BEAN PATHOLOGY

Activity 1. Characterizing and monitoring pathogen and insect diversity.

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

Rationale

Different studies have reported the presence of physiological races in the CBB pathogens while others have found to the contrary. The question of physiological races within the common bacterial blight has been the subject of much discussion, with one group reporting the presence of physiological races when the CBB pathogen is challenged onto different bean genotypes while another group has found to the contrary. The significance of such findings is that the presence of physiological races signifies the gene for gene interaction, which would have profound impacts or 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. 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. 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. In addition, these results are hopped to provide insights into the co-evolution of Xcp and Xcpf with Andean and Mesoamerican gene pools, and collaborate the results of Gilbertson et al. (2004). The objective was to detect if there existed differential reaction in the interaction of Xcp or Xcpf with Andean and Mesoamerican bean genotypes.

Materials and Methods: Bacteria isolates: Bacteria 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 belonged to the Andean gene pool and 24 to the Mesoamerican gene pool **(Table 1)**. 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* x *P. acutifolius* and embryo rescue techniques were included.

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.

Table 1. 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).

Genotype	Gene Pool[a]	Xcp		Xcpf	
		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
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

Genotype	Gene Pool[a]	Xcp		Xcpf	
		Incompatible[b]	Compatible[c]	Incompatible	Compatible
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.

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 1)**. A similar result was evident for Mesoamerican isolates 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 1)**. The VAX lines showed high levels of resistance to all isolates, in particular VAX 3, VAX 4, and VAX 6 **(Table 1)**.

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.

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.

Activity 2. 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. It is hoped that this assay can find wide application in quarantine and seed certification services.

Material and Methods: Previous studies reported the presence of a 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 1)**. The fragments were excised form 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 Brachariaa , *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 a 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 $MgCl_2$.

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, bacteria 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.

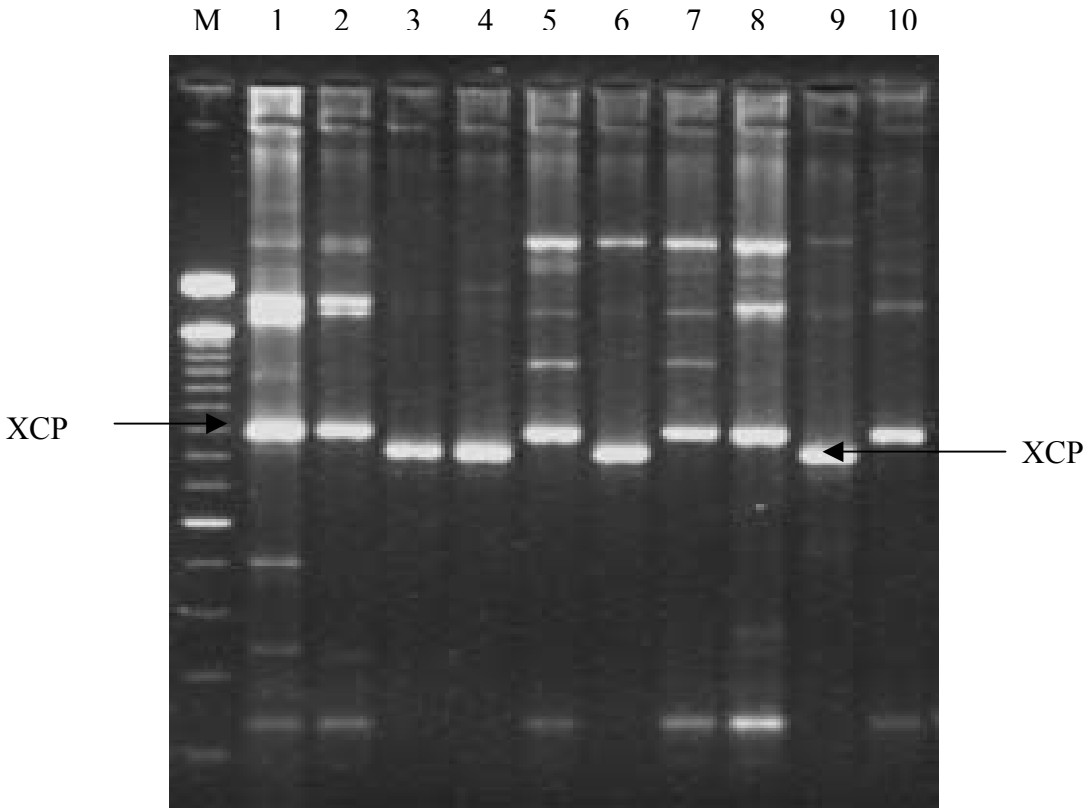

Figure 1. Amplification of *Xanthomonas campestris* pv. *phaseoli (*Xcp*)*, *Xanthomonas campestris* pv. *phaseoli* var. fuscans (Xcpf) using RAPD primer OPG11. Lanes 1, 2, 5, 7, 8, and 10 are Xcp isolates; lanes 3, 4, 6, and 9 are Xcpf isolates. Lane M is the 100 bp molecular size marker.

