Output 2: Pest-and-disease management components and strategies developed for key crops.

Activity 2.1. Levels of resistance to important insect pests confirmed in bean progenies

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Highlights:

- Resistance to the bean weevil (*Acanthoscelides obtectus*) was identified in *Phaseolus vulgaris* x *P. acutifolius hybrids*
- New accessions and lines with insect resistance were identified

Rationale

A novel Double Congruity Backcross technique developed at CIAT has permitted the development of fertile insterspecific *Phaseolus vulgaris* x *P. acutifolius* (common x tepary) bean hybrids. These crosses are made using the tepary genotype NI576 (a genotype competent to *Agrobacterium*-mediated genetic transformation). Some of these crosses involve the tepary accession G 40199 an excellent source of resistance to the bean weevil, *Acanthoscelides obtectus* and leafhoppers. In previous years we identified several progenies containing both *P. vulgaris* and *P. acutifolius* cytoplasm with very high levels of antibiosis resistance to *A. obtectus* and a handful showing acceptable levels of tolerance to the leafhopper, *E. kraemeri*.

Materials and Methods

Depending on the amount of seed available, previously selected genotypes were multiplied in the field or under greenhouse conditions. The seed was then utilized to screen the different nurseries for resistance to *A. obtectus* in the laboratory. In most cases, genotypes were replicated four to five times. Levels of infestation varied form 2 to 3 mature eggs per seed. Percentage adult emergence and days to adult emergence were recorded. In some cases, individual seeds were tested using a level of infestation of two mature eggs per seed. Tests for resistance to *E. kraemeri* were conducted in the field under high levels of natural infestation, usually with 3-4 replicates per genotype in randomized complete block designs. Evaluations for resistance include damage scores and bean production ratings, insect counts, damage counts; in a few cases, yields, and yield components.

Results and Discussion

Acanthoscelides obtectus: In 2005, emphasis was placed upon the reconfirmation of resistance in previously selected progenies. Seeds of resistant hybrids selected in 2004 were multiplied in the greenhouse. The materials were then tested in replicated nurseries. As shown in Table 2.1.1 all but five of the hybrids turned out to be resistant. Resistance in some cases was as high as that of G 40199, the original resistant parent.

Table 2.1.1. Levels of resistance to *Acanthoscelides obtectus* in selected F_5 – F_7 hybrid progenies derived from interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code and generation	Cross	Percentage adult emergence	Days to adult emergence	Rating
Interspecific *P. vulgaris* x *P. acutifolius* hybrids with *P. acutifolius* cytoplasm				
GNVAV 200A9 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	21.9	59.1	Intermediate
GNVAV 200D21 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	50.9	56.5	Susceptible
GNVAV 200D22 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	50.2	55.7	Susceptible
GNVAV 200G16 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	51.4	57.5	Susceptible
GNVAV 200G17 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	44.2	58.4	Intermediate
GNVAV 200G18 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	46.7	53.7	Intermediate
GNVAV 200G19 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	54.5	56.6	Susceptible
GNVAV 200H5 F_7	{[(G40022 x N1576) x V5] x A3} x VS42-7	25.3	61.8	Intermediate
GVV 110G F_6	{[(G40022 x N1576) x V5] x A3} x VS42-7	10.2	65.6	Resistant
GVV 110I F_6	{[(G40022 x N1576) x V5] x A3} x VS42-7	33.1	59.8	Intermediate
GVV 108N F_6	{[(G40022 x N1576) x V5] x A3} x VS42-7	19.4	63.9	Resistant
BWG 1F7 F_5	BW-1 FL x GKA-12 F_3 FB	10.3	64.8	Resistant
BWG 1F13 F_5	BW-1 FL x GKA-12 F_3 FB	76.1	51.7	Susceptible
BWG 1F14 F_5	BW-1 FL x GKA-12 F_3 FB	25.8	57.2	Intermediate
BWG 1F18 F_5	BW-1 FL x GKA-12 F_3 FB	21.4	61.3	Intermediate
BWG 5N1 F_5	BW-1 FL x GKA-12 F_3 FB	13.9	60.1	Resistant
BWG 5N4 F_5	BW-1 FL x GKA-12 F_3 FB	15.8	51.7	Resistant
BWG 6Y6 F_5	BW-1 FL x GKA-12 F_3 FB	18.7	62.2	Resistant
BWG 6Y15 F_5	BW-1 FL x GKA-12 F_3 FB	34.2	43.1	Intermediate
Checks				
G 40168	Susceptible *P. acutifolius* accession	82.2	43.1	Susceptible
G 25410	Susceptible *P. lunatus* accession	91.1	44.5	Susceptible
ICA Pijao	Susceptible *P. vulgaris* cultivar	94.9	32.8	Susceptible
G 40199	Resistant *P. acutifolius* accession	9.6	66.5	Resistant
G 25042	Resistant *P. lunatus* accession	1.5	76.0	Resistant

The important process of testing individual seeds to detect segregation and reconfirm resistance in interspecific hybrids continued in 2005. Results (Tables 2.1.2 and 2.1.3) showed that several of the genotypes tested do possess interesting levels of antibiosis resistance to *A. obtectus*. Seed multiplication of resistant genotypes is in progress.

We also tested several progenies derived from intraspecific crosses made in *P. lunatus.* These had been selected in 2004 for very high levels of resistance to *A. obtectus*. With one exception, all were highly resistant to the bruchid (Table 2.1.4)

Zabrotes subfasciatus (Mexican bean weevil): Resistance to the Mexican bean weevil (*Z. subfasciatus*) has been successfully incorporated into bean cultivars using a backcross breeding method that combines biochemical tests for the presence of arcelin and insect feeding bioassays. Preliminary observations suggested that the incorporation of arcelin in bruchid-resistant lines (coded RAZ) might affect yields. To test this hypothesis, selected RAZ lines and their recurrent parents were yield-tested under field conditions at CIAT headquarters.

Results (Table 2.1.5) suggest that the incorporation of arcelin does have a depressing effect on yields. Most RAZ lines tested yielded less than their respective recurrent parents. Differences in most cases were not significant but nevertheless important. Further testing is planned for 2006.

Table 2.1.2. Reconfirmation of resistance to *Acanthoscelides obtec*tus in pre-selected segregating hybrid progenies derived from single-seed selections performed in interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code and generation	No. of seeds tested	Percentage adult emergence	Days to adult emergence	Rating
Hybrids				
GKVGAG 1B 4D F_6	18	18.5	63.4	Resistant
GKVGAG 1B 4D F_6	13	20.5	64.2	Resistant
GKVGAG 1B 4D F_6	25	23.6	66.0	Intermediate
GKVGAG 1B 4D F_6	18	0.0	NEa	Resistant
GKVGAG 1B 4D F_6	30	36.6	57.6	Intermediate
Mean 5 resistant selections	Variable	19.8	62.8	Resistant
Mean 15 susceptible selections	Variable	72.0	58.3	Susceptible
GKVGAG 1E 2C F_6	23	44.1	63.5	Intermediate
GKVGAG 1E 2C F_6	30	7.9	58.9	Resistant
GKVGAG 1E 2C F_6	11	0.0	NE	Resistant
GKVGAG 1E 2C F_6	36	24.8	61.0	Intermediate
GKVGAG 1E 2C F_6	30	47.7	61.1	Intermediate
GKVGAG 1E 2C F_6	36	27.0	59.9	Intermediate
GKVGAG 1E 2C F_6	18	0.0	NE	Resistant
GKVGAG 1E 2C F_6	30	34.9	59.0	Intermediate
GKVGAG 1E 2C F_6	40	26.3	61.3	Intermediate
GKVGAG 1E 2C F_6	28	27.8	61.7	Intermediate
GKVGAG 1E 2C F_6	60	28.6	57.7	Intermediate
GKVGAG 1E 2C F_6	28	19.1	58.3	Resistant
GKVGAG 1E 2C F_6	15	44.2	57.9	Intermediate
GKVGAG 1E 2C F_6	11	21.2	49.4	Intermediate
GKVGAG 1E 2C F_6	15	22.2	56.2	Intermediate
GKVGAG 1E 2C F_6	20	41.7	65.1	Intermediate
Mean 16 resistant selections	Variable	26.1	59.3	Resistant
Mean 40 susceptible selections	Variable	71.3	56.4	Susceptible
Checks				
G 40168[b]	45	80.1	44.3	Susceptible
G 25410[c]	45	91.1	44.5	Susceptible
ICA Pijao[d]	125	94.9	33.0	Susceptible
G 40199[e]	45	9.6	66.5	Resistant
G 25042[f]	45	1.5	76.0	Resistant

