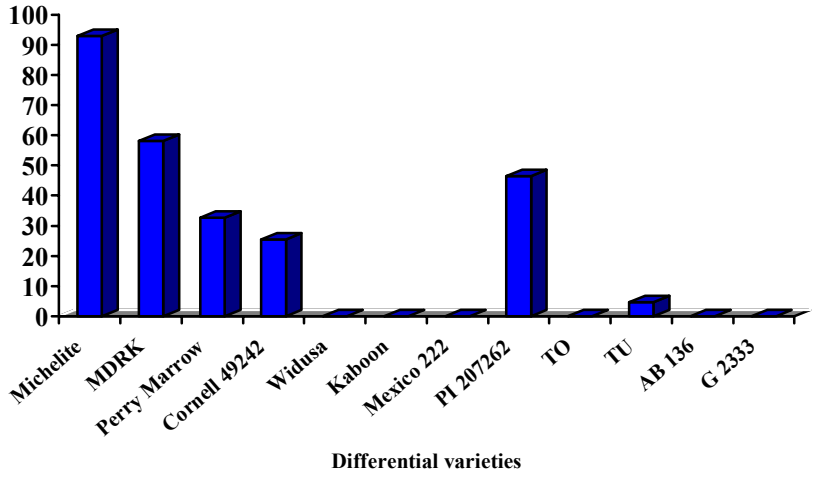

Figure 45. Percent susceptibility of anthracnose differential varieties inoculated with 43 isolates of *Colletotrichum lindemuthianum* from different zones in Colombia

Conclusion: Kaboon and G2333 continue to be immune to isolates from Colombia. The use of the resistance genes from these genotypes in bean improvement for anthracnose resistance in the different zones of Colombia should provide complete anthracnose resistance. Kaboon carries the Co-1^2 allele while G 2333 has three resistance genes (Co-4^2, Co-5, Co-7). Molecular markers linked to these resistance genes are available, making MAS possible in crosses involving these varieties.

References:

Mahuku, G., Jara, C., Cajiao, C., and Beebe, S. 2003. Sources of angular leaf spot (*Phaeoisariosis griseola*) in common bean core collection, wild *Phaseolus vulgaris* and secondary gene pool. Euphytica 130: 303-313

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3.1.7 Elucidating the infection process of resistant and susceptible bean genotypes by *Phaeoisariopsis griseola*

Rationale: Active resistance of plants to pathogens depends on recognition of the pathogens and initiation of defense mechanisms. Invasion by the pathogen triggers recognition and response in the plant leading to signaling cascades and the up- or down-regulation of numerous genes involved in the interaction that in turn may lead to adaptation or evasion by the pathogen. As host resistance is the best strategy to manage the angular leaf spot pathogen, several sources of resistance have been identified that can potentially be used in bean improvement for resistance to the angular leaf spot disease. These potential sources of resistance exhibit different types of resistance mechanisms that include (i) immunity – no symptom expression, (ii) hypersensitive (*HR*) response, and (ii) reduced or slow disease development and progression. The different types of resistance mechanisms can potentially be exploited in developing varieties that resist the angular leaf spot pathogen. To better understand the basis of immunity and HR response, we used light and electron microscopy to study the infection process in three genotypes differing in their response to infection by *P. griseola*, (G 10474 -immunity), G 19833 - *HR* response and PAN 72 susceptible). G 10474 has been found to have an immune response to over 99% of *P. griseola* races that we have tested, making the resistance gene(s) in this genotype a good candidate for managing bean angular leaf spot disease.

Material and Methods:

Fungal and Plant material: *P. griseola* pathotype 63-63, which overcomes the resistance in all angular leaf spot differential genotypes was inoculated onto three bean genotypes, G 10474 and PAN 72 (Mesoamerican), and G 19833 (Andean). G 10474 has an immune response when challenged with pathotype 63-63, G 19833 a hypersensitive response (*HR*) and PAN 72 a susceptible response. Inoculum production, inoculation, plant handling and disease evaluations where done in the greenhouse and as described previously (Mahuku et al., 2003).

Light microscopy: Samples of each genotype were collected 0, 4, 8, 12, 16, 20, 24, 28, 32, and 36 hours after inoculation (hai), and thereafter, daily until 14 days after inoculation (dai). For each sample, 10 leaf discs (1.5 cm in diameter) were cut using a cock borer, cleared and stained following the method described by Chongo et al. (2002), with the following modification: leaf discs were cleared by incubation in acetic acid:ethanol (1:2) for 24 hrs. The solution was changed, and the discs incubated for another 24 hrs. Leaf discs were stained using a lactophenol –Trypan blue (25% w/v phenol crystals, 50% lactic acid, 2.5 mg ml^{-1} trypan blue) for 4 minutes, mounted and fixed in a drop of 80% glycerol. The discs were viewed under light microscope Laborlux D (Leitz Wetzlar, Germany). To evaluate the infection process in each variety, data were collected for number of germinated conidia, size of conidia and length of the germ tube for the first 24 hours. Leaves collected 14 dai where put in humid chambers to induce pathogen sporulation, and evaluated 3 days later for the presence of fungal fruiting structures under a stereomicroscope.

Electron microscopy: Samples for scanning and transmission electron microscopy were collected 2, 3 and 7 dai. Samples were processed as described by Hsieh et al.(2001).

Results and Discussion:

Infection process: *P. griseola* spores started germinating 4 hours after inoculation (Figure 46A-C). Conidia germination occurred either on one side (unipolar) or both sides (bipolar) and occasionally, laterally. In some cases, the infection hyphae formed structures similar to aspersoria, and this was observed principally in the susceptible genotype, PAN 72 (Figure 46D; Figure 47A). The majority of the conidia germinated within the first 24 hrs after inoculation (Table 70). By 24 hours after inoculation, an average of 97% of the spores had germinated (Table 70). Germ tube length 24 hai was significantly short in G 19833 (69 µm) compared to the Mesoamerican genotypes, PAN 72 and G 10474 (88 and 89 µm**,** respectively) (Table 71). Duncan's multiple range test revealed significant differences in germ tube development for the three genotypes, with a coefficient of variation of 59.26.

Penetration: Infection started earlier in the Mesoamerican genotypes, G 10474 and PAN 72 (8 hours after inoculation) and was observed much later in G 19833 (12 hrs), probably signifying differences in leaf morphology of the two bean gene pools (Figure 46B and 46E). Infection was principally through stomata (Figure 46B and 46C; Figure 47B), and occasionally directly (Figure 47C). Only 1.8% of the spores had penetrated leaf tissue by 24 hai, and numerous events of penetration were observed much later, up to 3 days after inoculation.

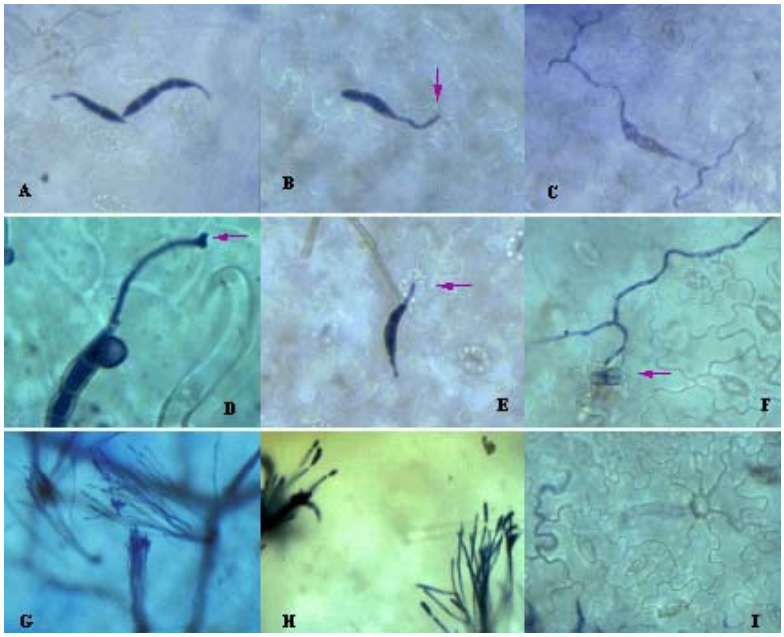

Figure 46. Light microscopy studies of the infection process of *Phaeoisariopsis griseola* (Pg) pathotype 63-63 on susceptible and resistant bean genotypes. (A) Pg conidia germinating on G 10474 at 4 hai; (B) Pg penetration through stomata of G 10474 at 8 hai (C); development of infection hyphae on leaf surface of G 10474 at 24 hai; (D) Pg infection hyphae showing aspersorium formation on PAN 72; (E) Pg penetration through stomata of G 19833 at 12 hai; (F) Necrosis of infected cells of G 19833 (HR response) 7 dai; (G) Pg fruiting structures (synema) on leaves of the susceptible genotype, PAN 72 following 3 day incubation of leaves collected 14 dai; (H) fruiting structures and sporulation on G 19833; (I) no fruiting structures or sporulation on leaves of G 10474 following incubation for 3 days.

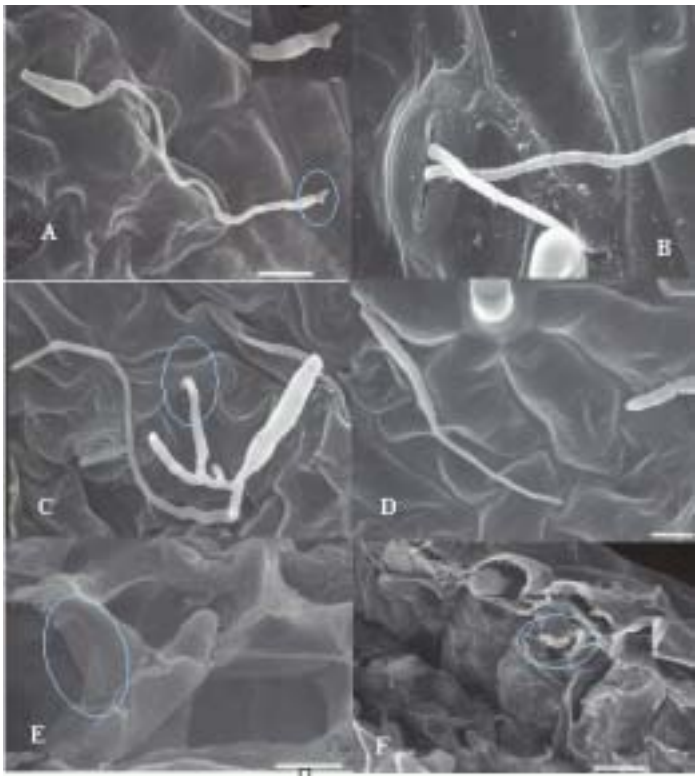

Figure 47. Electron microscopy pictures of the infection process of *P. griseola*. (A) Presence of an aspersorium-like structure in the germ tube of *P. griseola* isolate on PAN 72; (B) Penetration through stomata of the resistant genotype G 10474; (C) Direct penetration by *P. griseola* on G10474; (D) normal development of a compatible race of *P. griseola* on G 10474; (E) transverse section of compatible interaction of G 10474 with a compatible *P. griseola* race (7-55) showing normal mycelium development 7 dai; (F) and an incompatible interaction involving G 10474 at 7 dai with race 63-63.

Table 70. Percent germinated spores in three bean genotypes with different levels of resistance to *Phaeoisariopsis griseola* pathotype 63-63.

Genotype	% germinated conidia					
	4 hai	8 hai	12 hai	16 hai	20 hai	24 hai
G 10474	67.8	86.35	92.86	93.69	93.45	95.14
G 19833	72.8	61.65	82.02	92.39	82.76	97.67
PAN 72	69.04	79.66	89.76	92.52	97.75	98.15

Table 71. Mean germ tube length of conidia three bean genotypes with different levels of resistance to *Phaeoisariopsis griseola* pathotype 63-63.

Genotype	Mean germ tube length					
	4 hai	8 hai	12 hai	16 hai	20 hai	24 hai
G 10474	17.62	36.06	51.00	70.26	69.93	89.41
G 19833	8.17	8.88	45.23	51.19	53.00	68.82
PAN 72	11.44	19.40	52.06	56.83	60.24	88.36

The mean is based on 25 germ tubes of unipolar and 25 bipolar germinated conidias.

Disease progression: Disease development varied with the genotpype under investigation. In G 19833, small infection points were noticed 4-6 days after inoculation, as chlorotic points that later turned brown and necrotic (Figure 46F), a typical *HR* response. When inoculated leaves were placed under conditions that favor sporulation, the fungus formed synemma, and had typical fruiting structures (Figure 46G), showing that resistance to pathotype 63-63 in G 19833 is mediated through cell wall fortification, that confines the fungus to infected cells and limits its fungus. However, the fungus was still alive, as exemplified by sporulations from infected cells, when the fungus was put under conditions that favor sporulation. Although this type of response limits the spread of the fungus and restricts colonization of new tissue, the fungus remains alive and under favorable conditions, has the capacity to sporulate and could potentially serve as a source of inoculum.

In G 10474, although *P. griseola* successfully penetrated this genotype (Figure 46B), no symptoms were observed, showing lack of colonization. When inoculated leaves were put under conditions that favor sporulation, no synemma were observed and there was no sporulation (Figure 46I), revealing the absence of the fungus in plant tissues. Transverse electron microscopy studies of this incompatible interaction showed absence of colonization (Figure 47F). In a compatible interaction involving G 10474 with Pg pathotype 7-55, fungal colonization (Figure 47D, 47E) and sporulation were observed. These results point to the possibility that resistance in G 10474 is mediated through the production and involvement of an antifungal compound that leads to impaired fungal growth.

Normal disease progression and symptom development were observed in the susceptible genotype PAN 72, and profuse sporulation was observed when infected leaves were put under conditions that favored sporulation (Figure 46G).

Conclusion:

The mechanisms of resistance against *P. griseola* are diverse. Resistance in G 19833 is mediated through cell wall fortification that limits the spread of the fungus to infected cells. This could result from lignification and deposition of polyphenolic compounds that restricts the fungus to infected cells. Although the resistance in G 19833 is useful in limiting the colonization of leaf tissues, it might not be ideal, as this genotype could potentially be a source of inoculum, if planted in close proximity with susceptible genotypes, or in mixtures as often happens in Africa. Meanwhile, the immunity observed in G 10474 seems to result from the production of an antifungal compound that either impairs normal fungal growth or interferes with one of the biosynthetic pathway needed for successfully fungal development. To further confirm the presence of an antifungal compound mediating resistance in G 10474, we have transformed pathotype 63-63 to express the GFP protein. This system will be used to closely follow and monitor infection events involving compatible and incompatible interactions with G 10474.

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Contributors: M. Navia, G. Mahuku (IP-1), J.A. Arroyabe (Virology Unit)

3.1.8 *Agrobacterium*-mediated transformation of *Phaeoisariopsis griseola:* a tool for host/pathogen interaction studies

Rationale: The ability to transform an organism is a critical experimental tool. It can be used to test the function of cloned genes and, in systems in which the transforming DNA integrates into the target genome, it is a valuable way to create mutants in uncharacterized genes (Covert et al., 2001). The green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria,* has been used as a reporter molecule in many eukaryotes and prokaryotes. This protein is especially important for direct evaluation of events directly in living tissues, because only UV or blue light and oxygen is required for visualization. The transformation of *P. griseola* to express the GFP protein offers a tool that can be used to study the interaction of this pathogen with common bean, and facilitate studies to elucidate genes and associated pathways responsible for *P. griseola* pathogenicity. A visual marker of gene expression would be useful in detecting gene activity early in the infection process. In addition, the GFP system can be used to create mutants and further our understanding of the molecular basis of pathogenicity and host specificity in *P. griseola* by facilitating the isolation of novel virulence and avirulence genes The objective of this study was to develop a method that can be used for routine transformation of *P. griseola* and to have a system for elucidating the mechanism of resistance and nature of the interaction of *P. griseola* with its common bean host.

Materials and Methods:

Fungal isolates and binary vectors: The highly virulent *P. griseola* pathotype 63-63 was used in this study. Fungal growth and handling was as described previously (Mahuku et al., 2003). The *A. tumefaciens* AGL-1 strain was transformed using two binary vectors pPSK1019, (kindly provided by Dr. S. Kang) and pPK2 (kindly provided by Dr. Martin Rep). The GFP gene in the pPSK1019 vector is under the control of *gpd* from *Cochliobolus heterostrophus*, and the construction of the plasmid has been described before (Mullins et al., 2001). The pPK2-HPHGFP is derived from pPK2 (Covert et al. 2001) and the GFP gene is under the control of the *gpd* promoter from *Aspergillus nidulans.*

Transformation of *P. griseola*: The transformation procedure applied is based on the protocol described by Covert et al. (2001) with the following modifications: *A. tumefaciens* strain AGL 1

carrying either the pPK2-HPHGFP of pPSK1019 plasmid was grown overnight at 28°C in LB medium with appropriate antibiotics added to ensure maintenance of the plasmid. The following day, the cells were diluted in Induction Media (10 mM K_2HPO_4, 10 mM KH_2PO_4, 2.5 mM NaCl, 2 mM $MgSO_4.7H_2O$, 0.7 mM $CaCl_2.2H_2O$, 9 μM $FeSO_4.7H_2O$, 4mM $(NH_4)_2SO_4$, 10 mM Glucosa, 0.5% Glicerol, 40 mM MES) plus 200 μM acetosyringone (AS) to an OD_{660} equal to 0.30. The final volume was adjusted to 20 ml, incubated at 28°C with shaking (150 rpm) for approximately 6 hrs, until reaching the OD_{660} of 0.8. Conidia of *P. griseola* from a 12 day old culture on V8 juice medium (200 ml l^{-1} juice V8 Campbell, 3 g l^{-1} $CaCO_3$, 15 g l^{-1} agar) were collected by adding sterile water to the plates and rubbing the surface of the V8 juice medium with the end of a sterile micropipette tip. Conidial suspension was filtered through Miracloth to remove large fragments, and washed three times with sterile distilled water and the concentration adjusted to 10^6 conidias ml^{-1}. The conidia suspension was mixed with an equal volume of *A. tumefaciens* cells. This mix (200 μl per plate) was plated on a 0.45 μm pore, 45-mm diameter polycarbonate filter and placed on co-cultivation medium (same as IM except that it contains 5 mM glucose and 1.5% agar) with 200 μM AS. The plates were incubated for 1, 2 or 3 days at 25°C in the dark, after which the filter was transferred to plates with M-100 medium (Stevens 1974) containing hygromycin B (100 μg/ml) and cefotaxime (400 μg/ml) to kill the *A. tumefaciens* cells. The plates were incubated at 25°C and individual transformants were transferred to M-100 medium with hygromycin B (100 μg/ml) and appropriate antibiotics until conidiogenesis. Conidia of individual transformants were suspended in sterile water and plated on V8 juice medium supplemented with hygromycin B. To create monocultures, one germinated conidium from each transformant was picked and transferred to V8 juice medium with hygromycin B in small petri plate (60 x 15 mm). Spores from these monoconidial cultures were lyophilized for long-term storage or put on filter papers for short-medium term storage.

DNA extraction: Putative transformants were grown in V8 juice liquid medium (20% Jugo V8 Campbell) containing 400 μg/ml cefotaxime and hygromycin B (75 μg ml^{-1}) for 10 days at 25°C with shaking. Mycelium was collected by vacuum-filtration on sterile Whatman paper, washed several times with sterile water before drying between paper towels. The mycelium was lyophilized and macerated to a fine powder in liquid nitrogen and DNA was extracted as previously described (Mahuku, 2004).

Confirmation of transformation by PCR: To verify the integration of the T-DNA into fungal genomes, DNA extracted from putative transformants was amplified using primers targeting the hygromycine gene using the primers Hyg 1 (5'-GCGTGGATATGTCCTGCGGG-3') and Hyg 2 (5'-CCA TAC AAG CCA ACC ACG G-3') as described by Amey et al. (2002).

Confirmation of transformation by Southern hybridization: To determine the presence of the GFP protein, as well as determine the copy number of T-DNA, extracted DNA was digested with HindIII y KpnI and the products separated by electrophoresis on 0.7% gel, blotted onto Hybond-N+ membrane (Amersham Pharmacia Biotech) and fixed by UV cross-linking. Probes were labeled using the PCR DIG probe synthesis kit (Roche Diagnostics, Mannheim, Germany). Southern blot analysis was carried out as described by Sambrook et al. (1989) with 5 – 10μg of DNA in each sample. Prehybridization, hybridization, and high stringency washes of the membrane were completed at 65ºC as described by the manufacturer.