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 1)**. Southern blot analysis revealed that these fragments were unique to Xcp an Xcpf respectively **(Figures 4A and 4B)**. 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 2A)**. This primer pair did not amplify DNA from other pathogens (*Xanthomonas campestris* pv. *phaseoli*, *Xanthomonas campestris* pv. *phaseoli* var. fuscans, *Xanthomonas campestri* pv. *manihotis*, *Xanthomonas campestri* 36062, *Xanthomonas campestri* 1622/16, *Xanthomonas campestri* pv.o*ryzae, Agrobacterium tumefaciens, Pseudomonas fuscovaginae, Pythium ultimum*, *Colletotrichum lindemuthianum, Phaeoisariopsis griseola* and *Macrophomina phaseolina)* or bean DNA **(Figure 3)**. The primer pair xcpfG11-L1/xcpfG11-R1 was specific for Xcpf **(Figure 2B)**, while the primer pair xcpG11-L2/xcpG11-R1 was specific to Xcp **(Figure 2C)**. When tested on seed from infected pods of plants that had been inoculated with Xcpf under field conditions, only Xcpf was detected **(Figure 3C)**. Amplification with the CBB general primers **(Figure 3A)** revealed the presence of the bacteria, while amplification with Xcp specific primers revealed the absence of Xcp in seed **(Figure 3B)**. 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.

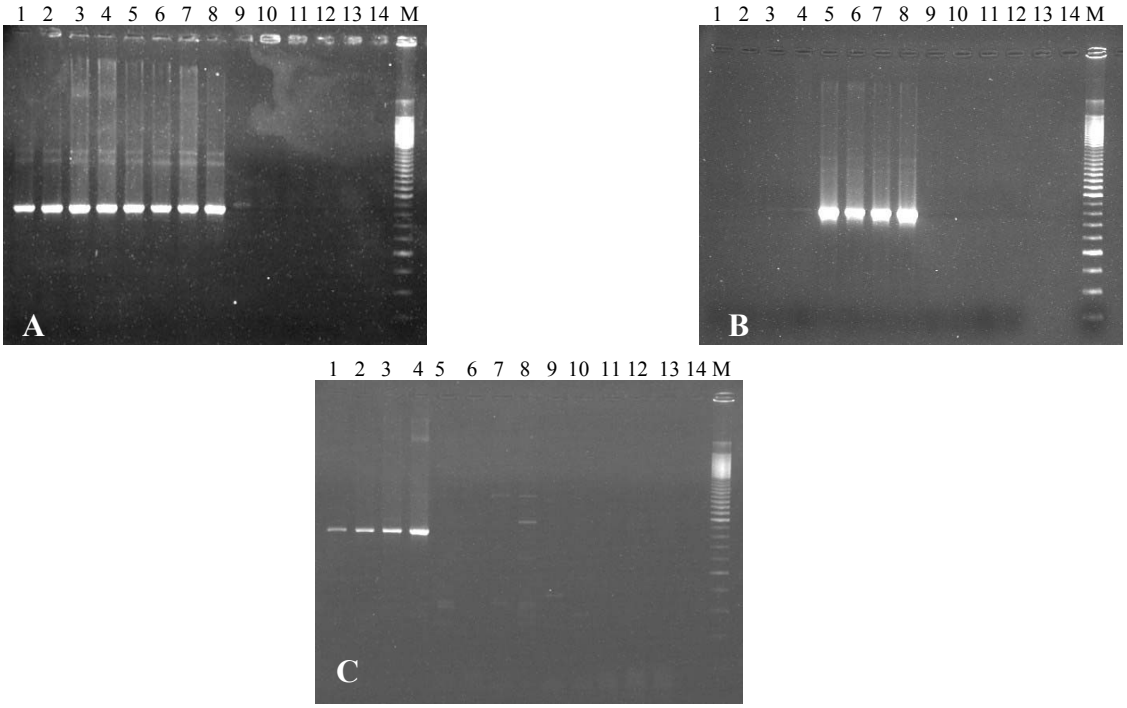

Figure 2. Specific detection of the common bacterial blight pathogens. Lane 1-4; *Xanthomonas campestris* pv. p*haseoli* , lane 5-8 *Xanthomonas campestris* pv. p*haseoli* var, fuscans; lane 9; *Xanthomonas campestri* pv. m*anihotis*, lane 10; *Xanthomonas campestri* pv.o*ryzae*, lane 11; *Phaeoisariopsis 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.