[a] N.E., no adult emergence from resistant seeds; [b] Susceptible *P. acutifolius* accession; [c] Susceptible *P. lunatus* accession; [d] Susceptible *P. vulgaris* cultivar; [e] Resistant *P. acutifolius* accession; [f] Resistant *P. lunatus* accession.

Table 2.1.3. Reconfirmation of resistance to *Acanthoscelides obtec*tus in pre-selected segregating hybrid progenies derived from single-seed selections performed in interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code and generation	No of seeds		Percentage adult emergence	Days to adult emergence	Rating
	Tested	Resistant			
Interspecific *P. vulgaris* x *P. acutifolius* hybrids with *P. acutifolius* cytoplasm					
NNQLAC 1G F_3	17	16	5.0	58.3	Resistant
Interspecific *P. vulgaris* x *P. acutifolius* hybrids with *P. vulgaris* cytoplasm					
T7K2C19 F_4	17	13	8.7	63.7	Resistant
T7K2C21 F_4	10	5	30.8	60.6	Intermediate
T7K2C23 F_4	6	5	12.5	64.0	Resistant
T7K2C25 F_4	4	2	55.6	52.5	Intermediate
T7K2F61 F_4	2	1	25.0	91.0	Intermediate
Checks					
G 40168[a]	20	0	76.8	56.5	Susceptible
G 25410[b]	20	0	90.9	47.3	Susceptible
ICA Pijao[c]	20	0	94.7	34.4	Susceptible
G 40199[d]	20	14	9.3	87.0	Resistant
G 25042[e]	20	18	1.7	91.0	Resistant

[a] Susceptible *P. acutifolius* accession; [b] Susceptible *P. lunatus* accession; [c] Susceptible *P. vulgaris* cultivar; [d] Resistant *P. acutifolius* accession; [e] Resistant *P. lunatus* accession.

Table 2.1.4. Resistance to *Acanthoscelides obtectus* in selected *Phaseolus lunatus* progenies.

Code and generation	Percentage adult emergence	Days to adult emergence	Rating
V5 F_3	0.0	NEa	Resistant
V5 F_3	85.5	57.6	Susceptible
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
V5 F_3	0.0	NE	Resistant
A6 F_4	33.3	57.5	Intermediate
A6 F_4	0.0	NE	Resistant
Checks			
G 40168[b]	67.4	45.2	Susceptible
G 25410c[c]	82.2	50.2	Susceptible
ICA Pijao[d]	90.0	32.1	Susceptible
G 40199[e]	1.1	77.0	Resistant
G 25042[f]	0.0	NE	Resistant

[a] NE, no adult emergence; [b] Susceptible *P. acutifolius* accession; [c] Susceptible *P. lunatus* accession;
[d] Susceptible *P. vulgaris* cultivar; [e] Resistant *P. acutifolius* accession; [f] Resistant *P. lunatus* accession.

Table 2.1.5. Yields of RAZ lines and corresponding recurrent parents. RAZ lines are selected for the presence of arcelin and high levels of resistance to the Mexican bean weevil.

Line or cultivar	Recurrent parent	Yield (Kg/ha)	Difference with respect to recurrent parent		Significance with respect to recurrent parent
			(Kg/ha)	Percentage	
RAZ 160	ICA Pijao	2148	68	3.1	ns[a]
RAZ 165	ICA Pijao	2014	202	9.1	ns
RAZ 166	ICA Pijao	1997	219	9.9	ns
RAZ 162	ICA Pijao	1947	269	12.1	ns
RAZ 159	ICA Pijao	1931	285	12.9	ns
RAZ 154	ICA Pijao	1893	323	14.6	ns
RAZ 161	ICA Pijao	1878	338	15.2	ns
RAZ 158	ICA Pijao	1833	383	17.3	ns
RAZ 157	ICA Pijao	1831	385	17.4	ns
RAZ 153	ICA Pijao	1827	389	17.6	ns
RAZ 156	ICA Pijao	1812	404	18.2	ns
RAZ 155	ICA Pijao	1793	423	19.1	ns
RAZ 164	ICA Pijao	1781	435	19.6	ns
RAZ 163	ICA Pijao	1779	437	19.7	ns
Mean backcrosses to Pijao		1890	326	14.7	ns
ICA Pijao		2216			
RAZ 34	Ex-Rico 23	1178	580	33.0	*
Ex-Rico 23		1758			
RAZ 190	Talamanca	2035	55	2.6	ns
Talamanca		2090			
RAZ 151	EMP 250	1970	0	0.0	ns
RAZ 152	EMP 250	1597	346	17.8	ns
Mean backcrosses to EMP 250		1783	160	8.2	ns
EMP 250		1943			
RAZ 63	EMP 175	1208	404	25.0	ns
EMP 175		1612			
RAZ 65	WAF 2	1641	0	0.0	ns
WAF 2		1545			

[a] ns, not significant; *, significant at the 5% level by Dunnett's test for comparing all treatment means with the mean of a control.

Leafhopper (Empoasca kraemeri): We continued the work on evaluation of interspecific *P. vulgaris* x *P. acutifolius* hybrids. Similar to the work with bruchids, these progenies were obtained by means of the Double Congruity Backcross technique developed at CIAT. We tested 53 progenies of crosses made with the tepary sources of resistance to leafhopper G 40019 and G 40036. Selected progenies and their reaction to leafhopper are shown in Table 2.1.6 In general, the best lines show an intermediate level of resistance comparable to that found in the tolerant check, ICA Pijao. It can also be said that resistance to leafhopper in interspecific hybrids is not as good as the resistance found in *P. acutifolius* accessions G 40036 and G 40019.

Table 2.1.6. Resistance to *Empoasca kraemeri* in selected progenies derived from interspecific *Phaseolus vulgaris* x *P. acutifolius* crosses.

Code	Damage scores[a]	Reproductive adaptation scores[b]	Yield (Kg/ha)		Percentage yield loss	Susceptibility index[c]
			Protected	Non-protected		
A99Y-15F2	5.5	6.3	1212	1109	8.5	0.7
A99Y-86F2	6.0	6.0	1270	1021	19.6	0.9
A99Y-90F2	5.7	6.3	1349	1153	14.5	0.7
TSC123	6.0	5.7	1019	917	10.0	0.8
EMP 558	6.2	6.3	1412	1216	13.9	0.7
EMP 586	4.5	6.3	1769	1655	6.4	0.5
ICA Pijao	5.7	7.7	1761	1380	21.6	0.8
BAT 41[d]	8.4	4.0	1237	752	39.2	1.3
LSD 5%	0.5	1.3	317	379	-	-

[a] On a 1-9 visual scale (1, no damage; 9, severe damage); [b] On a 1-9 visual scale (1, no yield, no pod formation; 9, excellent pod formation and filling, excellent yield); [c] Calculated with respect to the mean of the trial and the mean Pijao, the tolerant check; [d] Susceptible check.