Mitotic stability: To determine the stability of transformants, selected transformants were cultured on V8 juice medium without hygromycin B, and the culture repeatedly transferred (five times) to fresh V8 juice medium, after which they were grown on medium containing hygromycin B (75 μg/ml).

Virulence test of co-transformants: Two bean genotypes; G 10474 (resistant) and Sprite (susceptible) were established in the greenhouse. There were three plants of each genotype for each transformant. The wild-type *P. griseola* pathotype 63-63 was included as a control. Inoculum production, inoculation procedure and plant handling were done as described previously (CIAT, 2003). Disease progression and symptom development were monitored up to 14 days after inoculation. The development of the fungus within leaf tissue was monitored microscopically.

Expression of GFP: Putative GFP-expressing colonies were identified using an Eclipse E400 fluorescence microscope (Nikon). The microscope has a mercury lamp with an excitation filter of 450 – 490 nm.

Results:

Analysis of ATMT-mediated transformants: A total of 202 colonies resistant to hygromycin were obtained, 86 using the pPSK-1019 vector and 116 using the pPK2-HPHGFP (Table 72). The wild type pathotype 63-63 did not grow on medium with hygromycin B (100 μg ml^{-1}). Some of the transformants had color changes (lighter) when grown on V8 juice medium without hygromycin B, but the majority of the isolates were not affected, and did not show any notable differences when compared to the wild type (Figure 48).

Table 72. Number of hygromycin B – resistant (Hyg-R) fungal clones after *Agrobacterium tumefaciens*-mediated transformation for two binary vectors and three different days of co-cultivation.

Plasmid	Days of co-cultivation	Hyg-R / plate*
PPSK1019	1	20
	2	10
	3	13
pPK2-HPHGFP	1	7.5
	2	29
	3	22.5

* Values are mean of two replicates.

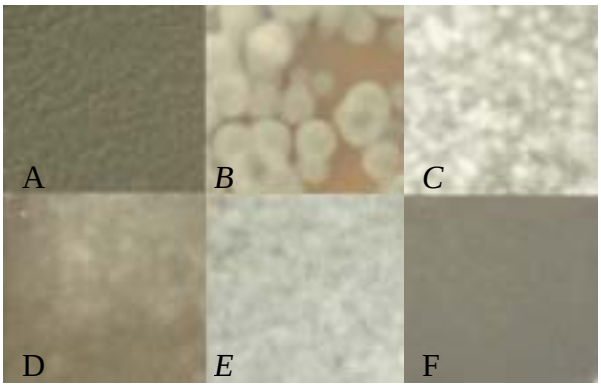

Figure 48. Morphology of hygromycin B –resistant *P. griseola* transformants compared to the wild-type isolate Pg 63-63. (A) Wild-type isolate, (B), (C), (D) and (F) are hygromycin B-resistant transformants. Some transformants are morphologically different from the wild type, while others are similar.

After initial PCR analysis to check for the presence of transforming DNA (Figure 49), Southern hybridization showed that the probe for the hygromycin B resistance gene did not hybridize to digested wild-type genomic DNA. The probe hybridized to all *Agrobacterium*-mediated transformations, indicating integration of the plasmid into genomes of the Pg pathotype 63-63 (Figure 50). The Hind III and Kpn I digest indicates both single and multiple integration events at random loci. The majority of the transformants had single integration. The differences in the sizes of the fragments upon hybridization indicate that the integration events were random. This was confirmed following hybridization with the GFP probe. None of the probes hybridized with the wild-type Pg pathotype 63-63. Hybridization with undigested genomic DNA (data not shown) showed that the signal was associated with DNA of high molecular weight, confirming that the T-DNA was integrated within chromosomal DNA.

GFP expression: Florescence microscopy revealed that the majority of transformants expressed GFP. Of the 202 transformants, 80% strongly expressed GFP, 7 did not fluoresce and the rest (20%) had weak fluorescence. Fluorescence was not linked to the number of GFP insertions, and some of the weakly fluorescing transformants had double insertions (Figure 51). As GFP was localized in the cytoplasm, it was possible to observed GFP expression in conidia (Figure 51 A, C, D); mycelium (Figure 51 F, I) and synemas (Figure 51G). The vacuoles did not fluoresce and these are seen as black areas of different sizes within the cytoplasm (Figure 51A, C). The wild-type *P. griseola* isolate did not fluoresce under UV light.

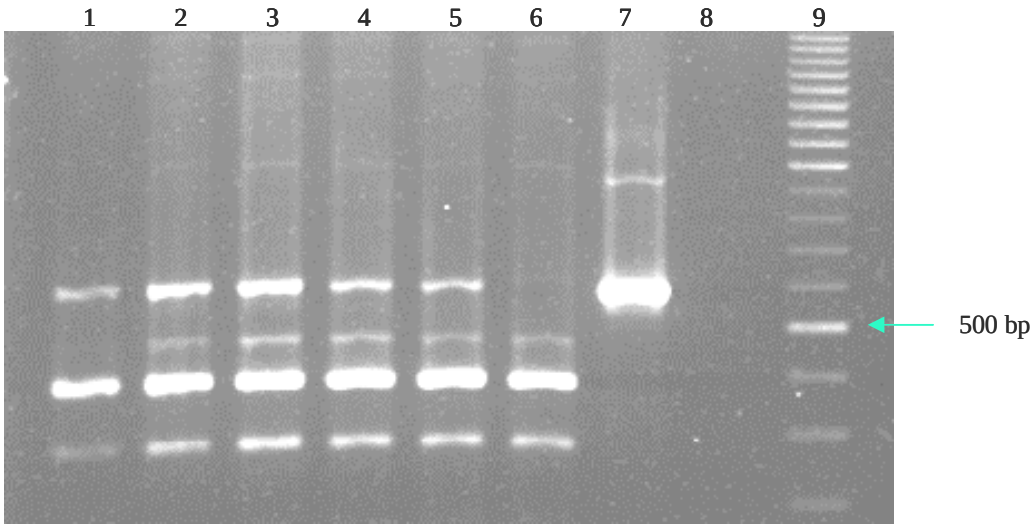

Figure 49. Polymerase chain reaction detection of the hph gene of randomly chosen HygB-resistant transformants, using the primers Hyg1 and Hyg2 primers (Amey et al., 2002) for amplification. Lanes 1-3 are transformants using the plasmid pPK2-HPHGFP; lanes 4 and 5 correspond to transfomants using the plasmid pPKS-1019; lane 6 is the wild type *P. griseola* pathotype 63-63; lane 7 is positive control (plasmid pPKS-1019); lane 8 is negative control and lane 9 is 100 bp ladder.

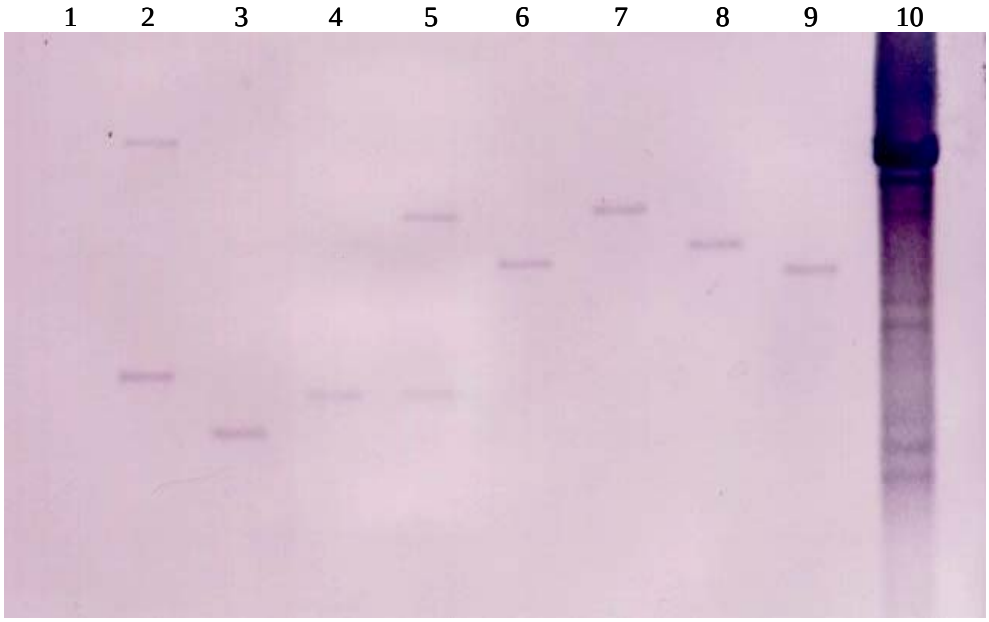

Figure 50. Transforming DNA integrating randomly and in single or double copy number into the target DNA. PCR amplified *hph* gene was used to probe the *Agrobacterium*-mediated transformants. All DNA was digested with Hind III. Lane 1 is wild-type DNA, lanes 2-9 are transformants generated with the pPSK-1019; lane 10 is digested pPSK-1019 plasmid

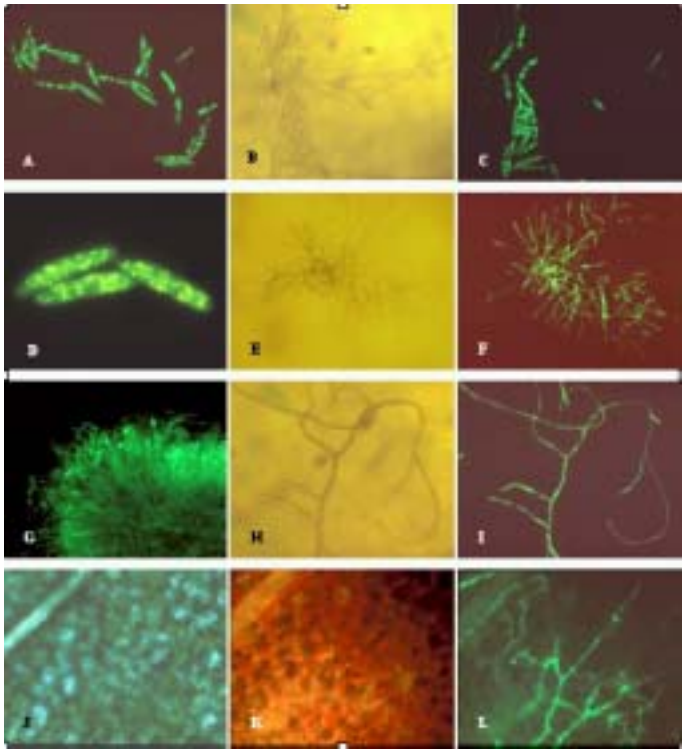

Figure 51. Fluorescence and light micrographs of GFP transformant and control strains. (A) conidia of a transformant carrying the pPK2-HPHGFP plasmid under fluorescent light; (B) conidia of a *P. griseola* transformant under white light; (C) conidia of *P. griseola* seen in (B) but under fluorescent light; (D) conidia of a transformant carrying the pPSK-1019 plasmid; (E) mycelium from transformation with plasmid pPSK-1019 under white light and (F) under fluorescent light; (G) mycelia from fungi transformed with pPK2-HPHGFP and grown on media on a slide; (H) white and (I) fluorescent light images of mycelium; (J) light micrograph of transformant growing inside plant tissue; (K) same leaf tissue under one filter and (L) under a different filter showing the transformant growing inside bean leaf tissue.

Assessment of virulence: It is important to know whether the constitutive promoters place an undesirable metabolic burden on the transformants, and to determine whether the transformants have been altered in their ability to cause disease in the bean host as a result of integration of transforming DNA within the genome. Apart from a difference in mycelium color observed within a few transformants, initial comparisons showed similarity to the wild-type. The rate of infection and symptom development on susceptible bean cultivar were the same as the wild-type Pg 63-63 pathotype for the majority of the transformants (Figure 52). Only three transformants had lost their ability to infect the susceptible bean genotype, Sprite, while another four developed very slowly compared to the wild type. These results are very interesting, as they seem to signify the presence of mutants with the pathogenicity gene disrupted through insertional mutagenesis.

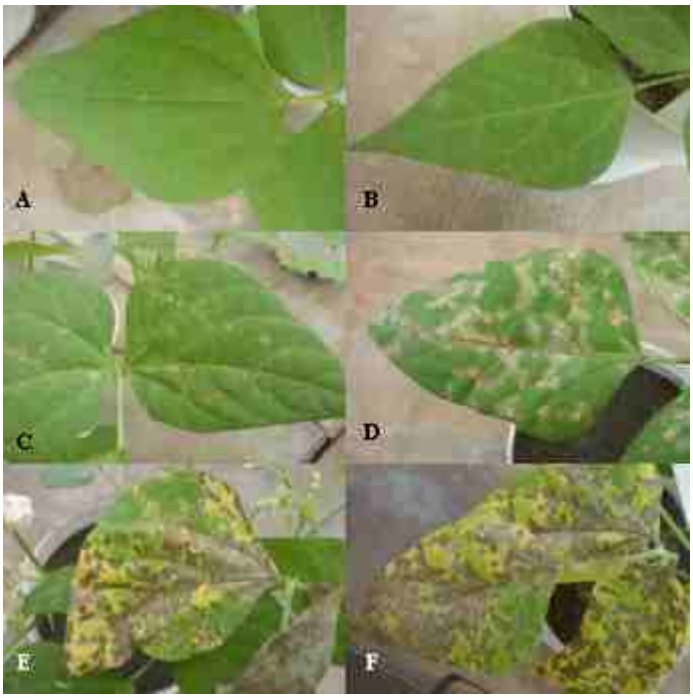

Figure 52. Angular leaf spot symptoms recorded 15 days after inoculating the universally susceptible genotype Sprite with transformed and wild type isolates of *Phaeoisariopsis griseola* pathotype 63-63. (A) control - inoculated with water, B to E represent bean leaves inoculated with transformants expressing the GFP protein, and F is wild type isolate. Some of the isolates have lost their ability to infect this variety, while in others, symptom development was delayed.

Conclusion: *Phaeoisariopsis griseola* was successfully transformed to express the GFP gene using the ATMT system. To the best of our knowledge, this is the first report of the transformation of this fungus. The two promoters driving GFP were expressed in *P. griseola*, but it appears that transformation with pPK2-HPHGFP was more efficient, giving the highest number of transformants. Southern hybridization showed that most of the transformants were a result of single integration events, and that the integration occurred in nuclear DNA. This is particularly important for insertional mutagenis and subsequent isolation of pathogenicity / virulence genes. The different levels of expression observed could reflect differences in the areas of integration in the genome. GFP expression was conserved following multiple transfer of isolates, revealing mitotic stability of GFP integration. In addition, the GFP protein was expressed in bean tissues, revealing that this system can be used to monitor infection events. A protocol for the transformation of *P. griseola* has been developed, including the subsequent analysis of the protein in fungal and plant material. This will be an important tool in providing information on the interaction of *P. griseola* with its common bean host.

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Contributors: M. Navia, G. Mahuku

3.1.9 Characterization and distribution of *Pythium* spp associated with bean root rot in East Africa

Rationale: There are approximately 100 known species of the genus *Pythium* include pathogenic, saprophytic and biological control groups. Our recent studies in Uganda have shown that over seven *Pythium* spp cause root rots on common beans, but their distribution and relative importance in other countries in East Africa are unknown. Characterization of *Pythium* species and their distribution is therefore considered a necessary pre-requisite in order to develop effective management strategies. However, identification of *Pythium* species using morphological or pathogenic characteristics is difficult given the large species numbers and their mixed occurrence in the soil. We have therefore continued with the characterization of *Pythium* spp., using molecular methods as a basis for developing simpler, accurate and rapid but reliable detection and characterization techniques. We therefore continued to characterize *Pythium* spp. prevalent in Kenya and Rwanda.

Methods: One hundred and thirty-four *Pythium* isolates obtained from root rot affected areas in Kenya and Rwanda were characterized by sequencing using the protocol of Levesque *et al*(1998). The DNA of isolates was amplified with universal eukaryotic primers targeting the internal transcribed spacer (ITS) regions and the 5.8S gene of nuclear ribosomal DNA. Purified template DNA was sequenced using an ABI prism automated sequencer. Sequences obtained were edited and compared to data of *Pythium* spp. managed by Dr. A. Levesque of the Agri-Food and Food and Agriculture Canada.

Results and Discussion: Out of 134 isolates characterized, 22 species were identified (Table 73). Thirteen of these have been reported in our previous pathogen characterization studies in Uganda and Kenya but nine were new additions. All except three (*P. macrosporum, P. zingiberis, P. graminicola*) were recovered from Rwanda with *P. ultimum* being the most frequent, followed by *P. torulosum* and *P. spinosum.* The three are pathogenic to beans. Fifteen of the 22 species were recovered from Kenya with *P. vexans* being the more frequent species, followed by *P. torulosum*, *P. irregular* and *P. ultimum.* Species distribution maps for Kenya and Rwanda are shown in Figures 53 and 54 respectively. These results are consistent with past observations that overall *P. ultimum* is the most frequent species in the region. Pathogenicity of some of the new species is being determined to establish their role in the bean root rot problem in the region.

Table 73. Identification by sequencing of *Pythium* isolates obtained from bean growing areas associated with bean root rots in Kenya and Rwanda.

	***Pythium* Isolates**		
Species	**Kenya**	**Rwanda**	**Total**
P. acanthicum	2	1	3
P. chamaehyphon	1	2	3
P. folliculosum	3	2	5
P. indigoferae	2	2	4
P. irregulare	9	1	10
P. lutarium	1	3	4
P. macrosporum	1	0	1
P. myriotylum	1	1	2
P. paroecandrum	3	3	6
P. torulosum	9	10	19
P. vexans	10	4	14
P. zingiberis	5	0	5
P. graminicola	4	0	4
P. spinosum	1	7	8
P. ultimum	5	23	28
P. arrhenomane	0	2	2
P. catenulatum	0	1	1
P. deliense	0	1	1
P. diclinum	0	2	2
P. dissotocum	0	3	3
P. rostratum	0	5	5
P. salpingophorum	0	4	4
Total	57	77	134

Contributors: R. Buruchara, S. Mayanja, G. Mahuku, A. Levesque.

Collaborators: R. Otsyula (KARI), L. Butare (ISAR).

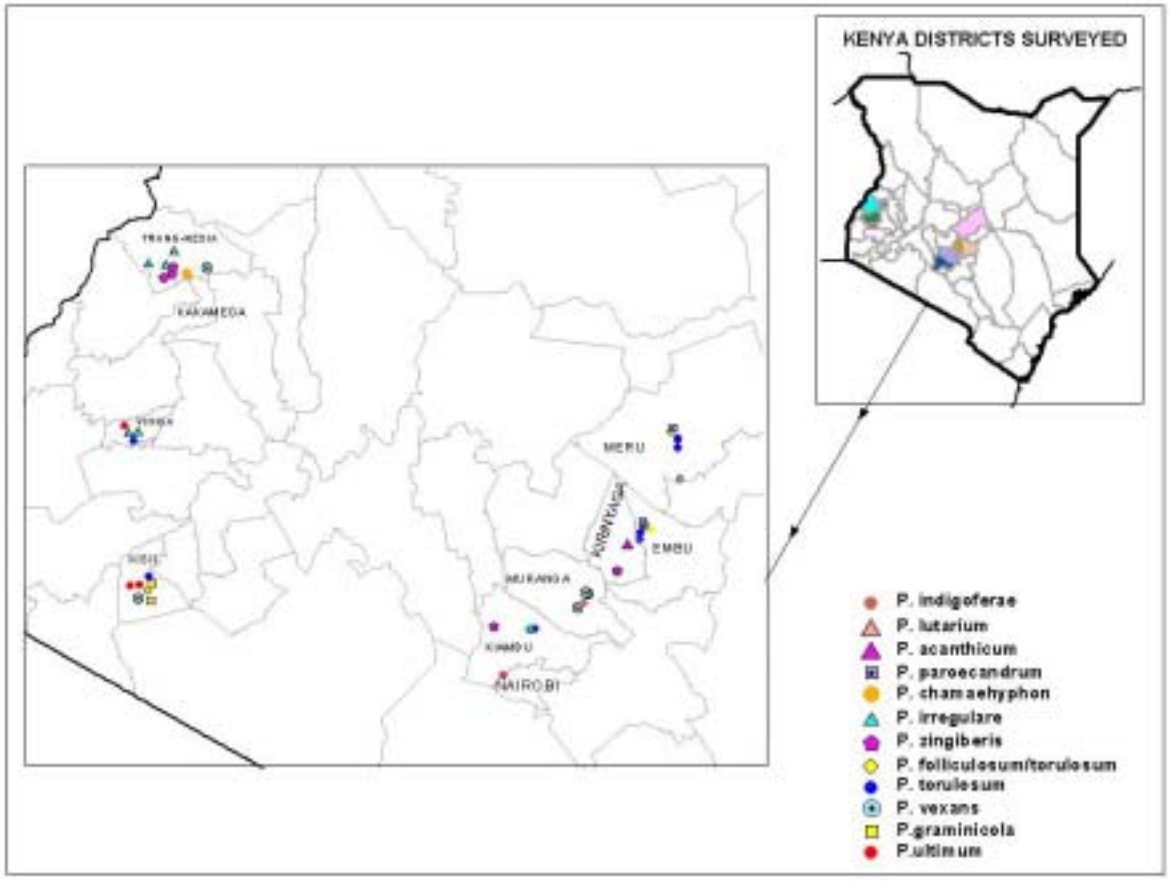

Figure 53. Distribution of *Pythium* species in some districts of Kenya where bean root rots are prevalent. Characterization was based on sequencing of *Pythium* isolates.