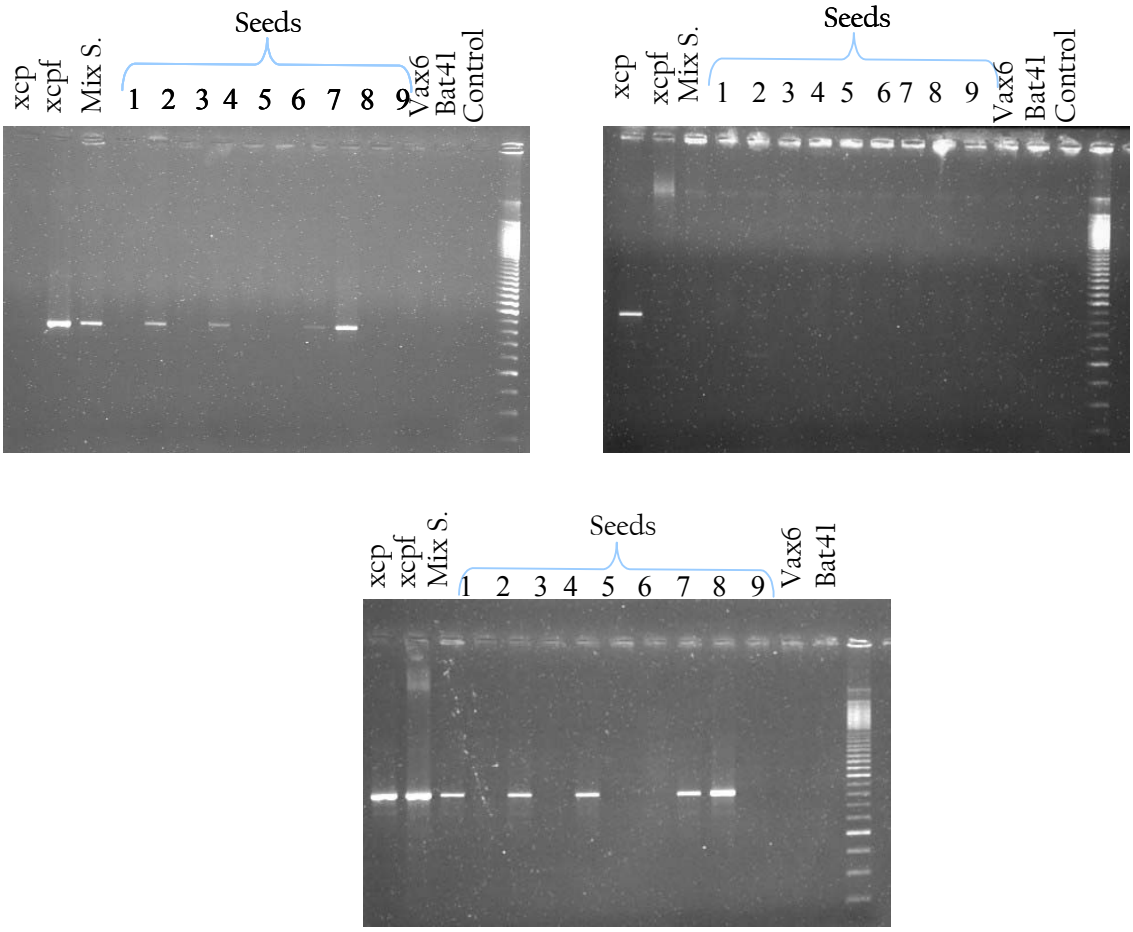

Figure 3. **Detection of CBB pathogens in common bean seed, collected from infected pods of BAT 41 inoculated with . *Xanthomonas campestris* pv. p*haseoli* var, fuscans (Xcpf). Lane 1 is Xcp, lane 2 Xcpf, lane 3 is DNA extracted from mixed seeds; lanes 4 individual seeds, lane 12 is bacteria free seed 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.**