Activity 2.2. Integrated soil fertility / Pest and disease management to address root rot problems in common beans.

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Highlight:

- Mulching with green manures increased bean yields of susceptible bean cultivars and this increase was associated with a reduction in root-rot incidence and the increased soil nutrient availability.

Rationale

The application of organic residues as mulches has been found effective in improving soil quality by increasing nutrient and water availability as well as controlling soil-borne pathogens by stimulating natural antagonistic organisms and/or producing toxic organic substances. Sources of green manure have different decomposition rates that regulate soil moisture and temperature while influencing the release of nutrients and and secondary compounds. This is likely to have different effects on the balance and relative population sizes of harmful and beneficial organisms. Our overall objective was to evaluate the impact organic residues of different qualities have on the abundance and diversity of soil nematodes, pathogenic and arbuscular mycorrhizal fungi (AMF).

Materials and Methods

The experiment was established in Ultisols at the CIAT's Santander de Quilichao Research Station, on an area that had a history of high incidence of root rot pathogens and used for selection of resistant bean genotypes. These soils have been systematically fertilized in the past and thus presented no nutrient limitations to bean plants during the experimental period (pH=6.8, total C = 2.5%, Bray-P II = 45 ppm, Exch. Ca = 10 meq/100 g soil). Experimental plots (size: 6x3m) were planted with the root-rot susceptible bean variety A70 during 4 consecutive seasons and here we report results for the last two seasons (2004A,

2004B). Figure 2.2.1 shows the climatic conditions during the period under report for CIAT's Santander de Quilichao Research Station.

Experimental treatments were covered with three green manures of contrasting quality (Table 2.2.1): (a) rapidly decomposing *Tithonia diversifolia* (TTH); (b) intermediate rate of decomposition by *Cratylia argentea* (CRA); (c) slow decomposing *Calliandra calothyrsus* (CAL) at a rate of 6 ton ha^{-1}; and (d) control (no green manure added). The experiment was replicated five times. The experimental layout is shown in Figure 2.2.2 Soil samples (0-10 cm) were collected within rows during the cropping season including planting and harvesting.

Table 2.2.1. Quality parameters for organic residues added to the soil as mulch. *C* carbon, *N* nitrogen, *ADF* acid detergent fiber, *NDF* neutral detergent fiber, *HEM* hemicellulose, *L* lignin, *PP* polyphenols (Cobo *et al*., 2002, Biol. Fertil. Soils 36:87-92).

Treatment	C %	N	ADF	NDF	HEM	L	PP	C/N	L/N	PP/N	(L+PP)/N
CAL	49.4	2.65	43.7	63.2	19	14.	18.44	18	5.47	6.9	12.43
CRA	44.3	3.28	42.6	64.2	21	17.	4.78	13	5.40	1.4	6.86
TTH	38.8	3.93	25.2	26.6	1	4.	8.65	9	1.16	2.2	3.36

Figure 2.2.2. Layout of the experimental plot at CIAT's Santander de Quilichao Research Station.

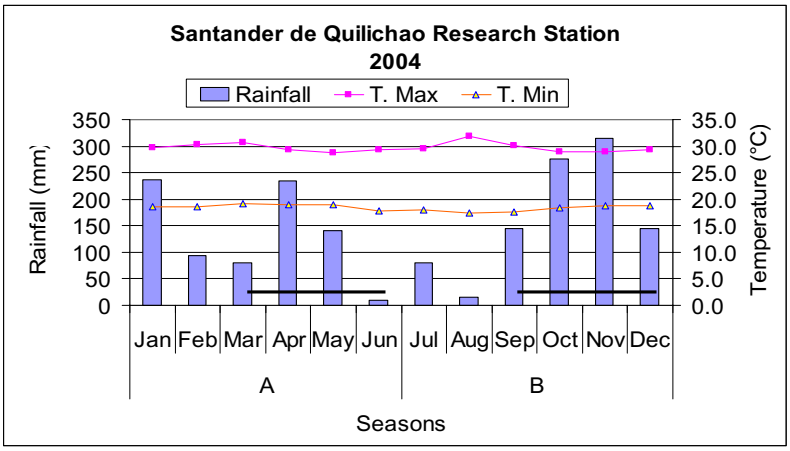

Figure 2.2.1. Climatic diagram during 2004 of CIAT's Santander de Quilichao Research Station. Two dark solid lines represent cropping seasons in 2004A and 2004B respectively.

Immediately after planting, the experimental plots were covered with three green manures of contrasting quality (Table 2.2.1): (a) rapidly decomposing *Tithonia diversifolia* (TTH); (b) intermediate rate of decomposition (but greater soil cover due to leaf morphology) by *Cratylia argentea* (CRA); (c) slow decomposing *Calliandra calothyrsus* (CAL) at a rate of 6 ton ha^{-1}; and (d) control (no green manure added). The experiment was replicated five times. Soil samples (0-10 cm) were collected during the cropping season, including at planting and harvesting. Samples were collected within rows and between rows, to measure the effect of the rhizosphere of bean plants on the soil biota studied.

Evaluation of soil microorganisms: Soil nematodes were extracted by density centrifugation with sucrose solution followed by careful separation by feeding habit. Abundance and diversity of soil pathogenic fungi were studied by serial dilution of soil and plating in selective media: PCNB for *Fusarium* and MA for *Macrophomina* and *Rhizoctonia*, complemented by evaluation of incidence of disease on susceptible plant genotypes. Arbuscular mycorrhizal fungi spores were extracted using density centrifugation with sucrose solution and hyphal lengths estimated using the membrane filtering technique (Miller & Jastrow, 1990, Sol Biol. Biochem. 22: 579-584). In addition, bean yield data was collected to estimate the overall effect of green manure application on crop productivity.

Results and Discussion

Bean yield and root-rot incidence following green manure treatments: Application of green manures consistently resulted in greater bean yields than the control largely as a result of lower root-rot incidence caused by *Macrophomina* (Figure 2.2.3). Significant yield differences among green manure treatments were only observed in the relatively drier 2004A season and CRA showed greatest differences with respect to the control. CAL consistently generated lower root-rot incidence than the control probably as a result of their slow decomposition (Stone *et al*., 2001, Soil Sci. Soc. Am. J. 65:761-770.) and high polyphenol content (Cobo *et al*., 2002, Biol. Fertil. Soils 36:87-92). Effective soil cover due to leaf architecture in CRA was beneficial during 2004A but actually led to increased incidence in 2004B, when soil moisture was greater, and resulted in lower relative yields. Application of TTH in 2004A showed low impact on incidence that contrasted with large incidence reduction during 2004B that was linked to highest relative bean yields. Soil analysis at the end of the 2004B season showed significantly higher total

C in CAL&CRA, total N in CRA&TTH, Bray-II P in CRA,TTH,CAL and exc. K in TTH while CON consistently presented the lowest value.

Changes in abundance and diversity of studied soil organisms: The mean abundance of *Fusarium* (Fus) in 2004B was about 1200 cfu g^{-1} soil and was not significantly affected by green manure treatment. *Macrophomina* (Mac), on the other hand, showed greater incidence in CRA and that is consistent with greater incidence observed in bean plants as shown in Figure 2.2.4. Nevertheless, despite high incidence of *Macrophomina* observed on infected plants we found relatively low abundances in the soil samples analyzed. Satellite experiments have been initiated to explore potential reasons for the limited detection of *Macrophomina* in the soil.