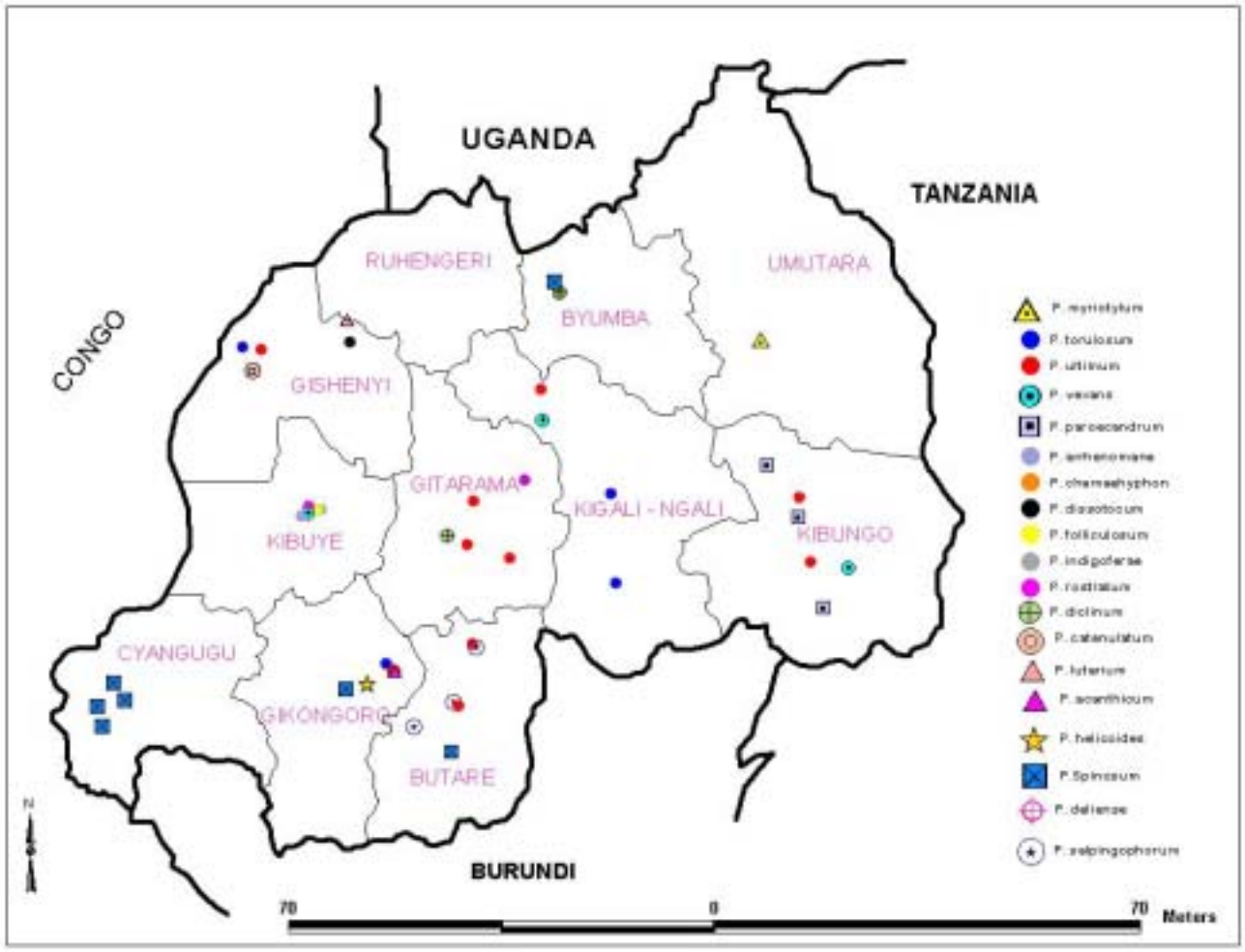

Figure 54. Distribution of *Pythium* species in Rwanda where bean root rots are prevalent Characterization was based on sequencing of *Pythium* isolates.

3.1.10 Monitoring of the changing situation with whitefly populations in the Andean zone

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 2004 we processed a total of 105 whitefly samples (adults and pupae) collected in the Cauca Valley and northern coast regions of Colombia. Samples were taken from beans, snap beans, cucurbits, tomatoes and several other annual crops. We used RAPD techniques (primer OPA-04) to identify pupae and adults. Identification was based on morphological characteristics of pupae and comparison between RAPD patterns in samples brought from the field with those of existing mass rearings of different whiteflies maintained at CIAT (Figure 55).

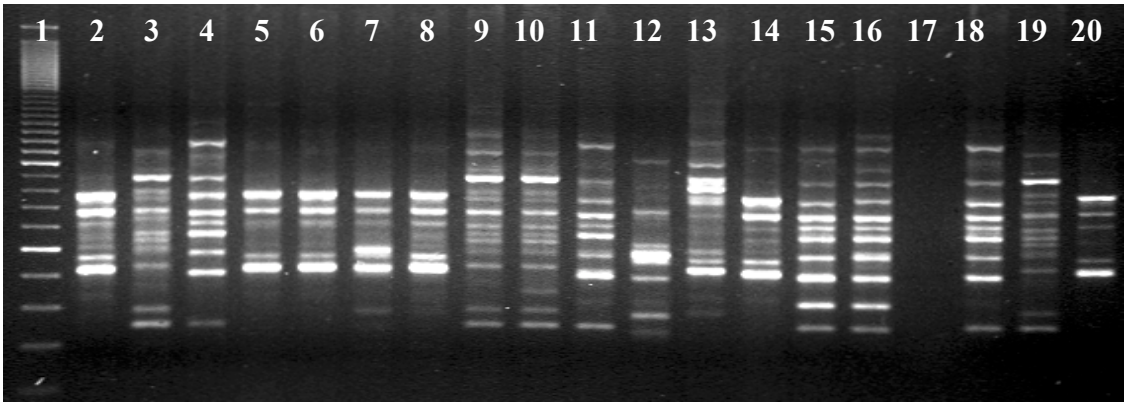

Figure 55. RAPD's for whitefly adults and pupae collected in the Cauca Valley. Amplifications using the OPA-04 primer; 1, DNA molecular marker (100 pb); 2, *T. vaporariorum* from reference rearing maintained at CIAT; 3, *B. tabaci* biotype A from reference rearing; 4, *B. tabaci* biotype B from reference rearing; 5-8 adults (5-6) and pupae (7-8) of *T. vaporariorum* collected in Darien on beans; 9-10, *B. tabaci* A adults collected on soybeans in Jamundi; 11, *B tabaci* biotype B collected on soybeans in Jamundi; 12, parasitized pupa of *B. tabaci* collected on soybeans in Jamundi; 13-14, *T. vaporariorum* adults on beans in Jamundi; 15-16, *B. tabaci* biotype B pupae on beans in Jamundi; 17, free; 18 *B. tabaci* biotype B from reference rearing; 19, *B. tabaci* biotype A from reference rearing; 20 *T. vaporariorum* from reference rearing.

Results and Discussion: Analysis of 105 samples taken in 24 locations in the Cauca Valley (Colombia) showed that 42% of the whiteflies collected belonged to the B biotype of *Bemisia tabaci*, the most aggressive form of whitefly known to date. This biotype was found affecting snap beans, tomatoes, cucumber, melon, soybeans, pepper, tobacco, and grapes. As in 2003, we found that the B biotype is now occupying niches previously reserved to the A biotype or to *T. vaporariorum*. As shown in Figure 56, species composition in the Cauca Valley has changed drastically in the past seven years. In 1997, *T. vaporariorum* was by far the most important species, representing 73% of the samples taken while the A biotype represented 15% of samples analyzed. At present, the A biotype is difficult to find (1.6% of samples), *T. vaporariorum* represents 11% of the samples and the B biotype is the predominant species with 42% of the samples. Up to 39% of crop samples examined were affected by a combination of *T. vaporariorum* and the B biotype of *B. tabaci*.

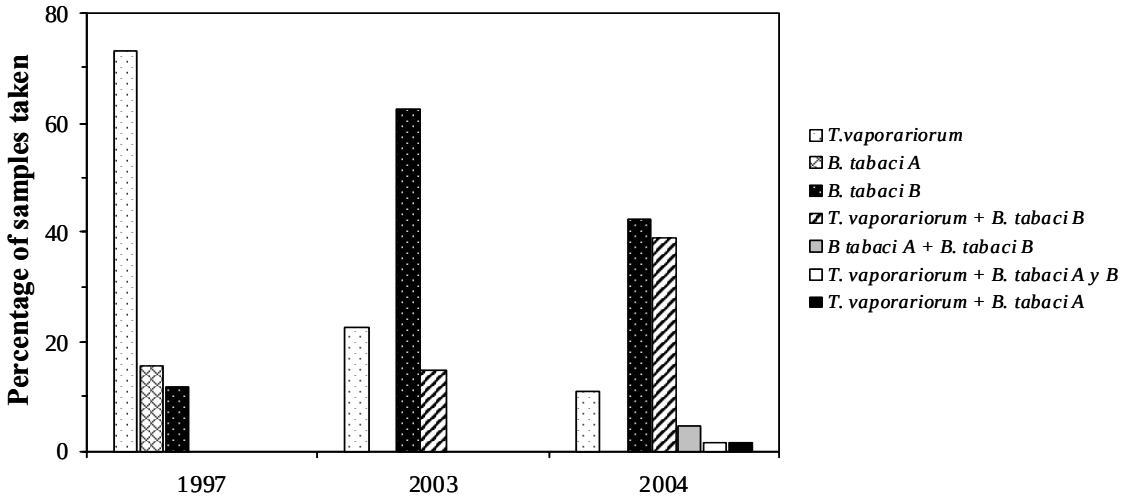

Figure 56. Changes in whitefly species composition in the Cauca Valley of Colombia (1997-2004)

Detailed monitoring of species composition on snap beans in the Pradera reference site revealed that at higher altitudes (1270-1840 masl) *T. vaporariorum* is still the dominant species (Figure 57). At altitudes ranging from 975 to 1120 masl, most individuals collected in the Pradera region belong to the B biotype of *B. tabaci* attacking different crops either alone (33.3% of samples taken) or in combination with *T. vaporariorum* (53.4% of samples). The B biotype is an aggressive form of whitefly that is causing all the serious problems described in our 2003 Report. In snap bean growing areas, it has become the causal agent of a physiological disorder known as pod chlorosis, which renders the produce useless. Most serious, it has become a very effective vector of a geminivirus that is devastating snap beans in the region.

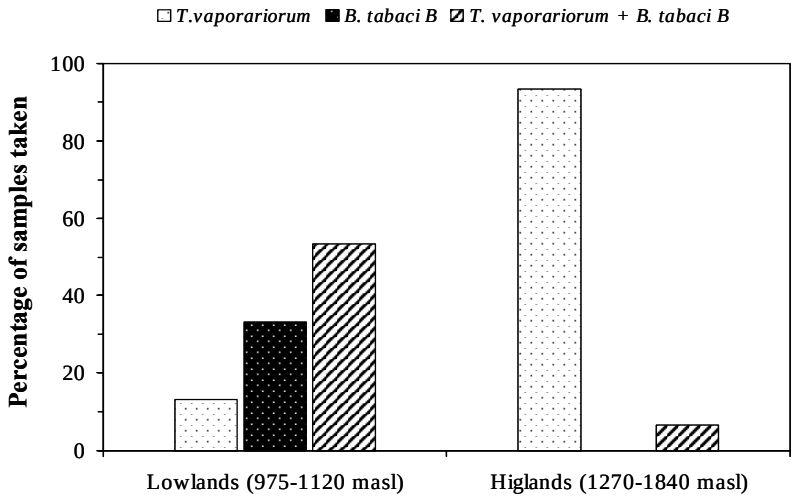

Figure 57. Whitefly species composition in the Pradera (Cauca Valley) reference site; 2004 survey. (masl = meters above sea level).

Contributors: I. Rodríguez, H. Morales, C. Cardona

3.1.11 Monitoring of insecticide resistance in whitefly populations

Rationale: Monitoring of insecticide resistance is another major objective of the DFID-funded project on Management of Whiteflies in the Tropics. Both major whitefly species and their biotypes in the Andean zone are the targets of excessive use of insecticides. This is reflected in ever increasing levels of resistance to insecticides and difficulties in control. The main purpose of a continuous monitoring of insecticide resistance is to develop alternative management strategies that will help to overcome resistance or delay the onset of this phenomenon.

Materials and Methods: In 2004 we established base-line data for five insecticides commonly used to control adults of the B biotype of *B. tabaci*: monocrotophos, carbofuran, carbosulfan, bifenthrin, and imidacloprid. These data will serve as the basis to establish diagnostic dosages for the species. These in turn will be used for periodic monitoring of resistance levels.

Using previously established diagnostic dosages for nymphs, we tested populations of whiteflies in the Cauca Valley in Colombia. Adult resistance levels were monitored under field conditions by means of the insecticide-coated glass vial technique. Resistance of first instar nymphs was measured using the foliage dipping technique. Systemic novel insecticides (mostly neonicotinoids) were tested using the petri dish technique (see 2003 Annual Report).

Results and Discussion: In general, it can be said that nymphal populations of both *T. vaporariorum* and *B. tabaci* biotype B are still susceptible to the insect growth regulators buprofezin and diafenthiuron and to imidacloprid, a novel neonicotinoid (Table 74). However, reduced responses to buprofezin in the Pradera site deserve further monitoring.

Future work on integrated pest management of whiteflies as pests of beans and snap beans in the Andean zone should include studies on the relative efficiency of the two most important parasitoids affecting whitefly populations in the region: *Encarsia nigricephala* and *Amitus fuscipennis*. Given the excessive use of insecticides, it is important to know what is the present response of these natural enemies to some of the most commonly used insecticides. The base-line data in Table 75 should in the future serve as the basis for possible development of insecticide tolerance in populations of these natural enemies, an optional strategy for management of the whitefly problem.

Comparison of toxicological responses of the whitefly and their parasitoids indicate that all of the insecticides tested are much more toxic to the parasitoids than to the whitefly (Table 76) with up to 100-fold higher tolerance in the herbivore. Nevertheless, the data show that both natural enemies studied do possess innate mechanisms of defense against toxic substances, which may be exploited by continuous mass rearing and selection for higher levels of tolerance followed by mass releases in the field. As such, resistant strains of one or both parasitoids would become management components in an integrated pest management system. .

Contributors: I. Rodríguez, H. Morales, M. F. Montenegro, and C. Cardona

Table 74. Response (percentage corrected mortality) of nymphs of *Trialeurodes vaporariorum* and *Bemisia tabaci* biotype B to three insecticides in three consecutive growing seasons. Cauca Valley (Colombia). Diagnostic dosages in ppm

Race	Percentage corrected mortality[a]		
	2001 B	**2002 B**	**2003 B**
	Trialeurodes vaporariorum		
		buprofezin (16 ppm)	
'CIAT'[b]	98.4 a A[b]	100.0 a A	97.6 a. A
La Cumbre	100.0 a A	100.0 a A	100.0 a A
Pradera	87.0 b A	77.4 a A	81.4 b A
		diafenthiuron (300 ppm)	
'CIAT'	98.2 a A	100.0 a A	96.2 b A
La Cumbre	92.6 a B	97.8 a A	100.0 a A
Pradera	88.6 a A	93.9 a A	90.5 c A
		imidacloprid (300 ppm)	
'CIAT'	100.0 a A	98.3 a A	93.7 a A
La Cumbre	92.8 b A	93.2 a A	99.0 a A
Pradera	84.9 b A	92.6 a A	93.2 a A
	***Bemisia tabaci* biotype B**		
		buprofezin (16 ppm)	
'CIAT'[c]	---	98.4 a A	96.9 a A
Rozo	---	80.6 b A	87.8 b A
La Unión	---	100.0 a A	87.7 b B
Santa Helena	---	100.0 a A	92.2 b B
		diafenthiuron (300 ppm)	
'CIAT'	---	100.0 a A	91.7 a B
Rozo	---	100.0 a A	91.5 a B
La Unión	---	98.2 a A	91.7 a B
Santa Helena	---	100.0 a A	95.1 a B
		imidacloprid (300 ppm)	
'CIAT'	---	91.1 b A	98.3 a A
Rozo	---	89.3 b A	90.1 b A
La Unión	---	100.0 a A	89.1 b B
Santa Helena	---	100.0 a A	98.6 a A

[a] For each species and product, means within a column followed by the same lowercase letter and means within a row followed by the same uppercase letter are not significantly different at the 5% level by LSD. Each species and product were analyzed separately

[b] A susceptible strain of *T. vaporariorum* maintained at CIAT

[c] A susceptible strain of *B. tabaci* biotype B maintained at CIAT. .

Table 75. Response[a] of adults of *Encarsia nigricephala* and *Amitus fuscipennis* to different insecticides

Insecticide	No. of individuals tested	CL_{50} (CL 95%)[b]	CL_{90} (CL 95%)	χ^2	b ± EEM	$P > \chi^2$
E. nigricephala						
methamidophos	400	0.67 (0.440 – 0.900)	4.04 (2.910-6.800)	1.72	1.64 ± 0.24	0.19 ns[c]
Methomyl	400	0.00915 (0.004 – 0.015)	0.062 (0.044 – 0.110)	0.56	1.54 ± 0.30	0.45 ns
carbosulfan	400	0.09 (0.060 – 0.120)	0.38 (0.280 – 0.670)	0.22	2.04 ± 0.38	0.64 ns
cypermethrin	400	0.65 (0.040 – 1.880)	11.09 (5.680 – 21.65)	0.57	1.04 ± 0.26	0.45 ns
A. fuscipennis						
Bifenthrin	400	0.023 (0.005 – 0.427)	0.171 (0.118 – 0.276)	1.00	1.47 ± 0.33	0.31 ns
Carbofuran	400	0.074 (0.050 – 0.097)	0.380 (0.286 – 0.576)	0.70	1.80 ± 0.24	0.40 ns

[a] Values of CL_{50} y CL_{90} in µg of active ingredient/ vial
[b] Confidence limits at 95%
[c] ns, not significant at the 5% level.

Table 76. Comparative responses of the whitefly *Trialeurodes vaporariorum* and its parasitoids *Encarsia nigricephala* and *Amitus fuscipennis* to different insecticides

Insecticide	CL_{50} *T. vaporariorum*	CL_{50} parasitoid	Response ratio
	E. nigricephala		
methamidophos	5.30[a]	0.670	7.91
methomyl	0.25[a]	0.009	27.77
carbosulfan	1.80[b]	0.090	20.00
cypermethrin	37.0[a]	0.650	56.92
	A. fuscipennis		
bifenthrin	2.40[b]	0.023	104.35
carbofuran	1.97[a]	0.074	26.62

[a] As determined by Cardona et al. (2001)
[b] As determined by Rodríguez et al. (2003).

Progress towards achieving output milestones:

Characterizing and monitoring pathogen diversity / stable strategies for managing pathogens developed.

- The varieties Widusa, Kaboon and G 2333 continue to be effective against all characterized races of *C. lindemuthianum* in Colombia.

- A transformation system for *P. griseola* was developed. This is an important tool in the elucidation of host-pathogen interaction and in pathogenicity gene isolation.

- The nature of immunity of G 10474 was elucidated. This information can be used to devise new ALS management strategies.

- *Pythium* isolates (134) from root rot affected areas in Kenya and Rwanda were characterized by sequencing of ITS-1 region. *P. ultimum* was the most frequent occurring species followed by *P. torulosum*.