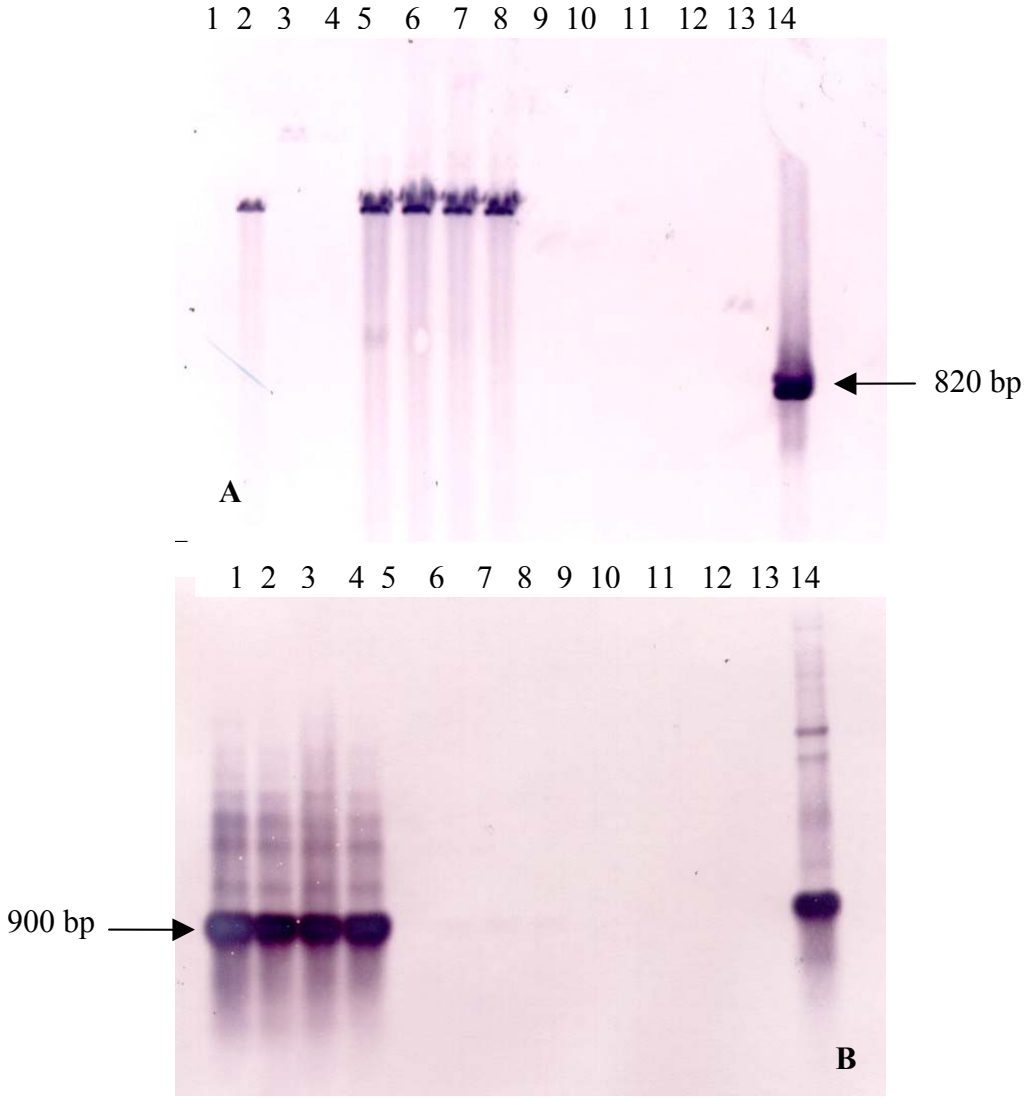

Figure 4. **Specificity of the CBB probes for *Xanthomonas campestris* pv. *phaseoli* (Xcp) and *X. campestris* pv. *phaseoli* var fuscans (Xcpf). (A): Genomic DNA was digested with EcoRI and hybridized using the DIG-labeled Xcpf specific fragment; Lane 1, 3 4 correspond to Xcp, lanes 2, 5, 6, 7, 8 are 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.**

References

Birch, P.R.J.,Hyman, L.J., Taylor, R., Opio, A.F., Bragard, C., Toth, I.K. 1997. Rapd PCR-based differentiation of *Xanthomonas campestris* pv. *phaseoli* and *Xanthomonas campestris* pv. *phaseoli* var. *fuscans*. Eur. J. Plant. Pathol. 103: 809-814.

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: Maria Antonia Henríquez, Monica Navia, George Mahuku.