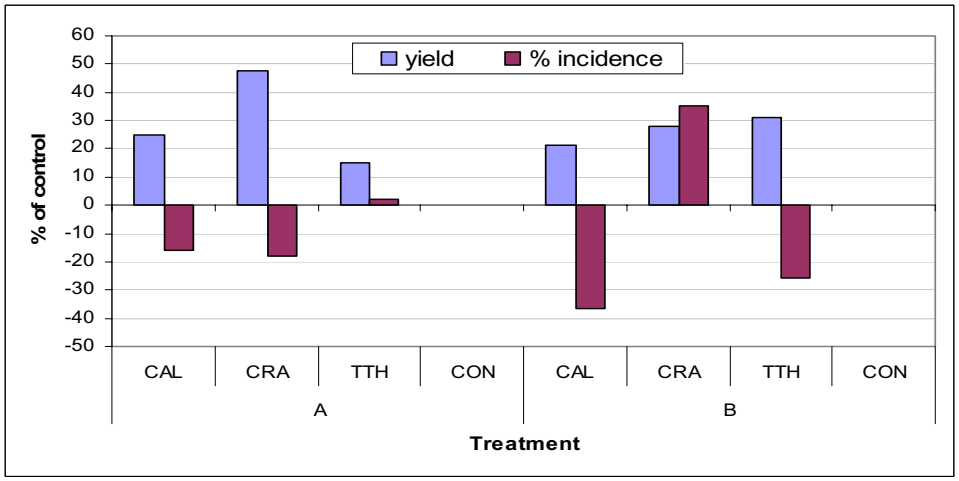

Figure 2.2.3. Crop yield and incidence of root rots of the bean genotype A70 expressed as a percent of the control treatment.

Abundance and diversity of nematodes: TTH consistently presented greater abundance of nematodes across both cropping seasons with values close to twice that of the control (Figure 2.2.5). This is likely a result of the high quality of tissues derived from this nutrient scavenging plant and their contribution to soil nutrient availability (Cobo *et al*., 2002, Biol. Fertil. Soils 36:87-92). Our results corroborate the quick response by bacterial feeding nematodes to increasing biological activity associated with nutrient additions to soil and faster decomposition rates. During 2004 greater differences existed between green manure treatments likely a result of the impact of different decomposition rates on soil moisture dynamics. Also a considerable overall reduction was observed in CON in 2004A but predatory and omnivorous nematodes populations were restored in 2004B when greater soil moisture was available.

Abundance of Arbuscular Mycorrhizal fungi (AMF): Mean AMF hyphal lengths were consistently higher in TTH compared to other treatments across both seasons (Figure 2.2.6). TTH has been previously reported to have high levels of colonization by AMF (Sharrock *et al*., 2004, Mycorrhiza 14:103-109.) and also grow on decomposing TTH shoots and leaves. Conversely, total AMF spore results were inconsistent and suggest the need for future analysis of isolated spores at the genus or species level.

Conclusions

Mulching with green manures generated increased yields of susceptible bean cultivars and this increase was associated with a reduction in root-rot incidence and the increased soil nutrient availability. This effect was most consistent in CAL and TTH, while in CRA it was only observed in the 2004A season. In the wetter 2004B season extended soil cover in CRA probably resulted in excess soil humidity/high root-rot incidence. The relative position of soil nematodes in the food chain and their rapid response to soil management, suggests their potential to influence through "ecological linkages" the population sizes of their food sources (i.e. fungi, bacteria, etc.). Having found greater abundance of fungivorous nematodes and AMF hyphae in TTH suggests no preferential feeding on AMF but rather on other soil fungi. Further studies in this long-term experiment will help testing this hypothesis.

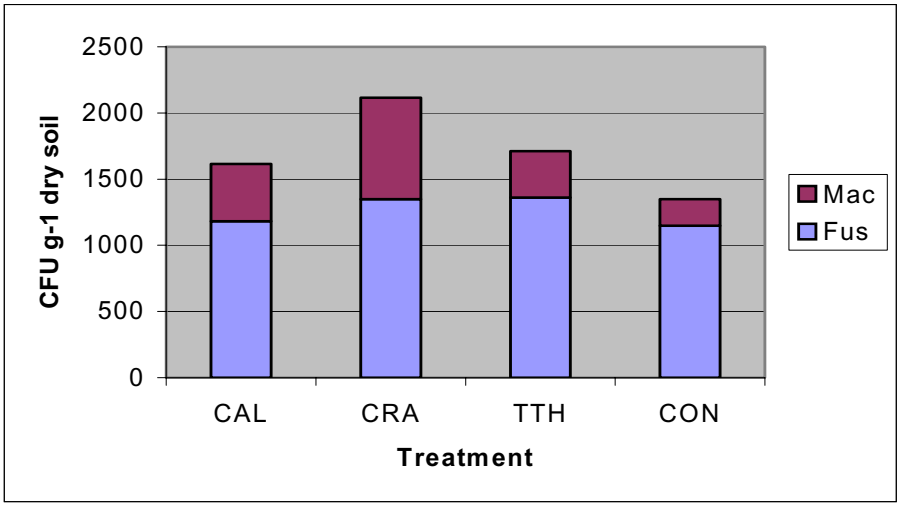

Figure 2.2.4. Abundance of *Fusarium* and *Macrophomina* across experimental treatments in 2004

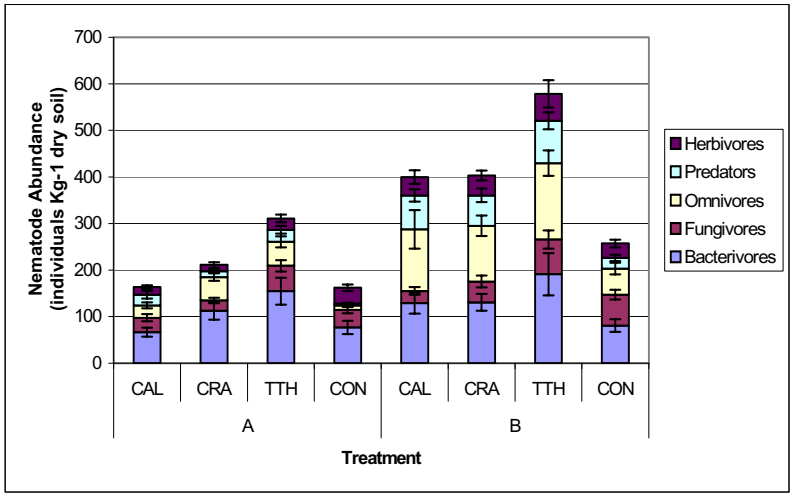

Figure 2.2.5. Abundance of soil nematodes discriminated by feeding habit across experimental treatments in 2004

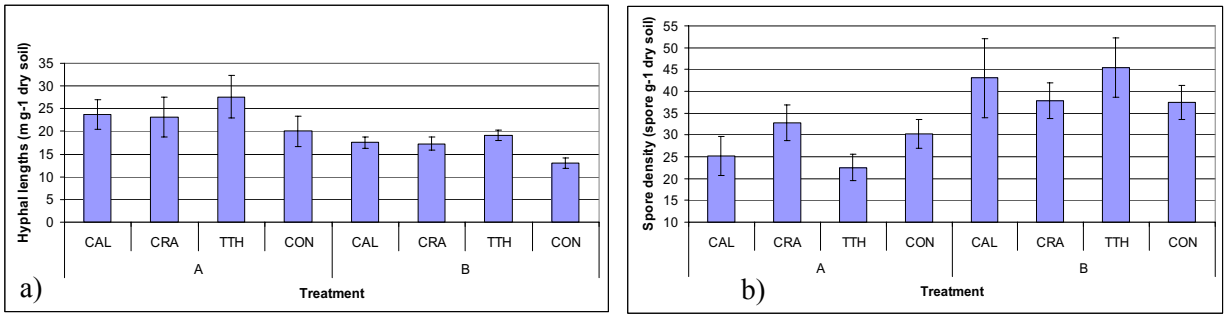

Figure 2.2.6. Activity and abundance of AMF estimated by a) hyphal lengths and b) spore counts in 2004.