- *Pythium* distribution maps showing relative importance of characterized species in Kenya and Rwanda were developed.

- White fly monitoring in the Cauca valley of Colombia demonstrates that *Bemisia tabaci* biotype B has displaced biotype A in most areas in a lapse of seven years.

- Natural enemies of *Bemisia* tend to be much more susceptible than *Bemisia* to common insecticides, although a directed effort to select resistant natural enemies might improve their ability to survive.

Activity 3.2 Characterizing disease and insect resistance genes

Highlights:

- At least two resistance genes condition resistance of G 19833 to four races of *C. lindemuthianum*. The resistance genes in G 19833 are distinct from those in the Andean genotypes Michigan dark red kidney, Kaboon and Perry Marrow, and might be a new Andean resistance locus.
- Two AFLP markers linked to angular leaf spot resistance in Mexico 54 and G 10474 were successfully converted to STS markers and protocols for their use in MAS were developed. Similarly, the RAPD marker $OPE4_{709}$ linked to ALS resistance gene in Mexico 54 was converted to a SCAR marker and a protocol was developed.
- Two AFLP-derived SCAR markers (PF9 and PF11) were developed for G 10474 and Mexico 54.
- The allelic relationship of ALS resistance genes in Mexico 54 with G 10474, G 10909, G 10613, and Cornell 49242 were elucidated.
- Inheritance of resistance to *Pythium* root rot in five resistant genotypes was shown to be conditioned by single dominant genes. Allelism test using the diallel mating scheme revealed that the resistance is conditioned by the same resistance locus.
- Further progress was made on developing a SCAR marker for resistance to *Apion godmani*. A total of seven RAPD bands were cloned and five single copy markers developed which mapped to the same locations as the original RAPDs. In addition a peroxidase fragment was cloned from one of the chromosomal regions with the most consistent Apion resistance gene. These markers provide potential assays for use in marker assisted selection.
- Microsatellite and SCAR markers were evaluated for their utility in populations segregating for geminivirus resistance in a collaborative project with the University of Puerto Rico.

3.2.1 Nature and inheritance of angular leaf spot resistance in G 9836 and G 10613

Rationale: Elucidating the nature and inheritance of resistance to angular leaf spot of common bean is one of the activities that has gained precedence in the bean program, because angular leaf spot has become one of the most important diseases in all bean-producing countries in the tropics. To effectively exploit the diversity in bean genes to combine and pyramid useful genes, sufficient characterization of the genetics of resistance is necessary. This is also a prerequisite to tagging these genes and developing an efficient marker assisted selection program. The ultimate objective of this study is to identify diverse sources of ALS resistance and to develop molecular markers that can be used to aid the transfer of ALS resistance to well-adapted market class type bean.

Materials and Methods: This year, we studied the nature of ALS inheritance in two genotypes, G 9836 and G 10613. These genotypes are currently being used in our breeding program. Populations (F_1, F_2, and F_1 backcrosses to resistant and susceptible parents) were made using the variety Sprite as the susceptible parents. Populations were developed as reported previously (CIAT 2003). Greenhouse disease evaluations, and data analysis were done as described

previously (CIAT 2003). Evaluations for disease severity were assessed using a CIAT 1 – 9 scale, where 1 represents no visible symptoms and 9 = severe symptoms and disease expression. Ratings of 1 to 3 were considered resistant and ratings > 4 as susceptible. Area under disease progress curves was calculated to assign genotypes to resistance and susceptibility classes. Several different genetic hypotheses were tested for each population using a Chi-squared test in the SAS program.

Results and Discussions:

G 9836: The observed segregation ratios from F_1, F_2, and back cross to resistant (BC1-R) and susceptible (BC1-S) parents revealed a tendency towards recessive expression of the genes conditioning resistance to *P. griseola* in G 9836 (Table 77). The majority of the F_1 plants and almost all backcross plants to the susceptible parent were infected, revealing that resistance is recessive. It is possible that resistance of G 9836 to race 63-63 is conditioned by a single recessive.

G 10613: All F_1 plants were resistant to pathotype 63-63, revealing a tendency towards a dominant gene conditioning resistance of G 10613 to Pg pathotype 63-63. A 3:1 segregation ratio in the F_2 population revealed that a single dominant gene conditioned ALS resistance of G 10613 to pathotype 63-63 (Table 77).

Table 77: Nature and inheritance of angular leaf spot resistance in some differential varieties and selected resistant sources.

Source	Generation	Observed (R:S)	Expected	X^2	P
G 9836 x Sprite	F_2	44: 110	1: 3	1.04	0.31
G 9836 x Sprite	F_1	14:26			
G 9836 x Sprite	BCS	1:39	0:1	0.93	0.34
G 10613 x Sprite	F_2	137:58	3:1	2.34	0.13
G 10613 x Sprite	F_1	24:0	1:0	0	1.0
G 10613 x Sprite	BCS	13:7	1:1	1.8	0.19

Conclusion: These results make it difficult to conclude with certainty the nature of inheritance in these two genotypes, especially in G 9836. That said, it is probable that a recessive gene(s) conditions ALS resistance in G 9836 and that in G 10613 is conditioned by a dominant gene(s). The segregation ratios observed could reflect two genes that are linked. More studies are needed before firm conclusions about the nature of inheritance in these genotypes can be drawn.

References:

CIAT 2003. Annual Report, Bean Program 2003. CIAT, Cali, Colombia.

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

3.2.2 Allelism test for angular leaf spot resistance genes from several potential sources

Rationale: Several source of ALS resistance have been identified. Last year, we elucidated the nature of ALS resistance in some of these sources. It is important to establish the allelic relation ships between these genes, and avoid using the same resistance gene that might be found in different genotypes. This study was carried out to test the independence of the resistance gene(s) in these potential sources of resistance.

Materials and Methods: Forty-five crosses were made between the different combinations of sources of resistance and the plants advanced to F_2 (Table 78). Because of the quantity of the populations involved, lack of greenhouse space, and the need to rapidly get information, we decided to first evaluate 100 individual plants from each cross, and in cases where a conclusive result was not obtained, to increase the number of F_2 plants with another 100 plants, until a conclusive result is obtained. During the course of this year, populations for 6 populations have been evaluated. Plant establishment, inoculum production, inoculations and disease evaluations were as described previously (CIAT 2003).

Table 78. Crosses for to test the independence of angular leaf spot resistance genes

	MEX 54	Cornell	MAR 1	MAR 2	AND 277	G 10613	G 10474	G 10909	G 4691
G 5686	X	X	X	X	X	X	X	X	X
MEX 54	-	X	X	**X**	X	X	X	X	X
Cornell		-	X	X	X	X	X	X	X
MAR 1			-	X	X	X	X	X	X
MAR 2				-	X	X	X	X	X
AND 277					-	X	X	X	X
G 10613						-	X	X	X
G 10474							-	X	X
G10909								-	X

Results and Discussion: Pathotypes that had an R x R reaction with the two genotypes involved in the cross were used for evaluations (Table 79). These results reveal that Mexico 54 carries different ALS resistance gene (s) from the one in G 10909 and probably Cornell 49242. A lack of segregation in Mexico 54 x G 10613 revealed that the gene conditioning resistance to pathotype 31-39 occupies the same locus in both genotypes. Allelism studies by Caixeta et al. (2002) using pathotype 31-55, revealed that Mexico 54 carried three ALS resistance genes *Phg-2*, *Phg-5*, and *Phg-6*. It is possible that one of these genes is the same as the one in G 10613. The segregation ratios observed in the G 10474 x Mexico 54 cross suggest the presence of as many as three resistance genes but there is a need to carry out progeny testing to confirm segregation. Inheritance studies have revealed that G 10474 carries a single dominant gene while Mexico 54 has three resistance genes, and one of them is recessive (Mahuku et al., 2003). In the cross MAR 1 x G 10474, no segregation was observed, revealing that the resistance genes in these two genotypes occupy the same loci. However, given the resistance spectrum displayed by these two genotypes, it appears that G 10474 contains a different allele that that found in MAR 1. Looking at the pedigree of MAR 1, the resistance source is derived from Cornell 49242. Allelism test involving G 10474 x Cornell 49242, once done, will confirm this conclusion. The segregation ratio of the G 10474 x G 10613 (63R: 1S) also suggest three independent genes segregating, but

this is pending progeny testing. A similar result was obtained in the Mexico 54 x G 10474 population, while Mexico 54 x G 10613 showed no segregation.

Table 79. Test for the independence of the resistance genes in F_2 populations derived from crossing different sources of angular leaf spot resistance

Cross	Race	Observed (R:S)	Expected	X^2	P	Conclusion
G10613*G10474	15-59	118:2	63:1	0.008	0.93	Three genes segregating
MAR1*G10474	7-7	117:0	1:0	0.000	1.00	A gene occupying the same loci
MEX54*CORNELL 49292	31-7	102:10	57:7	0.460	0.50	3 genes, 2 dominant, 1 recessive
MEX54*G10474	1-7	92:2	63:1	0.195	0.66	Three dominant genes
MEX54*G10613	31-39	109:0	1.0	0.000	1.00	A gene occupying the same loci
MEX54*G10909	7-35	72:21	3:1	0.290	0.59	Two independent genes

Conclusion:

More information regarding the relationship between the different sources of resistance will be obtained when the studies of independence are completed, and with progeny testing of the G 10474 x G 10613 and Mexico 54 x G 10474 crosses. However, it appears that G 10613 shares resistance loci with Mexico 54, while MAR 1 shares a resistance locus with G 10474.

References:

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

3.2.3 Inheritance of anthracnose resistance in the Andean germplasm accession G 19833

Rationale: Previous characterizations of resistance genes in G 19833 (Bean annual report 2003) revealed that this contains dominant and recessive genes for resistance against several Andean and Mesoamerican pathotypes of *C. lindemuthianum*, including 3481, a pathotype that overcomes the genotype G 2333, that contains three resistance genes (*Co-4*^{2}, *Co-5, Co-7*). We report further resistance gene characterization using another widely distributed C. *lindemuthianum* pathotype 65. In addition, this race was selected in order to confirm whether G 19833 carries an allele of the Co-1 locus, the only anthracnose resistance loci described in Andean genotype.

Materials and Methods: 200 F_2 plants from the G 19833 x La Victorie, were inoculated with *C. lindemuthianum* race 65 under greenhouse conditions. Plant establishment and handling, inoculum production, inoculation and disease evaluations were done as described previously (CIAT 2003).

Results and Discussion: Of the 200 F_2 inoculated with pathotype 65, 170 were resistant while 30 were susceptible. Chi-square (X^2) analysis revealed a segregation ratio of 57:7, suggesting that resistance to pathotype 65 is probably a result of three resistance genes, two dominant and one recessive.

Conclusion: These results suggest that G 19833 contains three independent resistance genes. However, we need to test the F_1 and backcross population to resistant and susceptible parents to confirm the presence of three resistant genes. If resistance genes are linked, this would confound conclusions.

References:

CIAT 2003. Annual Report, Bean Program 2003. CIAT, Cali, Colombia.

Contributors: G. Mahuku, C. Jara, J. Fory, G. Castellanos, H. Terán, S. Beebe

3.2.4 Allelism test for anthracnose resistance genes in the germplasm accession G 19833

Rationale: G 19833 has been highly resistant to *C. lindemuthianum* isolates known to infect genotypes that carry the only known anthracnose resistance gene of Andean origin (*Co-1*). Several alleles of this resistance gene have been characterized in Widusa (*Co-1*^{5}), Michigan Dark Red Kidney (*Co-1*); Perry Marrow (*Co-1*^{3}) and Kaboon (*Co-1*^{2}). This study was carried out to test the independence of the resistance gene(s) in G 19833 from the *Co-1* gene and establish if the resistance gene(s) in G 19833 were the same or different from the *Co-1* alleles in the cultivars Michigan Dark Red Kidney, Kaboon, Widusa and Perry Marrow (Melotto et al, 2000). Identifying another resistance loci in the Andean gene pool would be useful to diversify the Andean resistance genes to use in breeding programs and to avoid the dependence on a single multi-allelic gene.

Materials and Methods: Two hundred F_2 plants per population derived from crossing G 19833 x Kaboon, G 19833 x Perry Marrow, G 19833 x Michigan Dark Red Kidney (MDRK) and G 19833 x Widusa were inoculated independently using two *C. lindemuthianum* races (race 65 and 521) (Table 80). All bean varieties used in the crosses are resistant to these two races. Inoculum production, plant handling, inoculations and disease evaluations were done as described previously (CIAT, 2003). A plant with no visible symptoms or with only a few, very small lesions mostly on the primary leaf veins was scored as resistant (rating 1 to 3). A plant with numerous small or enlarged lesions, or with sunken cankers on both the lower sides of leaves and the stems was recorded as susceptible (rating 6.1 to 9). A plant with a rating score of (3.1 – 6) was considered as intermediate.

Table 80. Gene pool, described anthracnose resistance genes and disease reaction of bean cultivars inoculated with different races of *C. lindemuthianum*.

Genotype	Gene Pool	R-gene	Anthracnose race	
			65	521
MDRK	A	*Co-1*	R	R
Perry Marrow	A	*Co-1*^{3}	R	R
Kaboon	A	*Co-1*^{2}	R	R
Widusa	A	*Co-1*^{5}	R	R
G 19833	A	?	R	R

Results and Discussion: Inoculation of the G 19833 x MDRK, Perry Marrow and Kaboon with race 521 revealed that three independent resistance genes were segregating in this population (Table 81). A similar result was observed when the same population was inoculated with race 65 (X^2 = 0.12; P = 0.72) These results reveal that G 19833 carries three resistance genes that condition resistance to *C. lindemuthinanum* races 521 and 65. A similar result was observed in another background (G 19833 x Perry Marrow (*Co-1*^{3})). However, when G 19833 was crossed with Widusa (Co-1^5) and inoculated with race 65, a segregation ratio of 15:1 (P = 0.88) was observed, indicative of two independent resistance genes (Table 81).

Table 81. Test for the independence of the resistance genes in F_2 populations derived from crossing G 19833 with Kaboon, MDRK, Widusa and Perry Marrow

Cross	Race	Obs (R:S)	Esp	X^2	P	Conclusion
G 19833 x MDRK	65	198: 2	63: 1	0.12	0.72	3 dominant and independent genes
G 19833 x MDRK	521	194: 6	61: 3	1.27	0.26	3 genes,2 dominant; 1 recessive
G 19833 x PM	65	195: 5	63: 1	1.14	0.29	3 dominant and independent genes
G 19833 x PM	521	193: 7	61: 3	0.63	0.43	3 genes,2 dominant; 1 recessive
G 19833 x KAB	65	188: 12	15 : 1	0.02	0.88	2 dominant and independent genes
G 19833 x KAB	521	195: 5	63: .1	1.14	0.29	3 dominant and independent genes
G 19833 x Widusa	65	187: 13	15 : 1	0.02	0.88	2 dominant and independent genes

Conclusion: The segregation ratios observed reveal that G 19833 may carry three resistance genes that are different from the gene carried by MDRK (*Co-1*), Perry Marrow (*Co-1*^{3}), Kaboon (*Co-1*^{2}) and Widusa (Co-1^5). There is a need to test F_2 derived families of all crosses, to confirm that susceptible plants are segregating, and to be able to conclusively and confidently describe another Andean locus for anthracnose resistance in the Andean genotype G 19833.

References:

CIAT 2003. Annual Report of the Bean Program 2003. CIAT, Cali, Colombia

Melotto , M., and Kelly, J.D. 2000. An allelic series at the Co-1 locus conditioning resistance to anthracnose in common bean of Andean origin. Euphytica 116: 143-149.

Contributors: G. Mahuku, C. Jara, J. Fory, G. Castellanos, J.B. Cuasquer, H. Teran, S. Beebe

3.2.5 Inheritance of resistance to *Pythium* root rot and allelic relationship of resistance genes among resistant bean genotypes

Rationale: Last year we showed that resistance in three important sources (RWR 719, AND 1062 and MLB-49-89) against *Pythium* root rot was controlled by single dominant genes, which were simply inherited. This information is useful in designing effective strategies for cultivar improvement. Other potential resistant sources include AND 1055, SCAM 80-CM/15, RWR 1092 and MLB-40-89A. Because they consist of small, medium and large seeded materials, their utility is influenced by the genetic background (gene pool) of varieties to be improved. Small (e.g. RWR 719) or large (AND 1062) seeded types are better suited in the improvement of small or large seeded types respectively. The objective of this study was therefore to determine the nature and mode of inheritance of resistance in additional resistant lines (AND 1055 and SCAM 80-CM/15). To efficiently use the resistant genotypes, we also studied their allelic relationships.

Materials and Methods: More breeding populations (F_1, F_2, and F_1 backcrosses to resistant and susceptible parents) were generated for the three *Pythium* root rot resistant lines evaluated last year (RWR 719, AND 1062 and MLB-49-89A) using different susceptible parents (GLP 585, CAL 96 and Urugezi). Resistant cultivars AND 1055 and SCAM 80 –CM/15 were also used to generate breeding populations by crossing them to the four susceptible genotypes. Sixty seeds from each parent, 60 F_1, 300 F_2, 90 from backcross to susceptible parent (BCs) and 90 from backcross to resistant parent (BCr) were evaluated using *P. ultimum* as described under 2.1.1 above. Resistant (R) phenotype was assigned to plants with no or limited symptoms (score 1-3), whereas plants graded 5 or greater were considered to be susceptible (S). The data obtained was tested for goodness of fit to expected theoretical ratios with chi-square test.

Allelic relationships for resistance to *Pythium* root rot was determined in seven resistant genotypes, RWR 719, MLB-49-89A, AND 1062, AND 1055, SCAM 80-CM/15, RWR 1092 and MLB 40-89A. Twenty-one sets of crosses were made between the resistant cultivars to generate F_1 and F_2 populations. Ninety seed from each of the latter were evaluated against *P. ultimum* as already described above.

Results and Discussion: All the five cultivars, RWR 719, AND 1062, MLB-49-89A, AND 1055 and SCAM 80-CM/15 were resistant to *P. ultimum.* In contrast and as expected GLP 2, GLP 585, CAL 96 and Urugezi were susceptible under similar conditions. All F_1 families were resistant. Chi-square values for all resistant-susceptible combinations (Table 82) showed a good fit for segregation ratio of 3:1 (resistant to susceptible) in F_2, 1:1 in all F_1 backcrossed to susceptible cultivars (test cross population), and 1:0 in F_1 backcrossed to the five resistant genotypes. These results confirmed previous observations that resistance in AND 1062, RWR 719 and MLB-49-89A was controlled by a single dominant gene in each cultivar. Similar results were also observed for AND 1055 and SCAM 80-CM/15.

Table 82. Segregation for reaction to *Pythium ultimum* among F_2 plants, and test cross generations derived from crosses between four susceptible and five resistant bean genotypes, Kawanda, Uganda, 2004.

Cross	Number of plants segregating for *Pythium* reaction and X^2 values								
	F_2 [x]			BCs			BCr		
	R	S	X^{2y}	R	S	X^2	R	S	X^2
RWR719 x GLP 585	217	84	1.364^{ns}	41	49	0.722^{ns}	86	4	0.00
RWR 717 x CAL 96	228	71	0.296^{ns}	40	49	0.911^{ns}	88	1	0.00
RWR 719 x Urugezi	218	81	0.647^{ns}	48	42	0.411^{ns}	90	0	0.00
MLB-49-89A x GLP 585	212	87	2.485^{ns}	44	46	0.055^{ns}	81	9	0.00
MLB-49-89A x CAL 96	215	84	1.410^{ns}	42	47	0.322^{ns}	90	0	0.00
MLB-49-89A x Urugezi	223	76	0.021^{ns}	42	47	0.260^{ns}	90	0	0.00
AND 1062 x GLP585	210	81	1.471^{ns}	42	48	0.411^{ns}	88	2	0.00
AND 1062 x CAL 96	206	75	1.680^{ns}	43	46	0.084^{ns}	90	0	0.00
AND 1062 x Urugezi	210	90	3.871^{ns}	48	41	0.543^{ns}	90	0	0.00
AND 1055 x GLP2	216	84	1.364^{ns}	42	47	0.260^{ns}	90	0	0.00
AND 1055 x 585	233	67	1.213^{ns}	45	45	0.011^{ns}	87	3	0.00
AND 1055 x CAL 96	221	78	0.169^{ns}	38	45	1.072^{ns}	88	1	0.00
AND 1055 x Urugezi	218	81	0.770^{ns}	39	51	1.611^{ns}	88	2	0.00
SCAM 80-CM/15 x GLP 2	182	74	8.050^{ns}	53	36	2.986^{ns}	90	0	0.00
SCAM 80-CM/15 x GLP 585	222	77	0.067^{ns}	43	46	0.134^{ns}	90	0	0.00
SCAM 80-CM/15 x CAL 96	214	85	1.882^{ns}	40	49	0.911^{ns}	90	0	0.00
SCAM 80-CM/15 x Urugezi	221	78.	0.188^{ns}	43	46	0.167^{ns}	90	5	0.00

[x] Expected ratio for F_2, BCs and BCr is 3:1, 1:1 and 1:0 respectively.