Activity 3. 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: Samples (43) 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 Darien in the Cauca valley **(Table 1)**. Fungal characterization on a set of 12 differential varieties **(Table 2)** established for *C. lindemuthianum* was done in the greenhouse as described previously (Mahuku et al., 2003).

Table 1. Frequency distribution of *Colletotrichum lindemuthianum* races characterized from different departments of Colombia.

	Locality				
Pathotype	Altiplano Norte	Oriente Antioqueño	Santander	Cundinamarca	Valle
1		1[e]			
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

Results and Discussion: Sixteen pathotypes were identified among the 43 isolates and the most frequently characterized pathotype was 3 **(Table 1)**. 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%) **(Figure1).** 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.

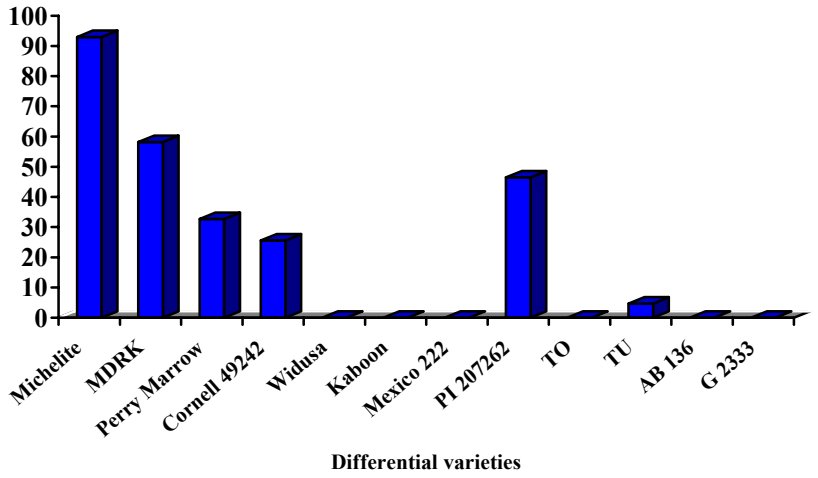

Figure 1. Percent susceptibility of anthracnose differential varieties inoculated with 43 isolates of *Colletotrichum lindemuthianum* from different zones in Colombia

Table 2. 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.

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.

Contributors: C. Jara, J. Fory, G. Castellanos, JB Cuasquer y G. Mahuku.

Activity 4. Developing integrated pest management components.

Identification of potential biocontrol bacterial agents

Rationale

Because of the increase in 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 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 using other diagnostic tools is under way. Meanwhile, characterization of these bacteria and establishment of their biocontrol activity and range is in progress.

Contributors: Carlos Jara, Guillermo Castellanos, Maria Antonia Henriquez, George Mahuku.

Activity 5. In vitro inhibition of Colletotrichum linemuthianum 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 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 bacteria 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 bacteria cells and then the fluids were passed through a 0.22 um pore-size nylon membrane to remove residual bacterial cells. The cell-free culture filtrate either heat inactivated by 100 C for five minutes or not heated were used to prepare medium for culturing *C. lindemuthianum* and test inhibition of conidia 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) 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 1, 2, and 3)**, 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 time that the bacteria was allowed to establish before inoculating the fungus **(Tables 1, 2 and 3)**. 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 dtermine 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.

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 protein in nature; where as the antimicrobial compound produced by B 3, 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 protein 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.

Table 1. 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 2. 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 3. 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

Contributors: Carlos Jara, Guillermo Castellanos, G. Mahuku.

Activity 6. 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&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 change 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 1)**. 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 make sound conclusions.