Activity 2.3. Developing germplasm with multiple resistances to diseases in beans

Contributors: R. Buruchara, P. Kimani, R. Otsyula (graduate student), S. Sebuliba, A. Male and C. Acam**,** S. Beebe and M. Blair.

Highlights:

- Several bean lines with multiple resistance to Pythium root rot and ALS have been selected and will be distributed to different countries for multi-locational evaluations. Some have already been distributed to Kenya and Malawi

- A SCAR marker associated with ALS resistance gene in Mex 54 has been successful used in selection for ALS resistance

- A collaborative mechanism has been set up to facilitate national partners to integrate application of marker assisted selection (MAS) in their breeding program.

- Several elite lines and germplasm were screened for the presence or absence of I-gene. Donor parents were identified and a breeding program initiated to improving resistance in selected useful materials against BCMNV.

Rationale

In Sub-Saharan Africa, beans are produced both for home consumption and for the market. Beans can contribute to a healthier diet being a major source of proteins and a potentially a good source of iron and zinc for a majority of resource poor communities. Great market potential exists for different market classes of beans. Market expansion for existing products is one means to encourage farmers to produce and market larger crop surpluses. This is significant given that bean production in Africa is almost entirely in the hands of resource poor farmers, usually women. However, achieving food security, nutritional and economic goals is limited in part by the effects of diseases on productivity and quality of the produce. Genetic improvement is considered the most appropriate strategy for poor resources farmers to overcome these constraints. Last year, we reported progress made in improving resistance against two major bean diseases; angular leaf spot (ALS) and Pythium root rots in commercial and adapted bush and climbing bean cultivars. This year we continued selections from segregating populations and lines focusing on seed types and resistance to Pythium root rot, ALS and bean common mosaic virus.

Materials and Methods

Several F_2 derived F_4, F_5, and F6 lines were evaluated at Kawanda for resistance to Pythium root rot, angular leaf spot, common bacterial blight and bean common mosaic virus.
Evaluation for BCMV and BCMNV resistance was done in the field at Kawanda Research Institute, Uganda using natural infection. A BCMV infected crop (through seed) of G 2333 was grown as a border crop and after every 10 test lines.

A key source of resistance to Pythium root rot, RWR 719, suffers from black root when exposed to bean common mosaic necrotic virus (BCMNV) because it carries the "*I*" gene. Lines were also screened for the presence or absence of *I*-gene on the basis of SW13 marker. This was to determine those that may have inherited the "*I*" gene and which would require protection. DNA was extracted from young trifoliate leaves of 2-week old plants using ammonium acetate method. The trifoliate leaves were ground with the aid of sterile sand in an extraction buffer consisting of Tris- sodium chloride, EDTA, proteinase K and SDS. The resulting slurry was incubated at 65C for 1 hour. Ammonium acetate was later added, the DNA precipitated using isopropanol and the resulting pellet washed with 70%ethanol. The yield was determined using a quantifying ladder (Bioline). Each PCR reaction consisted of 5ng of the DNA in a 12.5µl reaction volume consisting of 0.2mM dNTPS, 2mM magnesium chloride, 1XPCR buffer, 0.3mM of each of the primer and 0.1units of Taq polymerase. Each amplification cycle consisted of the following steps: 15 seconds at 94 C, 30 seconds at 53 C, and 1 minute at 72. After 35 cycles the samples were subjected to a final extension for 7min at 72 C and kept at 4 C. Amplicons were resolved on 1.2% agarose gel stained with 10mg/ml Ethidium bromide and the gel subsequently immersed in 0.5XTBE. Electrophoresis was performed at 70V and bands visualized under UV light and the image captured on a digital camera mounted on a computer.

Mex 54 has been shown to be resistant to most races of *Phaeoisariopsis griseola* in Africa. It has been used in several crosses as a key source of resistance against angular leaf spot. The nature of resistance has been characterized. A RAPD marker $OPE4_{709}$ that had been shown to be linked to a resistance gene in Mexico 54 was converted to a SCAR marker. The utility of the latter was validated and applied in marker assisted selection. Young trifoliate leaves from 14-day old plants were used. The protocol described for BCMV was also used except for the annealing temperature. Each amplification cycle consisted of the following steps 15 seconds at 94C, 30 seconds at 65C, and 1 minute at 72. After 35 cycles the samples were subjected to a final extension for 7min at 72C and kept at 4C.

Results and Discusión

Selection of recombinant inbred lines (RILs) for improved resistance to Pythium root rot: In addition to progenies selected last year from 14 crosses, 76 new progenies were selected this year from 6 additional crosses (Table 2.3.1.)

Last year progenies were selected from 16 crosses, 14 of which were used to develop populations by bulking all single plant selection harvested. Those with enough seed were planted at two sites (Rubaya and Kawanda) and others at only one site, giving priority to Rubaya (Table 2.3.1). Selection was based on plants with good architecture, seed types, yield and maturity in addition to resistance to Pythium root rots. A total of 242 progenies were selected from 13 crosses in Rubaya, while 320 progenies were selected from 10 crosses in Kawanda (Table 2.3.2).

Table 2.3.1. F_5 lines resistant to *P. ultimum* derived from F_2 resistant progenies by pedigree method. Kawanda, 2004A.

Pedigree	Line Code	Number of resistant progenies
GLP 585 x AND 1055	RF RO2-24	27
CAL 96 x AND 1062	RF RO2-35	13
URUGEZI x RWR 719	RF RO2-41	1
URUGEZI x SCAM 80CM/15	RF RO2-43	24
URUGEZI x AND 1055	RF RO2-44	7
URUGEZI x AND 1062	RF RO2-45	4

The trials in Rubaya were heavily infected with BCMNV, and therefore in 2005A they were re-grown at Kawanda under high incidences of BCMNV and selection was based on the resistance to this disease. From this, only 28 progenies were selected from 10 crosses (Table 2.3.2).

Selection of recombinant inbred lines (RILs) for improved resistance to angular leaf spot: Over 500 recombinant inbred lines (RILs) developed for resistance to Pythium root rots were also evaluated for ALS under field conditions at Kawanda. Selection was based on a score of less than 3.9 in the 1-9 CIAT disease severity scale. A total of 139 progenies were selected.

Multiple resistance crosses selected for ALS and Pythium root rot: Of the 285 F_6 progenies derived from crosses to combine resistance to Pythium and angular leaf spot, 196 were screened for Pythium resistance under screenhouse conditions. Of these, 85 had a score Ò3.9 on the 1-9 CIAT scale (Table 2.3.3).