In allelic studies, all parents were resistant to *P. ultimum.* Similarly, all F_1 families were resistant. There was no resistant–susceptible segregation in F_2 populations. These results imply that the seven cultivars have a common locus with a gene(s) conditioning resistance to *Pythium* root rot and inherited as a dominant character.

Contributors: R. Otsyula (graduate student), R. Buruchara, S. Sebuliba, P. Kimani

Collaborators: G. Mahuku, M. Blair.

3.2.6 Identifying and developing molecular markers linked to ALS resistance genes

Rationale: The emphasis in 2003 was to test and validate AFLP-derived markers that were identified in 2002 for G 10474, G 10909, Mexico 54 and MAR 1, and where possible, develop protocols for their use in marker assisted selection breeding programs. Our approach has been to first convert polymorphic AFLP markers to SCARs, then evaluating the potential SCAR markers on the entire F_2 segregating populations, so as to have markers that are useful to our NARS partners, as well as reducing the cost of marker development. This year, we concentrated on further refining these markers; identifying new markers and validating identified markers to establish their utility for MAS.

Materials and Methods:

$OPE4_{700}$ marker: Previous studies identified a RAPD marker $OPE4_{709}$ that is tightly linked (in coupling) to the resistance gene in Mexico 54 (0 cM). For easy of use of this marker, we cloned and sequenced the 700bp fragment and designed longer primers for specificity. These primers were tested in the entire F_2 population and used to amplify several parents that are routinely used in our breeding program (G 10474, G 10613, MAR 1, VAX 6 and G 10909), and genotypes belonging to the angular leaf spot differential series. For eventual mapping of this marker, both the $OPE4_{709}$ SCAR marker and RAPD primer were used to amplify the DOR 364 x G 19833 RIL population.

SCAR PF 11: An AFLP-derived SCAR marker was identified for Mexico 54. Subsequent tests and validation on the parents and respective bulks revealed that it was co-dominant. The SCAR was tested in 166 F_2 plants, differential varieties and breeding parents.

SCAR PF9 260 G1: The fragment of an AFLP marker linked in coupling with the resistance gene in G 10474 (E-AAC/M-CAT) that was previously identified (CIAT 2003), was cloned, sequenced and specific primers developed. The marker was used to amplify parental DNA, and DNA extracted from a bulk of 10 resistant and 10 susceptible individuals. Once polymorphism was confirmed, the marker were tested on 10 resistant and 10 susceptible individuals, before being evaluated on 246 F_2 individuals obtained from a G 10474 x Sprite cross, (111 inoculated with pathotype 7-35 and 135 inoculated with pathotype 63-63). The distance of the marker from the resistance gene was estimated using the program MAPMAKER. Validation of the SCAR marker outside the mapping population was done by amplifying DNA from genotypes representing the ALS differential series (constituting of 6 Mesoamerican genotypes and 6 Andean genotypes) and 14 additional genotypes that are currently being used as parents for the improvement of ALS resistance.

Results and Discussion: The SCAR-$OPE4_{700}$ was dominant and linked in coupling to the resistance gene in Mexico 54 (Figure 58). The marker was present in some susceptible F_2 plants and in all resistant F_2 plants (Figure 58). This is not surprising, as previous inheritance studies revealed that a single recessive gene conditioned resistance of Mexico 54 to race 31-55 of *P. griseola* (Mahuku et al., 2003). The marker was not present in any differential variety except Mexico 54 (Figure 59B).

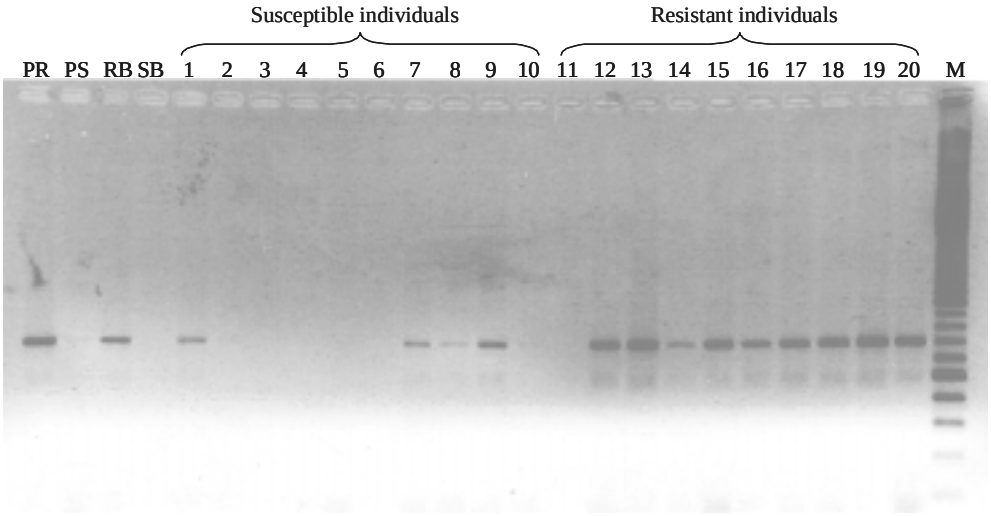

Figure 58. PCR amplification of F_2 individuals of a Mexico 54 x Sprite cross using the $OPE4_{709}$ SCAR marker. PR is resistant parent Mexico 54, PS is susceptible parent Sprite, RB is resistant bulk, SB is susceptible bulk and M is the 100 bp molecular size marker.

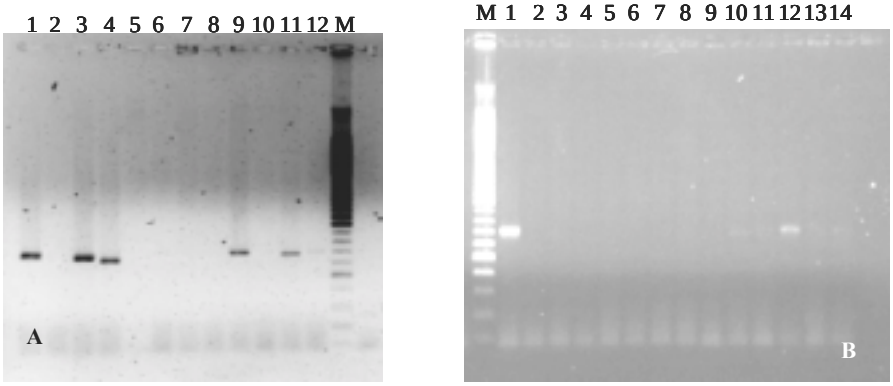

Figure 59. PCR amplification of sources of angular leaf spot resistance and parents used in improvement of ALS resistance in bean using the $OPE4_{709}$ SCAR marker. (A) From left to right, lanes 1–12 are Mex 54, Sprite, G10474, G10909, Mar 2, AND 277, AND 279, G 9603, G 4032, G 3991, G 5653, Mar 3. Lane M is the molecular size marker. (B) Mex54, Sprite, Timoteo, G11796, Bolon Bayo, Montcalm, Amendoim, G5686, PAN72, G5828, Flor de Mayo, Mex 54, BAT332, Cornell]

Amplification of other sources of ALS resistance including genotypes normally used in our breeding program, revealed the band in G 10909, G 10474, G 4032, G 5653 and MAR 2 (Figure 59A), but the fragment was smaller than the one in Mexico 54, revealing that this marker can be used in crosses involving Mexico 54 and these genotypes. Similar results were observed for MAR 1 and DOR 364.

On the cross of G 10909 and Sprite, the SCAR marker was co-dominant (Figure 60) and could distinguish between homozygous and heterozygous individuals. This marker is currently being evaluated on the entire G 10909 x Sprite mapping population to ascertain the distance of the marker from the resistance gene. However, results from this study reveal that this is another useful marker when G 10909 is used as a parent in ALS improvement.

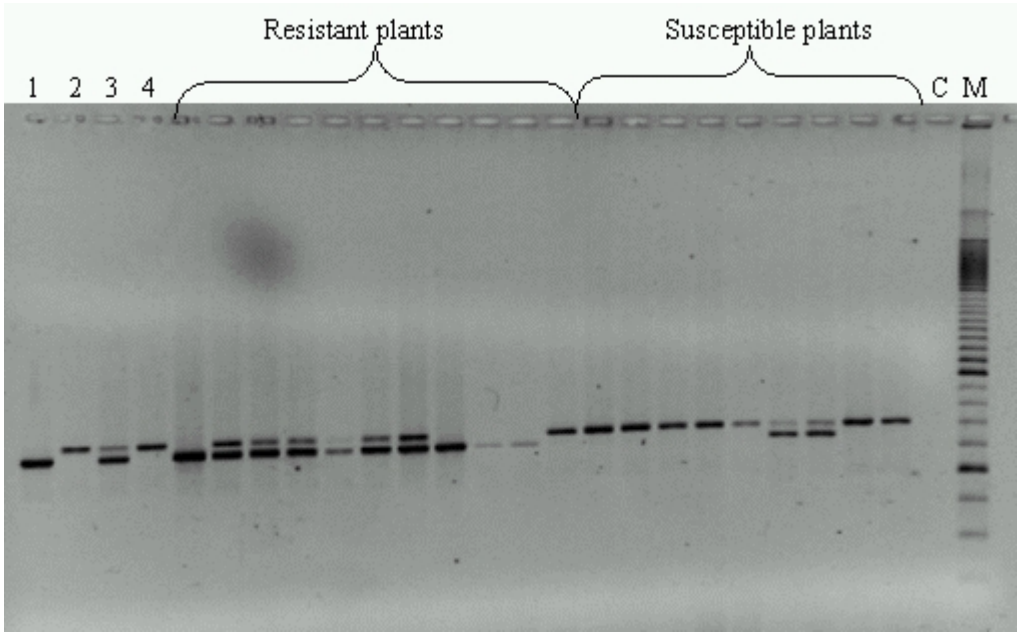

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Figure 60. PCR amplification of F_2 individuals of a G 10909 x Sprite cross using the $OPE4_{709}$ SCAR marker. Lane 1 is G 10909, Lane 2 is Sprite, Lane 3 Resistant bulk and Lane 4 is susceptible bulk., C is negative control and M is 100 bp molecular size marker.

SCAR PF 11: An AFLP derived SCAR marker (PF 11) was identified linked in coupling to the resistance gene in Mexico 54. When evaluated on F_2 segregating population, the marker was co-dominant (Figure 61). This marker is currently being evaluated in the entire F_2 mapping population, and outside the mapping population to establish its suitability for MAS.

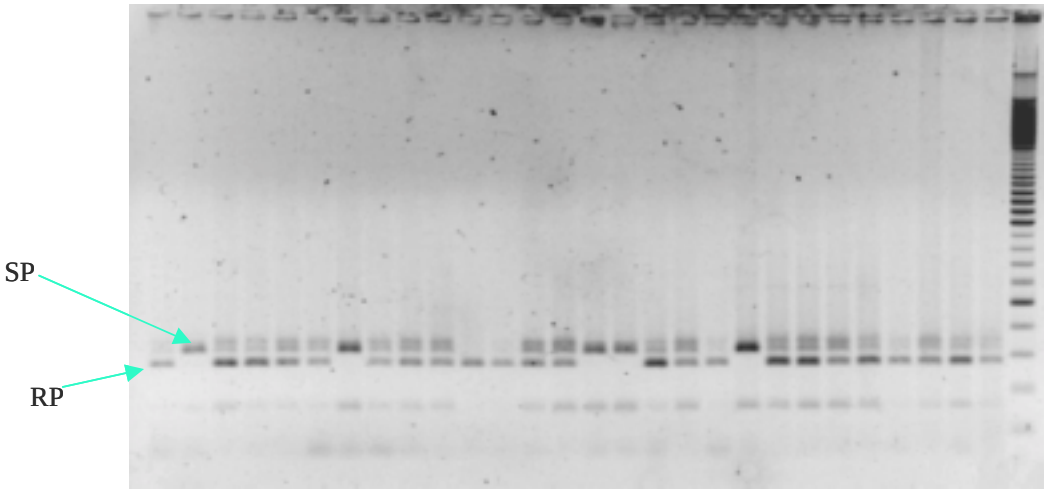

Figure 61. PCR amplification of F_2 plants from a Mexico 54 x Sprite cross using the AFLP-derived SCAR marker PF11. RP is resistant parent, Mexico 54, SP is susceptible parent, Sprite. The rest are individual F_2 plants that are either homozygous susceptible, homozygous resistant or heterozygous resistant.

SCAR PF9 260 G1: A second co-dominant SCAR marker was developed for G 10474. Analysis showed that this marker is 3.0 cM from the resistance gene. Amplification of susceptible and resistant individuals and parents produced a 280 bp band in the resistant parent and individuals and a 300 bp fragment in the susceptible parent and individuals (Figure 62). Evaluation of the differential varieties showed that this marker was present in some Andean genotypes and absent from others (Figure 62). However, it was largely absent from Mesoamerican differential genotypes, showing that this marker can be used to introgress the G 10474 gene into Mesoamerican genotypes. However, a parental survey should first be done to verify the utility of this marker in the parents that are being used before it is deployed in MAS involving G 10474. We are in the process of developing a protocol for the use of this marker in MAS.

Conclusion: A RAPD marker OPE4700, linked in coupling with the resistance gene in Mexico 54 was successfully converted to a SCAR marker. Validation of this marker showed that it was present in some genotypes, albeit with a different fragment size, revealing that it can be used to introgress this gene into Andean and Mesoamerican genotypes. The SCAR was polymorphic in the G 10909 mapping population, being co-dominant. Studies are underway to determine the location of the marker relative to the resistance genes are in progress. Another marker, PF11 was identified for Mexico 54 and successfully converted to a co-dominant SCAR. A third marker (PF9) located at 3.0 cM from the resistance gene in G 10474 was identified. The protocol for the use of this marker is currently being developed.

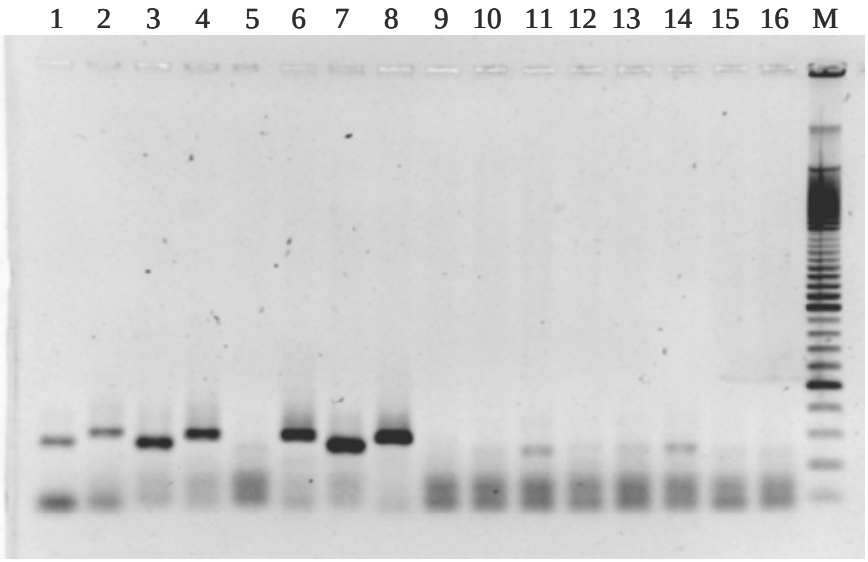

Figure 62. Amplification of the angular leaf spot differential genotypes using the AFLP-derived SCAR marker PF9 that is linked to the resistance gene in G 10474. Lanes 1-16 are G10474, Sprite, Bulk R, Bulk S Timoteo, G11796, Bolon Bayo, Montcalm, Amendoim, G5686, PAN72, G5828, Flor de Mayo, Mex 54, BAT332, Cornell.

References:

CIAT 2003. Annual Report, Bean Program 2003. CIAT, Cali, Colombia.

Mahuku, G., Jara, C., Cajiao, C., and Beebe, S. 2003. Sources of angular leaf spot (*Phaeoisariosis griseola*) in common bean core colletion, wild Phaseolus vulgaris and secondary gene pool. Euphytica 130: 303-313

Contributors: M.A. Henriquez, C. Montoya, G. Mahuku, H. Terán, S. Beebe

3.2.7 Development of SCAR markers for Apion resistance

Rationale: The bean pod weevil (*Apion goodmani* Wagner) is a destructive insect pest that damages beans grown in Mexico and Central America. Resistance is controlled by two possible mechanisms – either antibiosis involving a hypersensitive response that encapsulates the oviposition site – or antixenosis that affects the preference of oviposition sites. Epistasis between two independent genes, *Agr* and *Agm*, has been suggested to control the hypersensitive response. The fact that a few genes control resistance may explain why it has been relatively easy to transfer resistance from Mexican landraces where it is found to new breeding lines with Central American grain types. The objective of this research was to create additional SCAR markers linked to the genes controlling resistance in the recombinant inbred line (RIL) population derived from the cross Jamapa x J117.

Methodology:

Plant material: Plant material consisted in susceptible and resistant bulks (4 lines each) selected from 104 F_5-derived recombinant inbred lines (RILs) of the cross Jamapa x J117. Jamapa is a susceptible cultivar released in Mexico and J117 is a resistant landrace. The population has been evaluated in Mexico for Apion resistance over five seasons.

RAPD cloning: RAPD bands were purified from 1% low melting point agarose gels using Wizard PCR prep purification system (Promega). The purified insert DNA was cloned into the PGEM-T easy vector system for further analysis. Several recombinant clones were picked per ligation reaction, checked with *Eco*RI digestions, and their inserts sequenced using standard techniques, T7 and Sp6 primers, Big Dye sequencing kits and an ABI377 DNA sequencer.

SCAR primer design and CAPs assays: Specific primers were designed for each unique cloned RAPD sequence using Primer 3.0 software and these were tested for their ability to amplify SCAR products. Any monomorphic SCAR products were digested with frequent cutting restriction enzymes (*Alu*I, *Cfo*I, *Hae*III, *Hsp*92II, *Mbo*I, *Rsa*I and *Sau*3AI) to convert the markers into CAPS (Cleaved Amplified Polymorphisms).

Results and Discussion:

A total of seven RAPD bands, $U1_{1400}R$, $F10_{500}S$ (on linkage group b01), $M12_{800}S$ (b07), $C1_{800}S$ (b08), $B1_{1400}R$, $R20_{1200}CS$ (b11) and $W6_{800}R$ (unlinked), that were polymorphic from the parental survey and which were significantly associated with the resistance phenotype were selected for cloning. This brings to a total of nine the bands that have been targeted including $W9_{1300}S$ and $Z4_{800}R$ which were converted to SCARs last year. BLAST searches identified homologies for several of the nine cloned RAPD bands (Table 83). Several clones had homologies to retrotransposons from a range of dicotolydenous species. This is a common feature of cloned RAPD bands. Two of the clones were similar to gene sequences from soybean: 1) $Z4_{800}R$ with similarity to an unknown gene and 2) $F10_{500}S$ with similarity to a seed coat peroxidase.

Table 83. Development of SCAR and CAPS markers from RAPD bands that were significantly associated with *Apion godmani* resistance in common bean.

RAPD band	Linkage group	Aprox. Size	Blastx results	No. of primer sets	SCAR polym	CAPS polym.
$W9_{1300}S$	b01	1300	Retrotransposon	1	-	+ AluI
$Z4_{800}$	b01	800	Unknown gene Glycine max	1	-	-
$U1_{1400}R$	b01	1400	NS	1	-	-
$F10_{500}S$	b01	500	Peroxidase Glycine max	4	-	-
$M12_{800}S$	b07	800	NS	1	-	-
$C1_{800}S$	b08	800	NS	1	-	+ RsaI
$B1_{1400}R$	b11	1400	Retrotransposon Cicer	2	-	+ TaqI
$R20_{1200}CS$	b11	1200	Retrotransposon Arabidopsis	2	-	+ AluI,MboI
$W6_{800}R$	unlinked	800	NS	2	+	Na

SCAR and CAPS development is also summarized in Table 83. A total of 15 primer sets were designed for the nine RAPD band sequences and these were tested on the population parents and on the bulks. Most of the PCR products of these primer sets, except those derived from $W6_{800}R$, were monomorphic as SCARs. A single SCAR ($W6_{800}R$) showed a polymorphic fragment with clear positive and negative signals in PCR amplification (Figure 63). All monomorphic SCARs were tested with frequently-cutting restriction enzymes (all with 4 bp recognition sites). CAPs polymorphisms were revealed for four of the PCR fragments ($W9_{1300}S$, $C1_{800}S$, $B1_{1400}R$ and R2012CS) when digested with different restriction enzymes, two of the fragments being polymorphic with *Alu*I digestion while one each was polymorphic with *Rsa*I or *Taq*I digestion. All the polymorphic SCAR and CAPS markers genetically mapped to the same locations as the original RAPD bands from which they were derived.