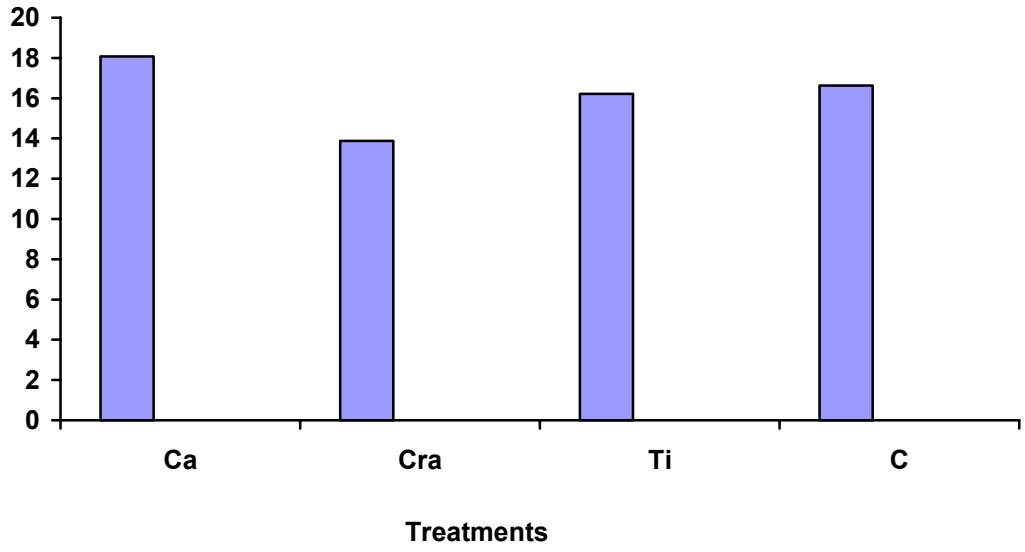

Figure 1. Diversity of soil-borne fungi in plots receiving or not receiving different sources of green manure. H represents the Shannon Wiener diversity index.

The most frequently isolated fungus was *Aspergillus* (A) in all treatments, while Macrophomina (Ma) and Rhizoctonia (R) were the least isolated fungi **(Figure 2)**. Other fungi that were isolated included *Fusarium* (F), *Penicillium* (P), *Humicola* (H) and *Mucor* (M) **(Figure 2)**. 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.

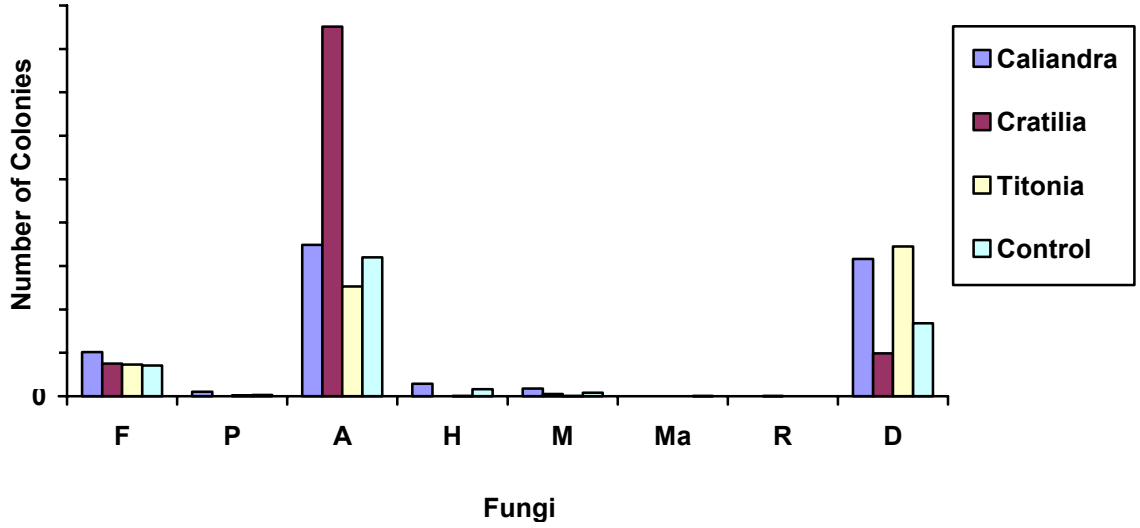

Figure 2. Frequency of different fungi isolated from plots receiving a fast, intermediate and slow decomposing green manure or the control.

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 3)**. 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.