Table 2.3.2. F_2 derived F_5 populations selected for Pythium root rot and BCMV, Uganda**.**

Crosses	Rubaya (2004B)		Kawanda (2004 B)		Rubaya lines selected in Kawanda (2005 A)	
	Lines planted	Lines selected	Lines planted	Lines selected	Lines planted	Lines selected
GLP-2 x RWR 719	200	17	1400	64	17	1
GLP-2 x MLB-49-89A	200	32	456	30	32	3
GLP-2 x SCAM 80CM/15	200	18	434	25	18	0
GLP-2 x AND 1055	23	7			7	1
GLP-2 X AND 1062	200	33	172	22	33	0
GLP 585 x RWR 719	200	39	780	7	39	4
GLP 585 x MLB-49-89A	182	55			55	1
GLP 585 x AND 1055		13			13	0
GLP 585 x AND 1062		10	126	10	10	1
CAL 96 x RWR 719		24	128	24	24	4
CAL 96 x AND 1055		11	45	11	11	0
URUGEZI x RWR 719		18	160	18	18	2
URUGEZI x MLB-49-89A		43	200	43	43	5
URUGEZI x AND 1062			200	0		6
Total selected		242		320		28

Single plant progenies selected for resistance to Pythium root rot and ALS are shown on Table 2.3.4. These consist of materials representing different market classes and types including the Calima, black, carioca, pink, grey, purple and cream; and large, medium and small seed types. It is interesting to note that 65 % of the selected progenies are of the preferred Calima seed types.

Application of (OPE4 $_{709}$) marker to select for ALS resistance and SW13 to determine presence of I-gene: Screening for I-gene on the 285 progenies using SW13 marker was initiated and is still on-going. Of these, 251 have already been screened, and 22 of have the "I" gene only, while, 122 were positive for the OPE4$_{709}$ SCAR marker, and 31 were positive for both the OPE4$_{709}$ SCAR and *I-* gene markers (Table 2.3.5).

Table 2.3.3. Evaluation (screenhouse for Pythium and OPE4 $_{709}$ SCAR marker for ALS) of progenies derived from crosses to combine Pythium and ALS resistance, Kawanda, 2005A.

	Pythium Evaluations				ALS Evaluations		
		Disease severity (on 1-9 CIAT scale) Screenhouse					
Combined Crosses	No. of progenies evaluated	1	1.1-3.9	Ò8.9	Number of progenies evaluated	Selected in screen house	Selected by marker (SCAR OPE4 $_{709}$)
CAL 96 x RWR 719) x CAL 96 x MEX 54)q	96	2	38	56	106	24	44
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	64	2	35	27	129	68	20
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	36	0	8	28	50	21	7
Total	196	4	81	101	285	113	71

Table 2.3.4. Selections made combing Pythium and ALS representing different seed types from crosses in 2005

Pedigree	Line Code	Selected plants	Seed color	Seed size within a market class	No of progenies within a seed size
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	13	black	small	6
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	5	black	medium	12
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	33	calima	small	38
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	41	calima	medium	47
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	14	calima	large	3
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	1	carioca	small	1
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	1	cream	small	7
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	21	cream	medium	14
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	1	cream	large	2
CAL 96 x MLB 49-89 A) x CAL 96 x MEX 54)	RF RA 02-3	1	grey	small	1
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	1	pink	medium	1
CAL 96 x SCAM 80 CM/15) x CAL 96 x MEX 54)	RF RA 02-6	1	pink	large	1
CAL 96 x RWR 719) x CAL 96 x MEX 54)	RF RA 02-1	1	purple	medium	1
Total selected plants		134			

Key to seed sizes, small =22 gm >, medium =23 – 35 gm, Large = 36 – 45 gm Market class colors are based on the primary colors.

Table 2.3.5. F_5 progenies screened for the SW13 and OPE_{709} SCAR marker.

Pedigree	Number of families with		
	$OPE4_{709}$ SCAR marker only	**SW13 marker only**	**$OPE4_{709}$ and SW13 markers**
F1 (CAL 96 x RW 719) x F1 (CAL 96 x MEX 54)	35	11	16
F1 (CAL 96 x RWR 719) x F1 (CAL 96 x BAT 332)	0		
F1(CAL 96 x MLB-49-89A) x F1(CAL 96 x MEX 54)	65	9	14
F1(CAL 96 x SCAM 80- CM/15) x F1 (CAL 96 x BAT 32	0		
F1(CAL 96 x MLB-49-89A) x F1(CAL 96 x BAT 332)	0		
F1(CAL 96 x SCAM 80CM/15) x F1CAL 96 x MEX 54)	22	2	1
Total	122	22	31

Development of backcross (BCs) populations with Pythium root rots resistance: Parallel to the development of RILs, a backcrossing program to transfer resistance into popular market class types (backgrounds) was initiated in 2004. Twenty (20) backcross populations were generated. Currently $BC_{s5}F_3$GLP2 x RWR 719 with 111 progenies have been given to partners in Kakamega and was also planted in Kawanda for seed multiplication and homogeneity studies. The other populations are in the field as bulk progenies and will be planted as single plant selections to develop progenies in the coming year. They will be made available to partners for heterogeneity test and for selecting lines of interest (resistance and farmer preferences) under different environments.

In addition, BC_{S3}F1 was advanced to BC_{S4}, and BC_{S3}F2 was advanced to F3 with the objective of trapping seed with the background of the susceptible parent before BC_{S4}

Seed distribution: A total of 557 RILS (F_6) were developed last year. This year the first set of RILS was distributed to Namulonge in Uganda, Kakamega in Kenya, and Malawi for multi-location evaluation with farmer participation (Table 2.3.6).

Conclusion: Considerable progress was made in efforts to improve resistance against two key constraints in Africa. The application of markers in progeny selection introduced a dimension which will enhance the efficiency and effectiveness of the process and allows the pyramiding of key traits.

Table 2.3.6. Seed distribution of recombinant inbred lines in 2005[a].

Crosses	Namulonge	Kakamega	Malawi
GLP-2 x RWR 719	29	29	14
GLP-2 x MLB 49-89A	31	31	14
GLP-2 x SCAM 80CM/15	5	5	2
GLP-2 x AND 1055	5	5	
GLP-2 X AND 1062	6	6	1
GLP 585 x RWR 719	7	7	2
GLP 585 x MLB 49-89A	54	54	43
GLP 585 x SCAM 80CM/15			
GLP 585 x AND 1055	12	12	3
GLP 585 x AND 1062	12	12	5
CAL 96 x MLB 49-89A	5	5	
CAL 96 x AND 1062	11	11	3
URUGEZI x RWR 719			
URUGEZI x SCAM 80CM/15	1	1	1
URUGEZI x AND 1055	1	1	1
URUGEZI x AND 1062	3	3	1

Activity 2.4. Application of MAS in progeny evaluation and selection within improved populations of beans

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Rationale

Marker-assisted selection (MAS) offers advantages in speeding and improving effectiveness of breeding and in pyramiding desired genes into commercial backgrounds. This is potentially useful within NARS breeding programs where breeders are taking on more challenging and complex breeding objectives and schemes. Since most NARS breeding programs have limited access to facilities for applying MAS, a mechanism is being developed to facilitate adoption and adaptation of MAS as a routine procedure in breeding programs through a regional collaborative (networking) approach. Networking has been successfully used to transfer technology, germplasm and experience horizontally across a number of countries. The objective of this activity was to facilitate NARS programs to integrate and apply MAS for certain traits of interest where markers have been identified, using the biotechnology laboratory at Kawanda, Uganda.

Mex 54 is resistant to most races of *Phaeoisariopsis griseola* found in Africa and has been extensively used to improve resistance of commercial but susceptible varieties. In previous years we showed that a RAPD primer OPE-04 is associated and segregates with a single resistant dominant gene in Mex 54 (detected when using race 63-39 of *P. griseola*). This RAPD marker has been converted to a SCAR marker ($OPE4_{709}$) offering possibilities for more reliable results in the application of MAS to select resistant progenies for one of the priority traits in Africa.