Unfortunately we were not able to make a polymorphic marker for the peroxidase gene that we found in the cloning process ($F10_{500}S$). The peroxidase represents an interesting candidate gene for providing insect resistance because peroxidases have been involved in hypersensitive response and have been shown to be up-regulated by wounding. Given that *Apion godmani* is a pod borer whose main site of action is at developing seed within the pod placenta and that beans resist the pod borer through a modified hypersensitive response, the peroxidase may be one of the mechanisms of resistance to this pest. Therefore, we will continue to pursue this mechanism of resistance and try to genetically map the peroxidase gene. Among other results worth highlighting is the potential of the new markers to dissect the inheritance of resistance and to help with marker assisted selection.

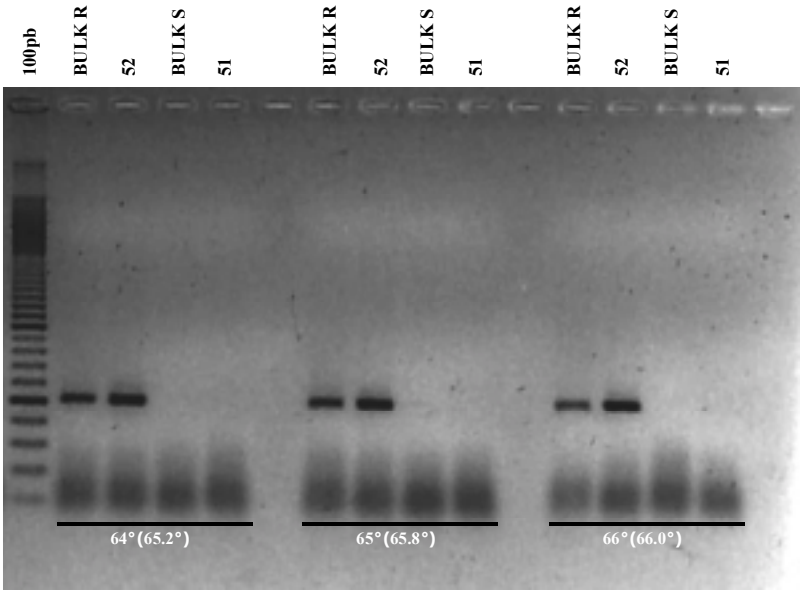

Figure 63. Dominant SCAR for resistance based on the W68C RAPD showing amplification in the resistant parent no. 52 and in the resistant bulk at a range of annealing temp. gradient = 55° -66°C.

Future Plans:

- The peroxidase gene markers will be converted into polymorphic SNP markers so as to genetically map this gene in the bean genome.
- QTL analysis will be carried out when phenotypic data is available for the entire set of recombinant inbred lines which is expected for 2005.
- We will test the ability of the markers to be used in marker assisted selection.

Contributors: MW Blair, C Muñoz, HF Buendia, (SB-2), C. Cardona (IP-1)
R. Garza (INIFAP-Sta. Lucia de Prias, Texcoco, Mexico).

3.2.8 Evaluation of geminivirus resistance sources for SCAR and microsatellite polymorphisms

Background: Bean golden yellow mosaic virus (BGYMV) is an important disease of tropical lowland bean production areas caused by a member of the Geminiviridae family. Symptoms include intense yellowing, pod deformation, stunting and flower abortion that cause important and often devastating yield losses. BGYMV is transmitted by the sweet-potato white fly (*Bemisia tabaci*) a widespread and cosmopolitan pest that is often found on a wide range of horticultural crops as well as tobacco, soybean and common beans. The disease is endemic in Central America and the Caribbean. Breeding for resistance to the virus has been the most effective strategy for controlling the disease since pest control is expensive and impractical given the rapid lifecycle of the insect and the repeated development of insecticide resistance. A few resistance genes have been identified in common beans (*P. vulgaris*) and these function by attenuating symptoms and yield losses when the plant is infected. In the case of a few related species (*P. acutifolius* and *P. coccineus*) there appears to be immunity. Most of these resistance genes remain under-utilized or have only been bred into a limited pool of advanced breeding materials or cultivars. For example several BGYMV resistance genes originated in the Mesoamerican genepool and these are only now beginning to be transferred to the Andean gene pool. Given this, there is an important role for marker assisted selection to play in encouraging the use and effecting the transfer of BGYMV resistance genes. Pyramiding of resistance genes is important for the greatest protection from the disease's symptoms. Our objectives for this study was to screen all the sources of geminivirus resistance for polymorphic microsatellites from the linkage groups which are thought to be involved in resistance and to evaluate all the SCARs developed for geminivirus resistance against this same panel of genotypes.

Methodology

Plant material and DNA extraction: A panel of genotypes was constructed with all the known sources of resistance to BGYMV (Table 84). A total of 16 genotypes were included and represent the parents of six populations that have been used to determine the genetic control of resistance (two at CIAT and four elsewhere). A miniprep DNA extraction technique was used.

Marker genotyping: Microsatellites were selected from linkage groups b04, b06, b07, b08, and b10 based on central CIAT map for the DOR364 x G19833 population and previous reports that suggested that these regions of the genome were important in virus resistance in common beans. The second set of markers consisted of SCARs related with virus resistance developed by other authors, including SR2 (CIAT, unpublished), SW12 (Miklas et al., 2000) and SAS 8 (Larsen et al., 2004). Microsatellites were run on 4% PAGE gels while SCARs were run on 1.5% agarose gels.

Table 84. Evaluation of SCAR markers on the geminivirus parental survey.

No.	Genotype	Gene pool[1]	BGYMV reaction	SAS8	SCAR[4] SR 2		SW 12
					530 (R)	570 (S)	
1	Morales	Meso	S	+	+	-	-
2	G35172	*P.coccineus*	R	-	-	+	-
3	Arroyo loro	Meso	S	+	-	+	+
4	X 015741	Meso	S	-	-	+	-
5	Bulk R1	--	R	+	-	+	+
6	Bulk S1	--	S	+	-	+	+
7	A55	Meso	S	+	-	+	NA[3]
8	G122[2]	Andean	--	-	-	+	+
9	Montcalm[2]	Andean	--	+	-	+	+
10	DOR476	Meso	R	+	+	-	+
11	SEL 1309	Meso	S	-	-	+	+
12	Bulk R2	--	R	-	+	-	+
13	Bulk S2	--	S	-	-	+	+
14	G19833	Andean	S	-	-	+	-
15	DOR364	Meso	R	+	-	+	+
16	XAN 176	Meso	S	+	-	+	-
17	DOR 303	Andean	S	+	-	+	+
18	IJR	Andean	S	-	-	+	-
19	Raven[4]	Meso	S	+	-	+	+
20	I 9365-31	Meso	--	-	-	+	-

[1] Meso = Mesoamerican, R: Resistant, S: Susceptible
[2]BCTV-resistant (G122) and partially resistant (Montcalm) checks
[3]new allele observed
[4]BCMV resistant check

Results and Discussion:

Among the SCAR markers, SR2, which is linked to the *bgm-1*gene, was co-dominant as expected, showing a resistant associated band (530 bp) and a susceptible associated band (570 bp) (Figure 64). The genotypes Morales and DOR476 had the resistant associated band, as did the BGYMV resistant bulk R2. All the other genotypes and bulks had the susceptible associated band for this marker. Since the *bgm-1* gene is known to have originated in a specific Durango landrace it is only found in genotypes derived from this original source through many of the CIAT breeding lines that were bred to contain this resistance gene which provided the best levels of control for severe yellowing symptom caused by BGYMV.

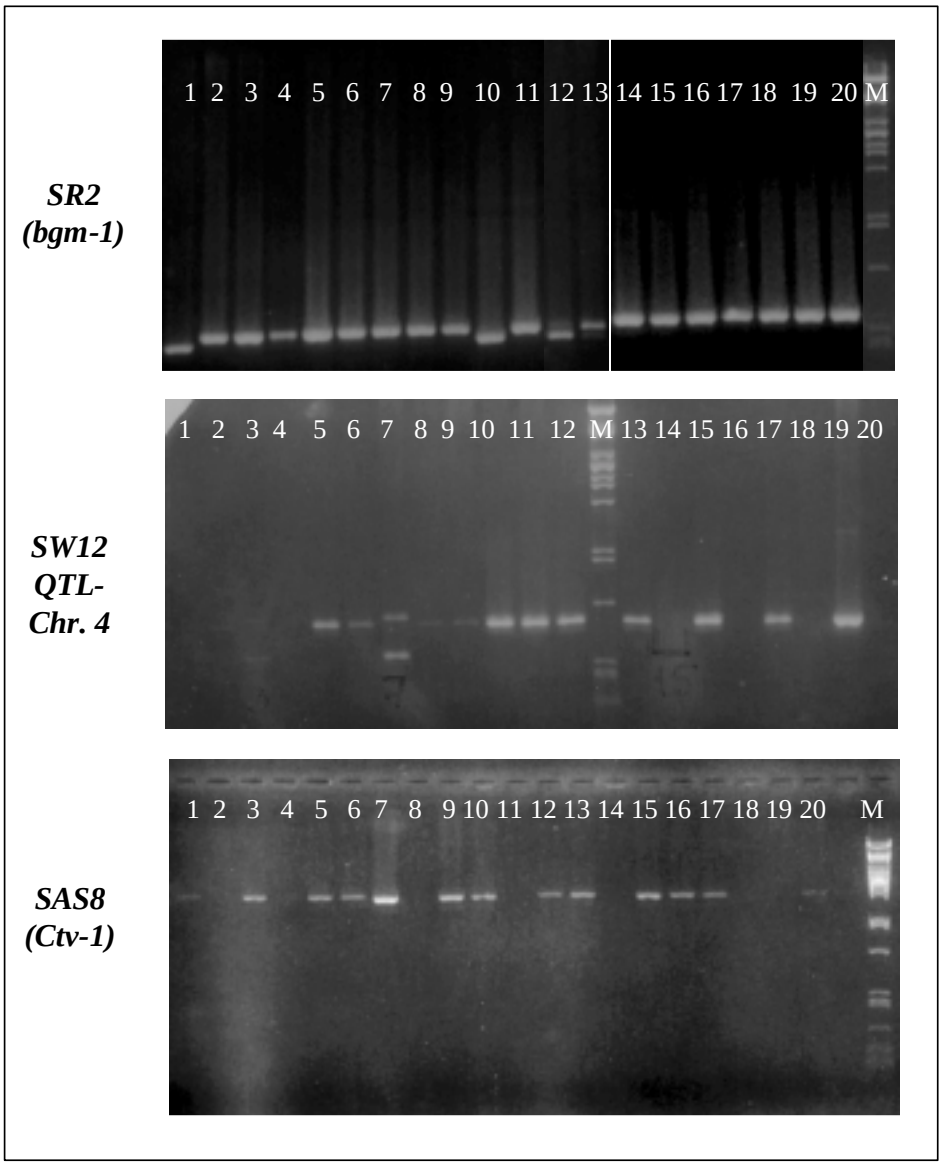

Figure 64. Parental survey of geminivirus resistance sources.

The SW12 marker, linked to a QTL for BGYMV resistance on linkage group b04 was dominant and present in the genotypes Arroyo Loro, DOR303, DOR364, DOR476 and SEL1309 and Raven most of which are genotypes that have been bred for BGYMV resistance (Figure 64). The same band was also present in both the resistant and susceptible bulks R1, S1, R2 and S2 (data not shown). Light bands were amplified for G122 and Montcalm, two Andean genotypes that have not been improved for BGYMV and would not be expected to have the band. A new banding pattern (allele) with two bands, one higher than the expected band and one lower than the expected band, was observed in A55.

The SAS8 marker developed for the *Ctv*-1 gene for resistance to beet curly top virus was dominant and was present in Morales, Arroyo Loro, A55, Montcalm, DOR476, DOR 364, XAN176, DOR303 and Raven. This marker was reported by Larsen et al. (2003) to amplify a band in all Mesoamerican genotypes and to not amplify a band for all Andeans. This seems to be the case in our survey where most of the positive genotypes are typically Mesoamerican and where the two positive Andean genotypes, Montcalm (S Phaseolin) and DOR303 (Pedigree A25 x Redkloud), are the result of Andean x Mesoamerican crosses.

In the microsatellite survey, the parental combinations varied in their level of polymorphism (Table 85). As expected the Andean x Mesoamerican cross DOR364 x G19833 was the most polymorphic. The cross DOR303 x IJR was almost as polymorphic as DOR476 x SEL1309, while the population Arroyo Loro x X0157-4 (G35172) had the lowest polymorphism rate. The number of microsatellite per linkage group varied from 5 to 12 and the relative polymorphism in different parts of the genome varied as well.

Table 85. Number of polymorphic microsatellite markers in each linkage group for each population and overall percentage population polymorphism.

Population	**Number of polymorphic markers**					**% polymorphism on each population**
Linkage Group	**b04**	**b06**	**b07**	**b08**	**b10**	
No. of markers tested	**n = 11**	**n =12**	**n=9**	**n = 5**	**n = 5**	
Arroyo Loro x X0157-4 (G35172)	2	2	2	2	1	23
DOR 476 x SEL 1309	2	7	7	2	2	49
DOR 364 x G19833	8	9	8	5	5	83
DOR 303 x IJR	4	4	5	2	2	41

Future work:

- Based on parental polymorphism and bulked segregant analysis, we will select microsatellite markers for mapping on the populations represented by this survey.
- QTL analysis on the selected populations.

Collaborators: MW Blair, LM Rodriguez, HF Buendia (SB2-CIAT), J. Beaver (UPR).

Progress towards achieving milestones:

Strategy developed for stable angular leaf spot resistance / markers for marker assisted selection developed for various biotic constraints

- Angular leaf spot resistance genes were identified and characterized in two genotypes that are potential sources of ALS resistance.

- The germplasm accession, G 19833 was identified as having 2 or 3 resistance genes for anthracnose. One of these genes appears to be an allele of the *Co-1* locus, while the other appears novel.

- Two AFLP markers for G 10474 and Mexico 54, and the RAPD marker $OPE4_{709}$ were successfully converted to SCAR markers and protocols for their use in MAS were developed.

- Nature of resistance to *Pythium* root rot and its inheritance in five major sources of resistance that have been used in crosses is better understood. One major gene at the same locus confers resistance in all sources studied.

- Several markers for resistance to *Apion godmani* have been developed and will be adapted for MAS.

Activity 3.3 Developing integrated disease and pest management components

Highlights:

- Diffusion of technology activities within the DFID-funded project on Sustainable Management of Whiteflies initiated.
- The second phase of the Tropical Whitefly IPM Project has demonstrated the importance of adopting IPM measures to recover common bean production in whitefly-stricken regions.
- Three potential biocontrol agents were identified and tentatively classified as *Paenibacillus polymyxa, Bacillus subtilus and Gluconobacter* spp. The antimicrobial compound is possibly proteinaceous in nature and one of the bacteria produces a heat resistant compound.
- Preliminary results showed *Calliandra calothyrsus* to be the best source of green manure for control of soil pathogens and to increase yields.
- Some of the *Pythium* species pathogenic to beans induced symptoms on and affected sorghum, millets, field peas and maize major crops grown in association with beans in south western Uganda.
- Some of the components useful in the integrated management of bean root rots were effective in reducing root damage and in increasing yield parameters on sorghum, millets, field peas and maize in bean based cropping system.

3.3.1 Management strategies for whiteflies

Rationale: Whiteflies have become the target of excessive pesticide use by snap bean and dry bean farmers in the Andean zone. A management system for whiteflies that contributes to reducing pesticide use has been developed and tested with farmers in Colombia and Ecuador (see 2002 and 2003 Annual Reports). In 2004 we tested other alternatives to further reduce the need for toxic insecticides and initiated diffusion of technology activities at both sites in Colombia and Ecuador.

Materials and Methods: Two large-scale trials were conducted in areas of the Pradera reference sites where *T. vaporariorum* is still the predominant species. We compared different approaches for whitefly control based upon judicious and less detrimental use of chemicals. Seed treatments and drench applications of novel systemic insecticides were compared with the timing of foliar applications of conventional (less costly) products, in some cases with applications based upon pre-established action thresholds developed in previous experiments (see 2002 and 2003 Annual Reports). These treatments were compared with farmers' practices. These trials were used as demonstration plots for farmers in the area.

Results and Discussion: As in previous trials, and as compared with farmers' practices, alternative management strategies based on judicious timing of applications and use of action thresholds resulted in yields that did not differ from those obtained by farmers with their traditional management approaches (Table 86). Crop appearance, damage (sooty mold) levels, and final produce quality (as judged by farmers attending field days) did not differ either. Use of

systemic insecticides as seed dressing and proper timing of foliar applications resulted in higher benefit/cost ratios with 60-70% less applications made per cropping cycle.

Table 86. Yields (tons ha^{-1}) and economic returns obtained with different approaches for control of the greenhouse whitefly *Trialeurodes vaporariorum* in Pradera, the reference site

Treatment	Yield (tons ha^{-1})		Benefit/cost ratios	
	Trial 1	Trial 2[a]	Trial 1	Trial 2[a]
Seed treatment with imidacloprid followed by two foliar applications of conventional insecticides at pre-established action thresholds	11.1a[b]	11.9	1.43	1.77
Seed treatment with imidacloprid followed by three foliar applications of conventional insecticides at pre-established crop growth stages	10.7a	11.5	1.38	1.62
Farmers' practices (6-7 foliar applications of conventional insecticides)	9.3a	11.9	1.14	1.65

[a] Un-replicated demonstrative trial. No statistical analysis performed
[b] Means followed by the same letter are not significantly different at the 5% level by LSD.

These trials were used to initiate diffusion of technology activities in the area. A field day was organized in collaboration with ICA and the Municipal Technical Assistance Unit. Attendance was good (76 people, 15 of them women). Farmers were informed on the purposes of the demonstration plots and received training on whitefly biology and safe management of insecticides. Farmer's schools activities were initiated with 12 farmers who received training on whitefly sampling, safe management of pesticides and use of action thresholds for rational whitefly control. Diffusion activities will be strengthened if the second phase of the special project on whiteflies is approved.

Contributors: J. M. Bueno, M. Castaño, F. Morales, C. Cardona

3.3.2 'Recovery of common bean production in the Valley of Zapotitán' Project

Until 1985, the Valley of Zapotitán was the main common bean production area to satisfy the demand of the capital city of San Salvador during the months of April, May and June, at the end of the prolonged dry season (November-March). The increasing incidence of *Bean golden yellow mosaic virus* (BGYMV), transmitted by the whitefly *B. tabaci*, gradually led to the abandonment of common bean production in this valley during the dry season. Although common bean is produced throughout Central America, the Salvadoran market demands a unique red-seeded bean type ('Rojo de Seda') only produced in this country. Thus common bean imports from neighbouring countries, did not satisfy the consumers and common bean prices and consumption fell (from 12 to 8 kg per capita) since 1985. In 1990, the collaborative project (PROFRIJOL) between CENTA and CIAT, led to the selection of a BGYMV-tolerant common bean variety (CENTA-Cuzcatleco). However, the commercial characteristics of this new variety were not adequate and, consequently, its market price was relatively low. Moreover, the BGYMV resistance of CENTA-Cuzcatleco has been breaking down even during the rainy months of the year, which has further contributed to its rejection by local farmers due to its high

protection costs. Hence, the TWFP and CENTA initiated activities towards the identification and validation of new improved common bean genotypes for the San Salvador market.