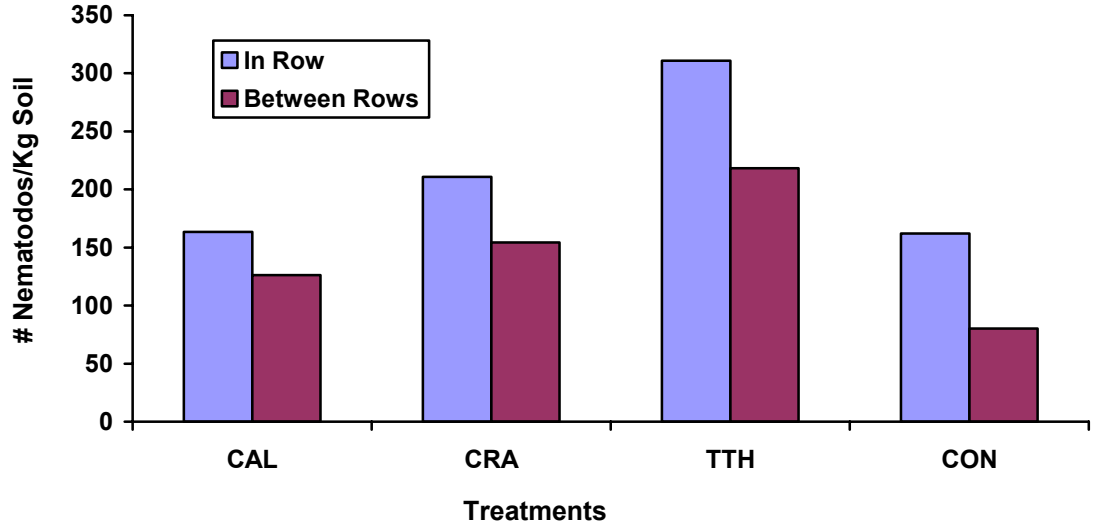

Figure 3. Total number of nematodes from plots receiving a fast, intermediate and slow decomposing green manure or the control.

Incidence of root rot pathogens: Significant differences were observed in the incidence of root rots in some treatments, when compared to the control **(Figure 4).** 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 ort 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 4)**. Although a slight increase in yield was observed (1.2%) for plots receiving *Cratylia*, this was not significantly different from the control plots.

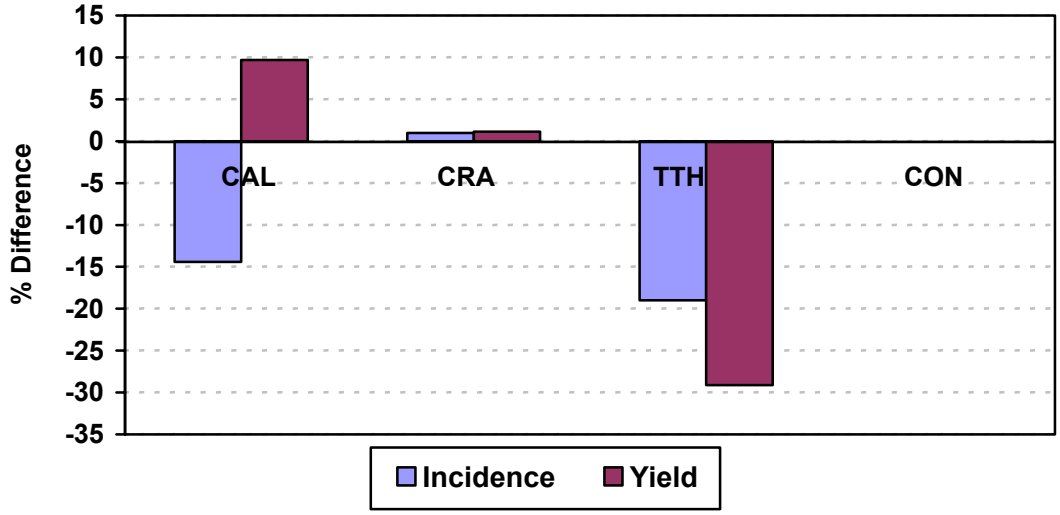

Figure 4. Incidence of root rots and yield of the bean genotype A 70, grown in plots with or without different types of green manures expressed as a percent of control treatment.

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.

Contributors: G. Mahuku (IP-1), E. Barrios (PE-2), Lorena Cortes (IP-1), C. Jara (IP-1), Asakawa N (PE-2), Jara C., Navia J. (PE-2).

Activity 7. Publications, book chapters, workshops.

Refereed Journals

Mahuku, G., Montoya, C., Henríquez, M.A., Jara, C., Teran, H., and Beebe, S. 2004. Inheritance and Characterization of the Angular Leaf Spot Resistance Gene in the Common Bean Accession, G 10474 and Identification of an AFLP Marker Linked to the Resistance Gene. Crop Science 44:1817-1824.

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

Mahuku, G.S. and Riascos, J. J. 2004. Virulence and molecular diversity within *Colletotrichum lindemuthianum* isolates from Andean and Mesoamerican bean varieties and regions. European Journal of Plant Pathology 110: 253-263.

Kelemu, S., Mahuku, G., Fregene, M., Pachico, D., Johnson, N., Calvert, L., Rao, I., Buruchara, R., Amede, T., Kimani, P., Kirkby, R., Kaaria, S., and Ampofo, K. 2003. Harmonizing the agricultural biotechnology debate for the benefit of African farmers. African Journal of Biotechnology 2:394-416.