There is also a growing interest to detect the presence or absence of "*I*" gene in progeny selections particularly where BCMNV is important and in using *bc-3* in breeding for resistance against BCMV and BCMNV.

Materials and Methods

Resistance to angular leaf spot (ALS), Pythium root rots and bean common mosaic necrotic virus are some of the traits of interest in varietal improvement programs of Rwanda, Uganda and Kenya. With the availability of markers and facilities at Kawanda, Uganda, there has been interest to introduce MAS, focusing on certain traits to ensure that priority genes are incorporated into the final products.

Advanced lines developed (F5 and F6) to improve or combine resistances which include angular leaf spot in commercial popular varieties were assayed for markers associated with resistance to the disease to verify the presence of desired alleles. Twenty-six lines were assayed from Rwanda and 75 from Uganda. DNA extraction and assaying were done as described in 2.4.1(i) above. Resistance to ALS was assayed using the SCAR marker associated with resistance gene in Mex 54 (Figure 2.4.1).

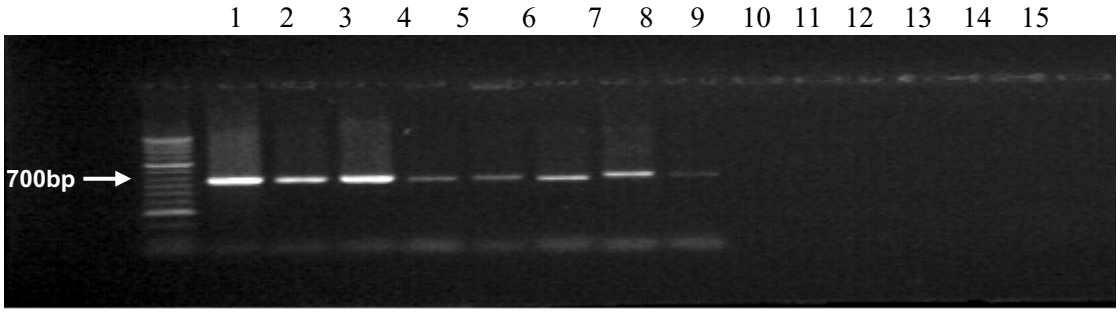

Figure 2.4.1. Results of PCR analysis using the SCAR primer $OPE4_{709}$. Sample 1 is Mex 54 while sample 15 is CAL 96. Samples 2 to 8 show presence of marker while lines and 9 to 14 show absence of the marker.

Results and Discussion

Out of the 26 lines assayed from Rwanda, 60% had the SCAR marker implying presence of the resistance gene. And of the 75 lines that have been assayed so far from Uganda, only 10 (13%) showed the presence of the marker (Table 2.4.1). The lines from Rwanda have previously undergone selection at the ALS hotspot in Rubona, and this probably explains the relatively high frequency of lines that seem to have the resistance gene. Using MAS, it was possible in a short time to repeatedly evaluate and identify lines with resistance to ALS. This approach will be used not only to make further selections from the two countries but will be accessible to other bean networks member countries.

Table 2.4.1. Advanced lines from Rwanda and Uganda screened for ALS resistance using the $OPE4_{709}$. SCAR marker, Kawanda, Uganda.

	Number of Lines		
Line-Code	**Evaluated**	**With Marker**	**Without Marker**
Rwanda Lines			
Urugezi x Puebla x Mex 54	2	2	0
Ngwinurare x SCAM 80CM/15 x Mex 54	4	4	0
Umubano x Mex 54	2	2	0
Vuninkingi x Mex 54	3	3	0
Umubano x Mex 54	6	2	4
Urugezi x Puebla x Mex 54	1	1	0
Ngwinurare x Puebla x Mex 54	3	1	2
Vuninkingi x Puebla x Mex 54	1	0	1
Mexico 54	1	1	0
Vuninkingi	1	0	1
Umubano	1	1	0
Urugezi	1	0	1
Sub-total	**26**	**17**	**9**
Uganda, Namulonge Lines			
Mex 54 x (Kanyebwa x Mex 54)	5	2	3
K132 x (K 132 x Mex 54)	4	0	4
K 132 x Mex 54	10	2	8
Mex 54 x (K 132 x Mex. 54)	14	3	11
Urugezi x Mexico 54	9	0	9
K 20 x Mexico 54	1	1	0
POA 2 x (Mex 54 x G 2333)	17	2	15
K 132 x (Mex 54 x G 2333)	1	0	1
AND 1055 x (Mex 54 x G 2333)	1	0	1
RWR 2075 x (Mex 54 x G 2333)	12	0	12
RWR 719 x (Mex 54 x G 2333)	1	0	1
Sub-total	**75**	**10**	**65**

Activity 2.5. Improvement of bean common mosaic necrotic virus resistance in common bean (*Phaseolus vulgaris)*

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Rationale

Bean common mosaic virus (BCMV) and bean common mosaic necrosis virus (BCMNV) are the most serious viral diseases affecting production of common beans in Africa. A dominant resistance ("*I")* gene confers resistance to a wide range of BCMV strains by inducing a hypersensitive resistant (HR) reaction

to BCMV. With the widespread occurrence of BCMNV in Africa, the potential of genotypes addressing different constraints or consumer objectives, cultivars and released varieties possessing the *I*-gene are at times never fully utilized and in some cases the *I*-gene becomes a liability. For example there are several lines or varieties which have been released and adopted by farmers because of their value against some of the most limiting factors in bean production in the region. These include RWR 719, RWR 2075, RWR 1946 and RWR 1873 which are all very good against root rots and have preferred seed characteristics. Unfortunately, these lines carry the *I*-gene limiting their usefulness to areas having no BCMNV strains or in seasons when occurrence or spread or BCMVN is low. To provide stable, broad-based resistance, a suitable strategy is to protect the *I*-gene in varieties by combining the *I*-gene and race-specific resistance genes (typically *bc-2*[2] or *bc-3*) and also to introgress resistance into key materials that neither have *I*-gene nor any type of resistance to BCMNV. The aim of this effort is to develop high yielding varieties of major market classes that combine and/or are resistant to bean common mosaic necrotic virus (BCMNV). Given that markers for *I* and *bc-3* genes have been identified and mapped, marker assisted selection has potential to speed and enhance effectiveness of the breeding effort.

Materials and Methods

This is a collaborative effort between three countries; Rwanda, Kenya and Uganda. The bean programs in the three countries have either released, or have used the *I*-gene materials in their breeding programs. This implies that several advanced, elite, promising and commercial bean lines have the *I*-gene that may causes the lethal black root reaction with BCMNV. The initial effort was therefore to characterize advanced, elite, promising and released lines for the presence or absence of *I*-gene and initiate protection of *I*-gene in elite and commercial varieties.

Characterization of advanced/elite/promising and released lines for the presence or absence of I-gene, which results in black root against BCMNV: Over 1025 genotypes consisting of advanced lines, released varieties and other germplasm where the *I*-gene was possibly incorporated or whose one of the parent may have contained the *I*-gene were assembled from National Agricultural Research Organization (NARO), Uganda; Kenya Agricultural Research Institute (KARI), ISAR, Rwanda, ECABREN, Regional Breeding programme, Nairobi and CIAT, Kawanda. Preliminary field screening for the presence of *I*-gene was done on about 400 entries at Senge, Kawanda, a hotspot for both BCMV and BCMNV. Lines were categorized as either susceptible with a mosaic reaction, *I*-gene or no symptoms. All plants that showed no symptoms (mosaic or black root) to BCMNV (may include possible escapees) were selected for confirmatory evaluations in the laboratory using the SW13 marker for the presence of *I*-gene. DNA was extracted and assayed as described in section 2.1.1(*i*) for the presence or absence of the *I*-gene. Genotypes of interest having the *I-gene* will be protected while lines or varieties without the *I*- gene will have *I and bc-3* genes incorporated and lines that combines *Ibc-3* genes will be evaluated by farmers or used in other breeding programs.