Research plan: A promising red-seeded common bean line possessing high levels of BGYMV resistance and adequate commercial characteristics was identified in field trials of materials developed by Dr. Juan Carlos Rosas, breeder of the Pan American School (Zamorano) in Honduras using parental materials selected through the PROFRIJOL project. The line selected, EAP 9510-77, was planted in September 2001, in five plots of 2,000 sq/m each, to cover the five districts of the Valley of Zapotitán. Half of the area was planted to the local susceptible common bean landrace, 'Rojo de Seda', and the other half with the new EAP line. The plots were planted and evaluated with local farmers in each district. The treatments consisted of minimum inputs: seed treatment (imidacloprid) and herbicide (Prowl). Yield was estimated per plant and per plot (Table 87).

Table 87. Comparative yield (kg ha^{-1}) of a new virus-resistant breeding line and the preferred local common bean landrace in the valley of Zapotitán, El Salvador

Zone	1	2	3	4	5	Average
Year	2001	2001	2002	2002	2003	
Virus rating	8	8	4	4	6	6
Rojo de Seda	120	150	350	408	230	251.6
EAP 9510-77	810	890	1,250	1,400	910	1,052

A demonstration plot was planted in 2001 in order to show farmers the superior yielding capacity of the new line EAP 9510-77, as compared with the previous cultivar CENTA-Cuzcatleco (DOR 364) and the preferred landrace 'Rojo de Seda' (Figure 65). DOR 364 was a CIAT-bred, virus-resistant cultivar released over a decade ago, and although its seed color was more purple than red, it was widely planted in various Central American countries. This cultivar is on its way out because of its increased susceptibility to BGYMV and dark red colour. The EAP line has a combination of different sources of BGYMV-resistance and better seed color. Given the clear preliminary results obtained in the first series of evaluation sites, which demonstrate that it is possible to grow common bean during the dry season (November-March) in the Valley of Zapotitán using minimum inputs, line EAP 9510-77 was evaluated at the national level by CENTA with complementary funding from DFID/PROFRIJOL/CRSP-USAID

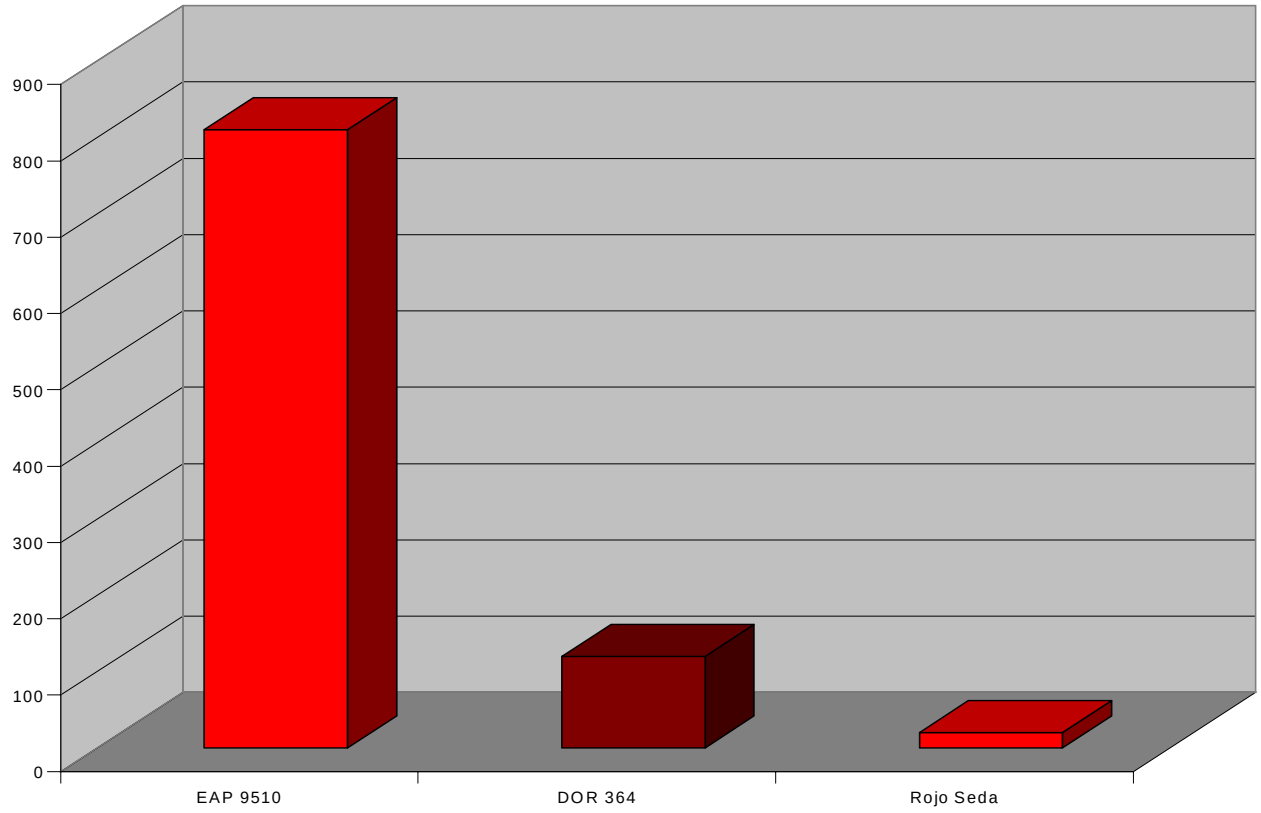

Figure 65. Yield of the new BGYMV-resistant EAP line and the preferred landrace 'Rojo de Seda'.

Principal national scientist : Ing. Carlos Atilio Perez (CENTA).
Collaborators: Agents (3) of the Zapotitán Extension Agency (CENTA) under the coordination of Ing. Mario Aragón.

Evaluation of the new line EAP 9510-77

A case study was conducted with 60 farmers in the western (6), central (23), para-central (22), and eastern (9) regions, during the second semester of 2003. Only 3 of the 60 farmers interviewed were women, which reflects the cultural characteristics of farming in Latin America. The age range of the farmers interviewed was 30-81 years, with 60% of the farmers being older than 50 years. This finding illustrates the migration of young people from rural to urban areas in search of jobs in commerce, industry and maquila, all activities that show positive growth in recent years, as well as an increase in minimum wages. Interestingly, 92% of the farmers interviewed were literate, although only 22% reached secondary school. Over 70% of the farmers owned their farms and 58% lived in the farm. 75% of the farmers do not have access to credit and most farmers have incomes between US $ 1.50 and 3.00/day.

The area of the validation plots varied according to the capabilities and willingness to collaborate of the participating farmers, from 200 m^2 to 1,750 m^2 for the new line, and from 200 m^2 to 2,598 m^2 for the local check (red-seeded cultivar chosen by the farmer).

Farmers also differ in relation to the cropping system used: monoculture (42%), association (20%) and relay (38%). The most popular common bean cultivars are: Rojo de Seda (30%), followed by two BGYMV-resistant cultivars (CENTA 2000 and DOR 585). 80% of the participating farmers registered higher yields with the new improved common bean EAP line. Only in the central region approximately 20% of the farmers concluded that they preferred their traditional bean cultivar. 62% of the farmers manifested that the new line had superior disease resistance qualities as compared to their own cultivars. 33% could not tell any difference (mainly those that already grow virus-resistant cultivars, such as CENTA 2000 and the DOR lines), and 5% concluded that the new material was more susceptible. However, it was later shown that the susceptibility of the new line was to 'web blight', a fungal disease present in isolated areas of El Salvador. 83% of the farmers considered that the commercial characteristics of the EAP line as excellent. The remaining 7% thought that their local material was better (mainly the local landrace 'Rojo de Seda' which is highly susceptible to BGYMV and cannot be grown in the dry season even under heavy chemical protection).

The most important result of this survey is that 87% of the farmers that planted the new improved bean line were willing to adopt it. This figure was almost 100% in areas affected by the whitefly-transmitted BGYM virus. Of all the seed obtained by the collaborating farmers, 37% was used for household consumption, 32% was saved as seed for the next planting, and 27% was sold to generate income.

Table 88 shows the statistical analysis of the different variables evaluated in order to determine the level of acceptance of the new line EAP 9510-77. Table 89 shows the superior yielding capacity of the new material EAP 9510-77 in the selected regions where it was evaluated, in relation to the local cultivar.

The line EAP 9510-77 was officially released in November 2003 as the new variety 'CENTA San Andres'. In the District of Zapotitán, the TWFP has financed two field days for 83 farmers (including 18 women), and 18 technicians, in order to promote the new variety.

Responsible scientist: Ing. Carlos Atilio Pérez C. (CENTA)

Table 88. Main variables that determine the adoption of a new bean cultivar

Variable	Coefficient	Standard Error	t-Statistic	Probability
Intercept	0.141624	0.260788	0.543063	0.5895
Growth Habit	0.14294	0.120277	1.188416	0.2403
Vegetative Cycle	0.115406	0.075519	1.528176	0.1328
Yield	-0.115342	0.087253	-1.321933	0.1922
Disease Resistance	-0.166001	0.099686	-1.665248	0.1021
Pest Resistance	0.101755	0.097583	1.042755	0.3021
HumidityTolerance	0.053299	0.049807	1.070111	0.2897
Market Price	-0.057198	0.054633	-1.04696	0.3002
Acceptance	0.693283	0.156329	4.434778	0.0001
R2	0.376418	Mean dep. var.		0.881356
Adjusted R2	0.276645	S.D. dep. var.		0.326145
St. Error Regress.	0.277387	F value		3.772743
Residual S.C.	3.847183	Probab (F)		0.001584

Model**:** Varietal Adoption = 0.141624 + 0.14294 contall + 0.115406 cveg-0.115342 rend-0.166001 resenf + 0.101755 respla + 0.053299 tolhum-0.057198 sale + 0.693283

Table 89. Yield results (kg ha^{-1}) of the validation trials of EAP 9510-77 in 4 regions of El Salvador

Region	EAP 9510-77	Local cv.	Yield Difference	Percentage
West	1815	1312	503	27.7
Central	1259	951	308	24.5
Para-Central	1088	875	213	19.6
East	1171	901	270	23.0
National Ave.	1240	952	288	23.2

3.3.3 Identification of potential biocontrol bacterial agents

Rationale: Because of the negative effects that synthetic chemicals have on the environment, it is apparent that the use of antagonistic microorganisms may be a good alternative in controlling diseases caused by pathogens with large pathogenic variability. If this is to be a viable and reliable alternative, it is important to understand why, when and how these microorganisms and their products affect the development of pathogens. Several bacteria that exhibit a biocontrol effect on some common bean pathogens have been isolated in our laboratory. Preliminary experiments revealed the antagonistic effects of these bacteria. Three bacteria have been of particular interest. We report the identification of these bacteria.

Bacteria identification:

Bacteria 1: The bacterium was isolated from the phyloplane of common bean leaves. The bacterium is gram positive, non-motile, with large spores and produces acid in the presence of manitol, maltose and cellobiose, does not utilize urea, has the ability to utilize catalase and oxidase. Based on the biochemical and morphological analysis, this bacterium was identified as a Bacillus. Analysis of the partial sequence of the 16S rDNA gene revealed that this bacterium was 98% similar to *Paenibacillus polymyxa*. Based on sequence analysis of the partial 16S ribosomal gene, morphological and biochemical tests, bacterium 1 was tentatively classified as *Paenibacillus polymyxa*.

Bacterium 2: The bacterium was isolated as a contaminant from petri plates of V8 juice medium. Morphologically, this bacterium is irregular in shape, convex, translucent with colonies that are $\leq$ 1 mm. It is gram positive with small Bacillus type spores. Based on morphological and biochemical tests, this bacterium was identified as *Bacillus subtilus.* This identification was confirmed following partial sequence of the 16S rDNA gene and blast sequence search that showed that the sequence of this bacterium was 98% similar to that of *Bacillus subtilus.*

Bacteria 3: The bacterium was isolated from the phyloplane of *Morinda citrifolia.* Morphological tests revealed that this bacterium is a gram-positive cocos with circular colonies < 1 mm, transparent and convex. Partial analysis of the 16S rDNA sequences revealed that this bacterium is 100% similar to *Gluconobacter* spp. This bacterial has been tentatively labeled as *Gluconobacter* spp.

Conclusion: Further identification and classification of the bacteria by an independent bacteriologist is under way. Meanwhile, characterization of these bacteria and establishment of their biocontrol activity and range is under way.

Contributors: C. Jara, G. Castellanos, M.A. Henriquez, G. Mahuku

3.3.4 *In vitro* inhibition of *Colletotrichum lindemuthianum* by three potential biocontrol bacterial species *(Paenibacillus polimixa, Bacillus subtilus* and *Gluconobacter spp.)*

Rationale: Biological control is an alternative sound strategy for the management of plant pathogens because it is environmentally safe while promoting build up of natural enemies, thus creating a sustainable production system. For this reason, we are involved in studies to identify and evaluate the efficacy of potential biological control agents. We report the effect of cell-free culture filtrates of three potential bicontrol bacteria on mycelial growth and germination of *Colletotrichum lindemuthianum* conidia.

Materials and Methods: Three potential bacterial biocontrol agents (tentatively classified as *Paenibacillus polimixa* (B1); *Bacillus subtilus* (B2); and *Gluconobacter* spp (B3)) were used in this study. The bacteria were grown on either PDA or nutrient agar, unless otherwise specified.

Inhibition of *C. lindemuthianum* growth

To establish the effect of the bacterium on the growth of *C. lindemuthianum*, bacterium from a 48 hr culture on PDA was inoculated onto PDA medium in a circle at different distances (2, 3, 4, and 6 cm) diameters from the center of the petri plate. A plug of mycelium (4 mm diameter) cut from the edges of an actively growing *C. lindemuthianum* isolate was placed in the center of each plate. To establish the nature of the potential antifungal compound, the fungus was inoculated immediately after culturing the bacteria (0 hrs), and at varying times 24, 48 72 and 96 hrs after culturing the bacteria. There were five plates for each treatment and the experiment was repeated. Radial growth of the fungus was evaluated 7, 14 and 21 days after culturing. Control plates contained the fungus on the same media, and inoculated at the same time but without the bacteria.

Preparation of cell free culture filtrates

Cell-free culture filtrates of the bacteria were produced by culturing the bacteria in 250 mL of nutrient broth (Difco) in 750 mL flasks and incubating at 28°C with shaking at 200 rpm until an OD_{600} of 1.1 was reached. The culture filtrate was centrifuged at 7000 g to remove bacterial cells and then the fluids were passed through a 0.22 µm pore-size nylon membrane to remove residual bacterial cells. The cell-free culture filtrate either heat inactivated by 100 C^o for five minutes or not heated were used to prepare medium for culturing *C. lindemuthianum* conidia and test inhibition of conidial germination.

Conidia germination assay

Cell-free culture filtrates were mixed with autoclaved and cooled PDA agar to a final concentration of 60% (v/v). *C. lindemuthianum* conidia suspension (10^6 conidia ml^{-1}) in sterile distilled and deionized water was plated on to PDA medium amended or non–amended PDA medium (control) with cell-free culture filtrates. Inoculated plates were incubated at 24°C and evaluated for the growth and development of *C. lindemuthianum*.

Results and Discussion:

All bacteria were effective in inhibiting the growth of *C. lindemuthianum* (Tables 90, 91, and 92), however, *Gluconobacter* spp. and *Paenibacillus polimixa* were the most effective. *C. lindemuthianum* spores plated on medium containing cell-free culture filtrates did not germinate, where as on control plates (lacking bacterial filtrates), normal fungal growth was observed (data not shown). The antimicrobial compound is diffusible, and was more potent with increased time that the bacteria was allowed to establish before inoculating the fungus (Tables 90-92). The rate of fungal growth inhibition dependent on the distance that *C. lindemuthianum* was from the bacteria. This was more evident for *B. subtilis* and *Paenibacillus polimixa*. This might reflect the nature of the antimicrobial compound, which in turn, might determine the rate of diffusion through the medium. Conversely, this might also reflect the rate of antimicrobial production, with *Gluconobacter* producing and reaching high concentrations faster than the other bacteria. The compound produced by *Gluconobacter* appears to be in high concentrations and diffuses through the medium rapidly. More studies are under way to optimize the production media and culturing conditions, as a means of verifying this assertion.

Table 90. Radial growth of *Colletotrichum lindemuthianum* challenged with the potential biocontrol bacteria *Paenibacillus polimixa* inoculated at different times and distance.

	Distance of bacteria from center of plate (cm)				
Time (hrs)	**2**	**3**	**4**	**6**	**Control**
0	0.28	0.41	0.92	1.54	4.54
24	0.10	0.26	0.45	1.29	4.45
48	0.10	0.1	0.24	1.08	6.71
72	0.10	0.1	0.16	0.47	4.58
96	0.10	0.1	0.20	0.48	5.50

Table 91. Radial growth of *Colletotrichum lindemuthianum* challenged with the potential biocontrol bacteria *Bacillus subtilus* inoculated at different times and distance.

	Distance of bacteria from center of plate (cm)				
Time (hrs)	**2**	**3**	**4**	**6**	**Control**
0	0.80	1.14	0.97	2.17	6.80
24	1.31	1.16	1.25	1.71	4.50
48	0.59	0.54	0.29	1.17	4.43
72	0.41	0.54	0.49	1.30	5.14
96	0.41	0.44	0.30	1.24	4.80

Table 92. Radial growth of *Colletotrichum lindemuthianum* challenged with the potential biocontrol bacteria *Gluconobacter* spp. inoculated at different times and distance.

	Distance of bacteria from center of plate (cm)				
Time (hrs)	**2**	**3**	**4**	**6**	**Control**
0	0.13	0.13	0.23	0.32	4.54
24	0.10	0.10	0.10	0.10	7.5
48	0.10	0.10	0.10	0.10	6.71
72	0.10	0.10	0.10	0.10	4.58
96	0.10	0.10	0.10	0.10	5.60

Heating the culture filtrate destroyed the activity of the antifungal compound, produced by *B. subtilis* and *Paenibacillus polimixa* but not that produced by *Gluconobacter*. The antimicrobial compounds seem to be different. For B1 and B2 it appears that the antifungal compound is proteinaceous in nature; where as the antimicrobial compound produced by B3, is either a heat resistant protein or some other type of compound. Further tests are needed to definitely identify the antimicrobial compounds in these bacteria.

Conclusion: Three bacteria with potential to manage fungal pathogens have been identified. Preliminary results have revealed at least two compounds; a heat susceptible and a heat stable compound. It is probable that one these compounds are proteinaceous in nature. However, this assertion needs to be confirmed. There is a need to optimize culturing conditions, and to test the efficacy of these compounds on a diverse range of plant pathogens.

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3.3.5 Integrated soil fertility/Pest & disease management approaches to address root-rot problems in common beans

Rationale: Consensus about societal demands for agricultural sustainability and biodiversity conservation has been reached in the past decade (UNCED,1992). New approaches to continuing problems, like soil degradation and soil pest and diseases, are then needed in order to achieve agricultural sustainability. Our overall working hypothesis in this study is that combining soil fertility and pest management approaches would provide a unique opportunity to exploit synergies allowing a better control of soil fertility, pest and disease limitations to crop productivity than either approach alone.

The management of organic matter is crucial to the activities of the soil biota. Use of green manures can have a multi-faceted beneficial effect on crop productivity arising from (i) protection of the soil from erosion; (ii) increased nutrient cycling; (iii) synchronized nutrient release and uptake by the plants; and (iv) increase in soil biological activity and diversity of

microorganisms, which in turn can lead to minimized damage and loss from soil borne pathogens, and increased activity of beneficial microorganisms. However, different sources of green manure can have different effects on the balance between populations of harmful and beneficial organisms because they have different rates of decomposition and nutrient release as well as different impact on soil moisture and temperature that invariably affects relative population sizes. For this reason, it is important to evaluate the effect of different sources of green manure on three key functional groups of soil biota: 1) pathogens, 2) microregulators and 3) microsymbionts. We are studying the population dynamics of soil pathogenic fungi (*Fusarium, Sclerotium, Macrophomina, Rhizoctonia* and *Pythium*), soil nematodes (discriminated by feeding habit), soil microsymbionts (mycorrhiza, rhizobia) during cultivation of common bean in soils infested with pathogenic fungi. Evaluations were carried out by: a) directly identifying and quantifying different soil biota from functional groups mentioned above and b) indirectly, by evaluating the incidence of disease on susceptible plant genotypes, by plant infection test for native rhizobia symbiotic potential and AMF activity in soil through hyphal lengths. The relative position of these three groups in the soil food web suggests the potential for soil organic management to reduce soil pathogenic fungi populations and incidence in bean plants by changes induced in soil moisture and temperature, nutrient availability and interaction with other soil organisms.