Non-refereed Journals

Riascos, J.H., Mahuku, G., and Cárdenas, H. 2004. Diversidad genética del agente causal de la antracnosis del fríjol común (*Colletotrichum lindemuthianum*) (ASCOLFI Newsletter).

Henriquez, M.A., Mahuku, G., Muñoz, J.E., Castellanos, G., y Jara, C. 2004. Determinaciones de la diversidad genética del agente causal de la mancha angular del fríjol, *Phaeoisariopsis griseola* (Sacc.) Ferraris, mediante el uso de marcadores moleculares. (ASCOLFI Newsletter).

Book Chapters

Mahuku, G.S. 2003. Angular leaf spot. In: Compendia of Bean Diseases. American Phytopathological Society, St. Paul. MN. **(In press).**

Workshop Presentations

Jara, C., Castellanos, G., Cuasquer, J.B., y Mahuku G. 2004. Determinación de la variabilidad patogénica en diferentes cepas de *Xhanthomonas capestris* pv. *phaseoli* y *Xanthomonas campestris* pv. *phaseoli* var fuscans en 56 genotipos de fríjol común. XXV Congreso de la Asociación Colombiana de Fitopatología y Ciencias afines (ASCOLFI) , Agosto 11-13 de 2004, CIAT, Palmira.

Navia, M., Mahuku, G., y Arroyave, J.A. 2004. Evaluación del proceso de infección de *Phaeoisariopsis griseola* en interacciones compartibles e incompatibles con el fríjol común, *Phaseolus vulgaris*. . XXV Congreso de la Asociación Colombiana de Fitopatología y Ciencias afines (ASCOLFI) , Agosto 11-13 de 2004, CIAT, Palmira.

Montoya, c., Mahuku, G., Henríquez, M. A., y Jara, C. 2004. Identificación de marcadores moleculares ligados a genes de resistencia a mancha angular de fríjol. . XXV Congreso de la Asociación Colombiana de Fitopatología y Ciencias afines (ASCOLFI) , Agosto 11-13 de 2004, CIAT, Palmira.

Henríquez, M.A., Mahuku, G., y Navia, M. 2004. Cebadores específicos para la detección y diferenciación de *Xanthomonas campestris* pv *phaseoli* y *Xanthomonas campestris* pv. *phaseoli* var fuscans en el fríjol común. . XXV Congreso de la Asociación Colombiana de Fitopatología y Ciencias afines (ASCOLFI) , Agosto 11-13 de 2004, CIAT, Palmira.

Students

Name	Degree	Status	University	Title
Monica Navia	BS.	Completed	Universidad del Valle	Elucidación del proceso y eventos de infección del frijol común Phaseolus vulgaris (Fabaceae) con el hongo Phaeoisariospsis griseola (Stilbaceae) agente causante de la mancha angular.
Lorena Cortes	BS.	Continuing	Universidad del Valle	Efecto de las diferentes fuentes de abono verde en el suelo sobre el manejo de hongos causantes de pudriciones en el frijol (Phaseolus vulgaris, L. Fabaceae).
Maria Antonia Henriquez	Ms.	Continuing	Universidad Nacional de Colombia, Palmira	ESTs para entender la interacción entre genotipos del frijol común (Phaseolus vulgaris) y Phaeoisariopsis griseola, el agente causal de la mancha angular.

Trips

Date	Destination	Event or Purpose
June 4, 2004	Ibagué-Tolima	Workshop to train technicians (65) on the agronomic management of snap and dry beans and integrated disease management.
June 24-25, 2004	Bogotá, D.C.	Workshop to train technicians (110) on the agronomic management of snap and dry beans and integrated disease management.
July 30, 2004	Pitalito-Huila	Workshop to train technicians (65) on the agronomic management of snap and dry beans and integrated disease management.
July 11-16, 2004	Kampala, Uganda	Discussion on the workplan to implement molecular techniques for detection of Pythium species that cause bean root rots.

Special Projects

Title	Donor	Comments	Funding Period	Total Amount
Improving rural livelihoods in Rwanda: Promoting integrated crop, disease, and pest management (ICDPM) strategies for intensification and diversification of agricultural systems.	Bilateral project for Belgium	Concept note	3 years (2005-2007)	3 million Euros
Iniciativa Peruana de Rhizobiología : Fijación biológica de nitrógeno para el establecimiento de sistemas agrícolas sustentables y el progreso de los pequeños productores del Perú.	IDRC	Rejected	5 years	CAN$ 999 625