Materials and Methods: An experiment was established in CIAT's Santander de Quilichao Research Station, using a plot that has a history of high incidence of root rot pathogens. The plots were planted with a root rot susceptible bean variety A 70. Immediately after planting, the plots were covered with three green manures treatments: (1) rapidly decomposing *Tithonia diversifolia* (TTH); (2) intermediate rate of decomposition but greater soil cover due to leaf morphology by *Cratylia argentea* (CRA); (3) slow decomposing *Calliandra calothyrsus* (CAL) at a rate of 6 ton ha^{-1}; and (4) control (no green manure added). The experiment was replicated five times. Soil samples (0-10 cm) collected during the cropping season included at least planting and harvesting time. Samples were collected within rows and between rows, to measure the effect of the rhizosphere of bean plants on the soil biota studied.

Results and Discussion:

Diversity of soil pathogenic fungi

Preliminary data revealed that plots receiving CRA had a significantly less fungal diversity ($P<0.05$) than plots receiving the other sources of green manure or the control (Figure 66). No differences were observed between the other treatments and the control. However, since this is the second season after initiation of the experiment, it is still too early to draw sound conclusions.

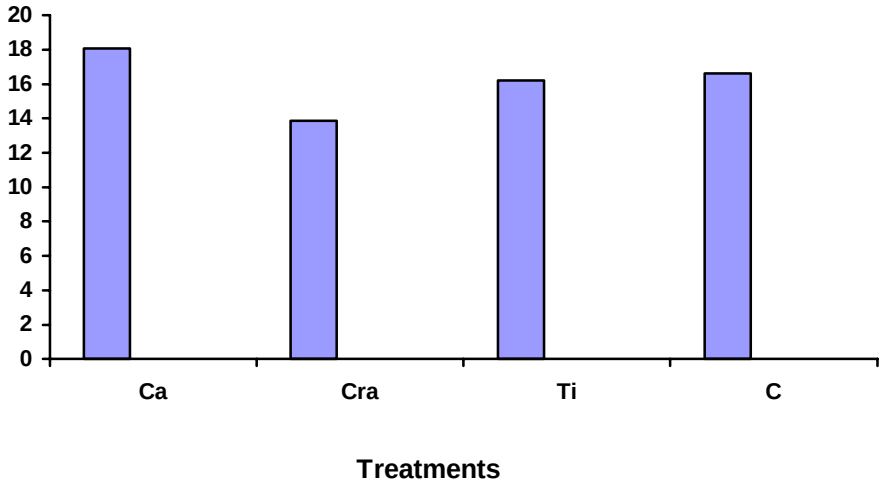

Figure 66. Diversity of soil-borne fungi in plots receiving or not receiving different sources of green manure. Value on Y-axis represents H, the Shannon Wiener diversity index.

The most frequently isolated fungus was *Aspergillus* (A) in all treatments, while *Macrophomina* (Ma) and *Rhizoctonia* (R) were the least isolated fungi (Figure 67). Other fungi that were isolated included *Fusarium* (F), *Penicillium* (P), *Humicola* (H) and *Mucor* (M) (Figure 67). The presence of *Penicillium* is interesting, as some species of this fungus are known to solubilize phosphorus. *Humicola* is a fungus that has been found to be involved in decomposing organic matter, and this was found in abundance in plots receiving *Calliandra*. Several fungi were isolated that are currently being classified. These were tentatively placed under the "unknown" group (D). It is possible that some of these fungi could be potential biological control agents. Although *Macrophomina* has been observed in the past in high frequencies and incidence on infected plants, this fungus was not detected in the soil samples analyzed thus far. It is possible that the method of analysis that is used leads to the exclusion of this fungus, or the high incidences observed under field conditions results from seed-borne inoculum.

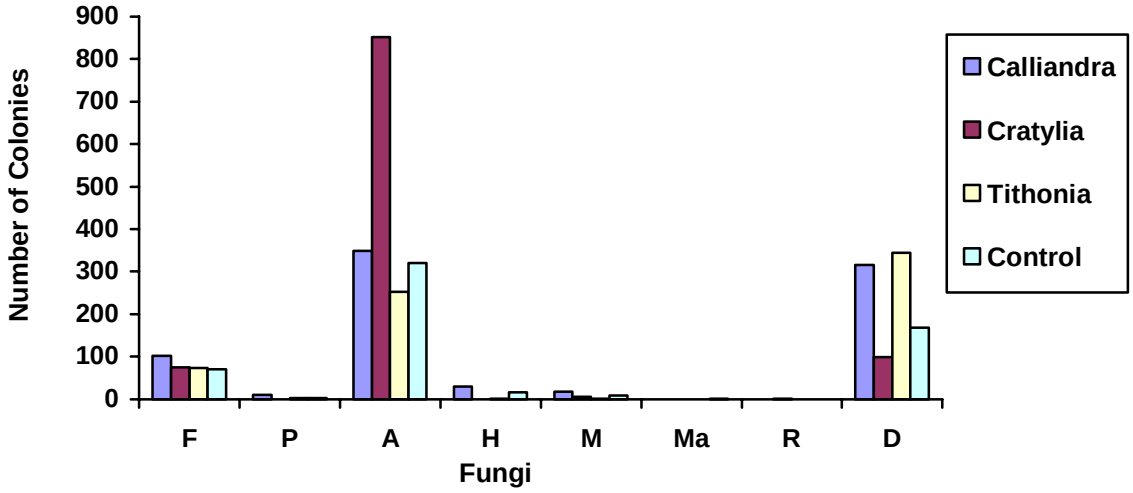

Figure 67. Frequency of different fungi isolated from plots receiving a slow (CAL), intermediate (CRA) and fast (TTH) decomposing green manure or the control.

Abundance of soil nematodes

Total number of soil nematodes was always higher in the row than between the rows highlighting the importance of the bean plant rhizosphere effect (Figure 68). On average greater number of nematodes were found when Tithonia pruning was applied to the soil and the overall order was TTH>CRA>CON=CAL . Taxonomic identification of nematodes and classification into feeding groups is on going and should help in the interpretation of abundance trends observed.

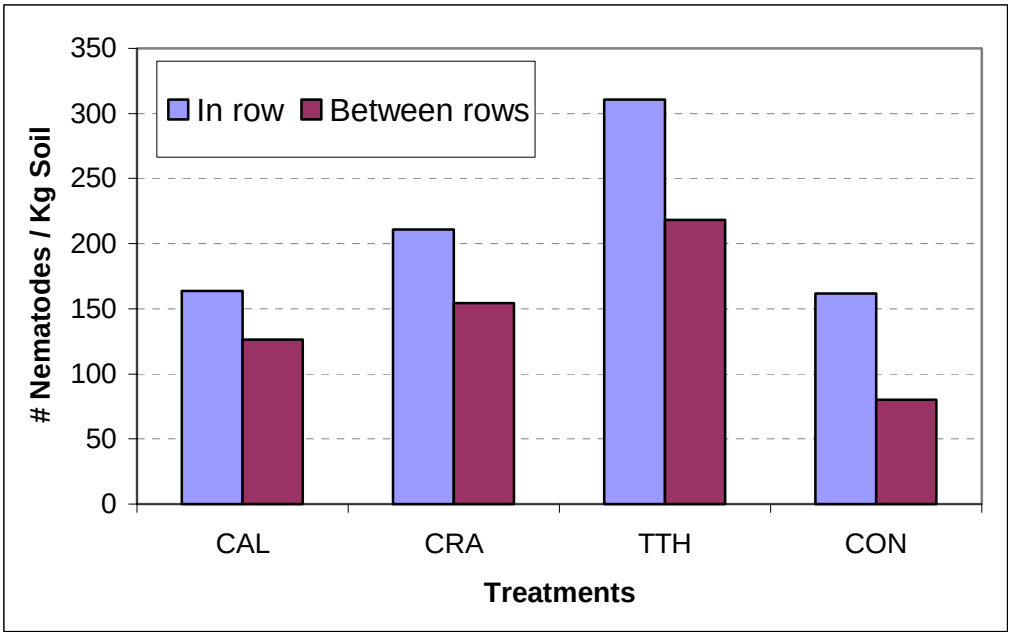

Figure 68. Total number of nematodes from plots receiving a slow (CAL), intermediate (CRA) and fast (TTH) decomposing green manure or the control.

Incidence of root rot pathogens: Significant differences were observed in the incidence of root rots in some treatments, when compared to the control (Figure 69). Application of *Calliandra*, and *Tithonia* significantly reduced disease incidence ($P<0.05$), where as a slight increase in disease incidence was observed in plots receiving *Cratylia.* Analysis of the samples collected from these plots revealed that most of the root rot symptoms were caused by *Macrophomina phaseolina* and *Fusarium solani*, while *Rhizoctonia solani* was occasionally isolated. Significant yield increases were observed for plots treated with *Calliandra* (10%) and lowest for plots receiving *Tithonia* (-29%) (Figure 69). Although a slight increase in yield was observed (1.2%) for plots receiving *Cratylia*, this was not significantly different from the control plots.

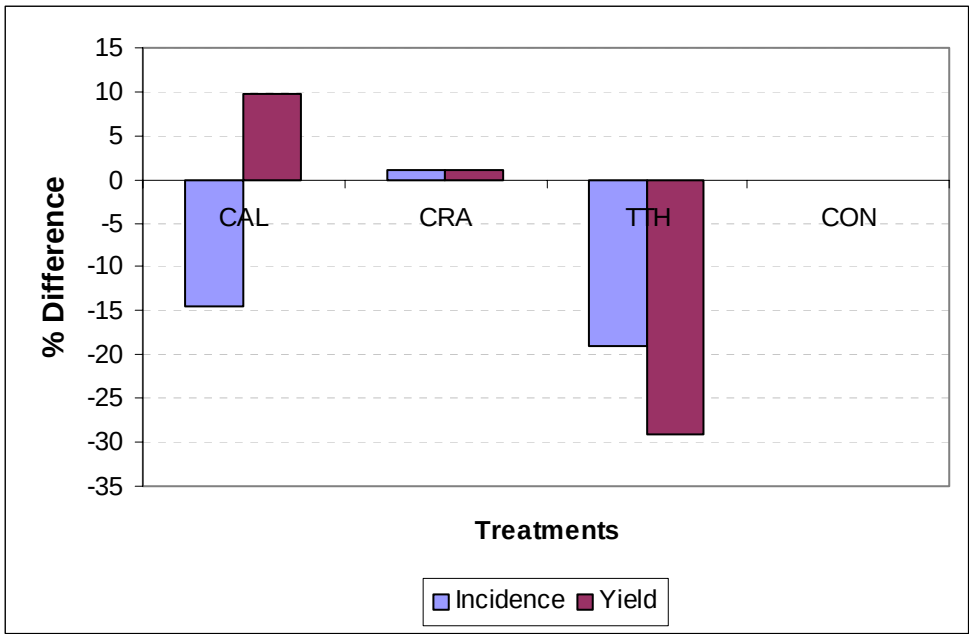

Figure 69. Incidence of root rots and yield of the bean genotype A 70, grown in plots with or without different types of green manures expressed as a percent of control treatment.

Conclusion: First results indicate that despite the relatively limited time of green manure treatments some initial trends can be identified. Compared with the control, application of *Calliandra* resulted in increased bean yield, reduced incidence of root rots and low nematode abundance. In the case of *Cratylia*, there were minor differences root rot incidence, yield and nematode abundance (in row) when compared to the control. Although disease incidence was low in plots receiving *Tithonia*, bean yield was also negatively affected. Taxonomic identification of nematodes would help to understand if high nematode populations in TTH were involved in reducing bean yield. In addition, the impact of treatments on the bean plant symbiosis with mycorrhiza and rhizobia needs to be included for a more complete explanation of yield differences encountered. Nevertheless, yield differences were likely also influenced by a combination of physico-chemical factors including differences in nutrient release by the three green manure sources.

While at this early stage application of *Calliandra* seems to offer the best results we need to examine how transient or cumulative these effects are and the mechanisms of action involved. The potential exists that unknown beneficial microorganisms are promoted in the soil by green manures and thus can potentially be used to manage root rot pathogens and/or for promoting plant growth. We are currently evaluating fungi that have tentatively been grouped under the "unknown" group for potential antagosistic effects, as well as *Penicillium* species for their ability to solubilize phosphorus.

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3.3.6 Pathogenicity of *Pythium* spp and effects of management options for root rots on crops grown in association with beans in southwest Uganda

Rationale: Bean is one of the crops grown under the intensive agricultural system in southwest Uganda. Others include sorghum, maize, sweet potatoes, Irish potatoes, bananas and peas. Crop rotation in the strict sense is rare. Dominance of crops in the field shifts according to season. Rotations commonly practiced include beans-maize-sorghum, beans-maize-beans and beans-Irish potato/maize-sweet potato (Edidah, 2003). Maize and sorghum are also intercropped with beans and/or Irish potatoes such that the bean crop appears in the field season after season. However, of all these crops, beans are most affected by root rots. In recent years this has resulted in the decline in bean production in the area. Given that some of the root rot causing pathogens (e.g. *Pythium* spp) are known to have a wide host range, some of the questions asked are: do crops grown in association or in rotation with beans play any role in the pathogen survival, inoculum density and severity of root rots in beans?; is bean the only crop in the system that is affected or is it simply a good indicator of the level of root rot pathogens?; to what extent are other crops in the system affected by bean pathogens?; what are the effects of management options for bean root rots on other crops? To address these questions, we initiated studies to characterize *Pythium* spp associated with major crops found in the bean based systems; to determine pathogenicity of some *Pythium* species on these crops; and to determine the effects of management options for bean root rot on crops grown in association with beans.

Materials and Methods:

Pathogenicity studies: Three *Pythium* species pathogenic to beans (*P. ultimum, P. chamaehyphon, P. pachycaule)* were artificially inoculated on three crops commonly associated with beans namely: sorghum, millets and maize. Autoclaved millet (100 g) was mixed with 200 ml of water in a 500-ml bottle and subsequently used to raise the fungi. After two weeks of incubation, the infested millet was mixed with pre-sterilized soil at a ratio 1:10 v/v in wooden trays. Maize, sorghum and millet were planted in two rows of twelve plants and replicated in three trays. Bean varieties CAL 96 and RWR 719 were used as susceptible and resistant checks respectively. Cumulative emergence and plant stand was recorded one week after germination. Three weeks after germination, plants were assessed for any root and shoot symptoms that may be associated with *Pythium* infection.

Effect of management practices: Four crops; beans (B), sorghum (S), maize (M), and peas (P) were subjected to four amendments i.e., farm yard manure (FYM), green manure (GM), inorganic fertilizer (NPK), fungicide (Metalaxyl + Mancozeb 63.5% WP) in farmers fields in Rubaya, Kabale district, southwest Uganda. Sorghum, maize and peas seed were obtained locally from farmers. A root rot susceptible bean variety (CAL 96) was used as a check. Farmyard manure and green manure (*Crotalaria*) were applied on a dry weight basis at a rate of 5t ha^{-1} and their nutrient level determined. NPK fertilizer was applied at a rate of 50 kg of N ha^{-1}. Ridomil was applied as seed treatment (slurry) at a rate of 2.5 kg ha^{-1}. Qualitative data was obtained through field observations and photography. Quantitative data collected included: emergence, plant stand, disease incidence and severity at different times during the growing season, plant vigor and yield parameters (dry matter production). Disease severity was evaluated according to

a CIAT nine-point scale where 1 is resistant and 9 susceptible (Abawi and Pastor Corrales, 1990)..

Results and Discussion:

Pathogenicity studies

The different *Pythium* species invoked typical root rot symptoms on susceptible bean cultivar CAL 96 in screen house studies. As expected, cultivar RWR 719 was resistant. Sorghum exhibited severe stunting and purple color on leaves. These features were more pronounced with isolate KAK 5 B (*P. pachycaule)*. Similarly, millet exhibited stunting as well as yellowing and drying of the leaf tips, unlike plants in un-inoculated control trays. Maize showed less pronounced effects characterized by reduced plant vigor and size.

Symptoms on roots of sorghum were comprised of red-black lesions and discolorations, reduced root mass and length. Millets displayed some lesions and reduced root mass. Maize exhibited little if any lesions on roots 3 weeks after emergence although root mass was relatively lower than in the control trays. *Pythium* was re-isolated from roots of all crops grown in infected soil.

These screen house results showed that *Pythium* species used had an effect on the different crops tested. The most affected crop was beans and then sorghum, millet and maize in that decreasing order. Maize exhibited an interesting reaction in that there was some reduction in both shoot and root mass but little necrosis on the latter. Stunting in crops is attributed to reduced capacity of roots (either due to damage or reduced amount) to support adequate water and food uptake. We can tentatively conclude from these preliminary observations that *Pythium* species pathogenic to beans cause damage to sorghum, millets and maize to varying degrees. Further investigation to elucidate these interactions is on-going.

Effects of management options on incidence and severity of root rots

The management options evaluated affected the crops in different ways. FYM and Metalaxyl/Mancozeb significantly reduced initial root rot infection on beans. High incidence of root rots was observed with GM and attributed to interactions between the root rot pathogens and soil micro-organisms. But FYM, GM and NPK enhanced root (mass) growth in beans, compared to control plots.

As in screenhouse studies, infected sorghum plants exhibited stunted growth, purple leaves, shoot death and dark-red to black root lesions (Figure 70). Significantly high incidence and severities were observed in control plots particularly 54 and 72 days after planting. Amendments reduced these effects and plant recovery was evident in plots amended with GM, FYM and NPK. Symptoms on maize were expressed as grey lesions on roots (Figure 71), stunting and poor establishment. However, incidence and severity were low indicating that maize was less affected (Figure 72A). As with sorghum, amendments and particularly FYM reduced severity (Figure 72B) and improved plant vigor and growth.

A B

Figure 70. (A) Severely affected sorghum roots (control plots); (B) Sorghum root with prop root development in plots amended with NPK.

Figure 71. Maize roots showing root lesions

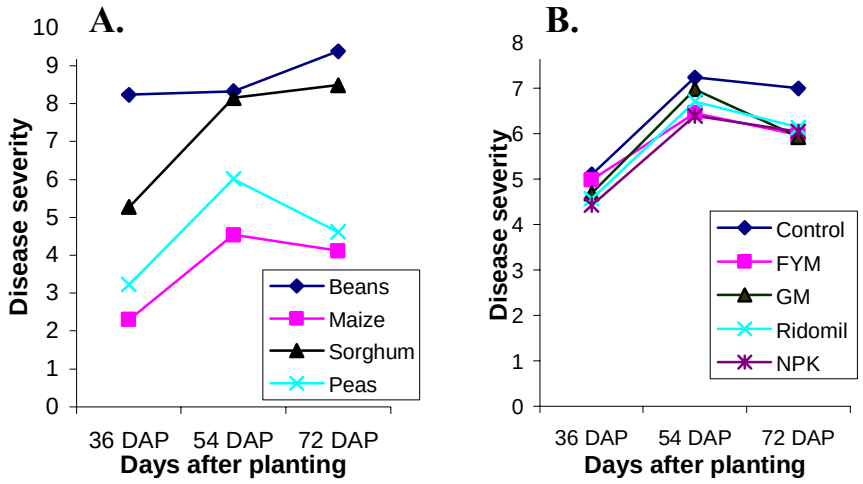

Figure 72. Disease severity in crops over the season (A.), and effects of different root rot management practices on root rots over the season (B.)

NPK, GM, FYM and ridomil (in sorghum) improved dry matter production (DMP) in both maize and sorghum (Table 93). Improved DMP in sorghum due to ridomil is probably due to its protective effect against *Pythium* species.

Table 93. The effect of different soil amendments on mean dry matter production (72 days after planting) for maize and sorghum. Rubaya, Kabale, 2004 season A.

Crop / Treatment	**Dry matter (g)**	
	Maize	**Sorghum**
Control	106.9	18.5
Farm yard Manure	128.3	42.7
Green Manure	138.4	38.9
Metalaxyl+Mancozeb 63.5% WP	112.5	46.1
NPK	163.2	48.8
	L.S.D at P < 0.05 (32.20)	

Overall the different management options evaluated influenced severity of root damage and other growth parameters on crops grown in association with beans. This implies that the use these options do not only contribute in the management of bean root rots, but are also beneficial to other crops. Studies are underway to further define this contribution.

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Collaborators: N. Spence (CSL, UK), G. Tusiime (Makerere University)

Progress towards achieving output milestones:

- IPM measures have served to recover common bean production in an important whitefly-stricken production region of El Salvador.

- Some of the components useful in the integrated management of bean root rots were effective in reducing root damage and in increasing yield parameters on sorghum, millets, field peas and maize in bean based cropping system.