Protection of I-gene in elite and commercial varieties from the lethal hypersensitive reaction induced by BCMNV: The focus is to protect released varieties and advanced lines, which have desirable agronomic or consumer traits but have the *I*-gene. Seed of sources of resistance (USWK-6, TARS VAR -7, USCR-7, USCR-9, TARS VAR -1) having combinations of *I* and *bc-3* genes were obtained from Miklas (USA) and increased in the greenhouse. Introgression was started with RWR 719 and MLB-49-89A, both important varieties in root rot management. Introgression of resistance genes in more commercial varieties will be done when adequate seed of donor parents is harvested.

Results and Discussions

Field screening for resistance to BCMNV and black root: Three groups of germplasm and lines were evaluated for resistance to BCMV and presence of *I*-gene under field conditions at Senge, Kawanda Agricultural Research Institute. Each entry was planted in plots of 2 rows, 3m long. Plots were separated by 50cm and spreaders were planted after every 10^{th} plot. Plant reaction was assessed on the basis of presence or absence of mosaic and black root at 4 different times during the growth period of the plant.

Of the nearly 400 entries constituting mainly of lines from NARO, Uganda, CIAT Kawanda and Rwanda, about 53 % showed black root (Table 2.5.1) implying a high proportion of materials with *I*-gene.

Table 2.5.1. Reaction of groups of germplasm to natural infection of BCMNV under field conditions.

Germplasm / Lines	Source	Number of entries	Number showing black root
Bush lines	NARO-Uganda	170	94
PPB IYT	CIAT-Uganda	63	38
Climbers (complex crosses)	ISAR, Rwanda	72	28

Screening germplasm for I-gene using SW13 marker: Entries that had been evaluated in the field were also evaluated using the SW 13 marker to compare and confirm observations made in the field. DNA extraction and assaying was done as described above on about 100 lines (Figure 2.5.1) The use of markers was more efficient as it was possible to detect lines that had escaped detection in field evaluations. Out of 65 lines derived from 11 climbing bean population developed in Rwanda, 35 were found to be positive for the *"I"* gene. This allows the bean program to make decisions on how to proceed with these lines given the fact that, much as it conditions resistance to the non-necrosis inducing strains of the virus, the presence of the (*I*) gene renders varieties carrying it to be susceptible to the necrosis inducing strains that are very prevalent in Africa.

Screening of donor materials for the presence of I and bc-3 genes: The objective of this activity was to screen potential donor materials and verify the presence of *I* and *bc*-3 genes. Five donor varieties (expected to contain both the *I* and *bc*-3 genes) namely: USWK-6, TARS VAR-7, USCR-7, USCR-9, TARS VAR-1 were grown in the screenhouse at Kawanda. CAL 96 was included as a negative check. DNA extraction and amplification was done as described above. Amplification was done using SW13 and ROC 11 primers. With the SW13 primer, all donor varieties gave one band while the check variety CAL 96 gave none (Figure 2.5.2). When the ROC 11 primer was used, the reverse was true; the susceptible variety (CAL 96) gave a band while all the rest showed none (Figure 2.5.3). This confirmed presence of *I* and *bc*-3 genes in theses varieties but which lacked in the susceptible variety CAL 96.

Introgressing bc-3 gene into commercial cultivars with I-gene through a backcrossing breeding programme: Because of inadequate seed of donor resistant parents, this activity was initiated by making crosses with two *I*-gene parents RWR 719 and MLB-49-89A. The F_1 populations were generated and will be advanced to F_2 generation by selfing. The F_2 populations will then be evaluated for the presence of *I* and *bc-3* genes.

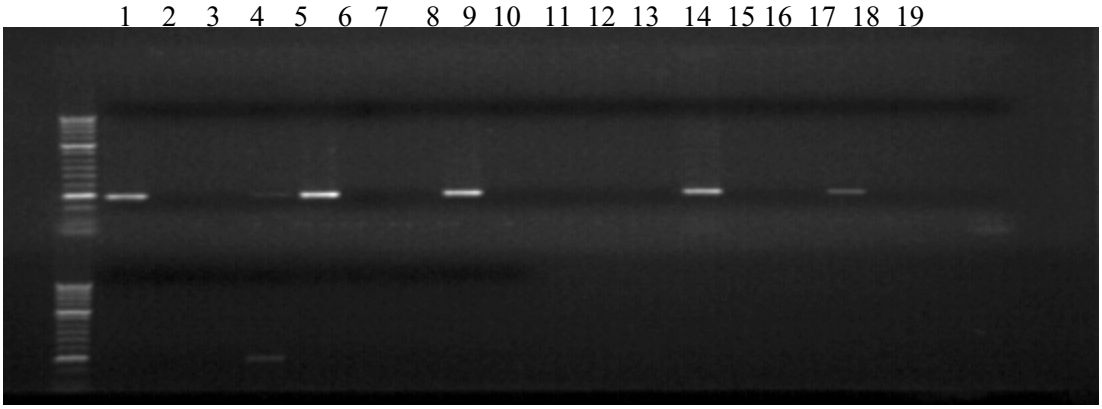

Figure 2.5.1. Left lane is 100 bp ladder. Lane 1 is a positive control variety (USWK 6). Lanes 5, 8, 13, 16 are positive for *I*-gene while the rest are negative.

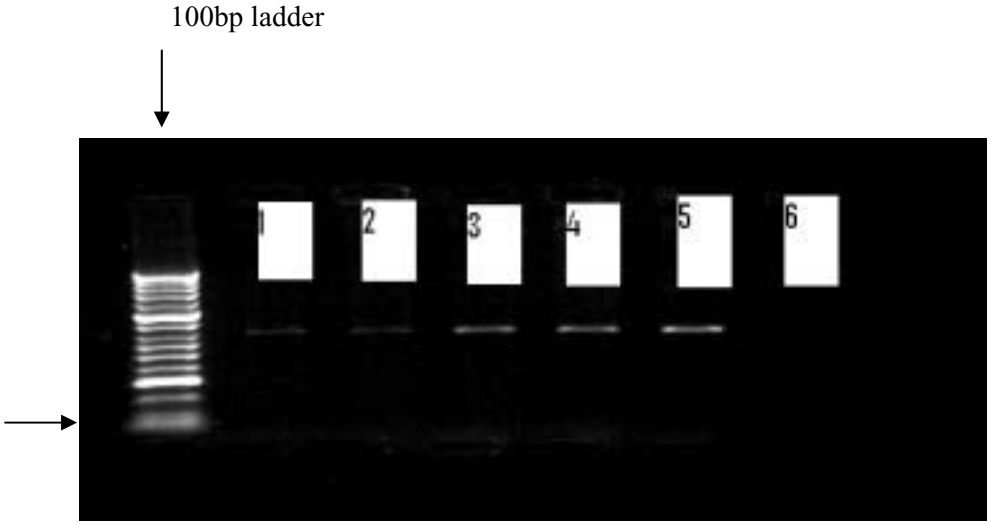

Figure 2.5.2. Amplification of donor parents using SW 13 primer. Lane 1= USWK-6; Lane 2 = TARS VAR-7; Lane 3 = USCR-7; Lane 4 = USCR-9; Lane 5 = TARS VAR-1 and Lane 6 = CAL 96.

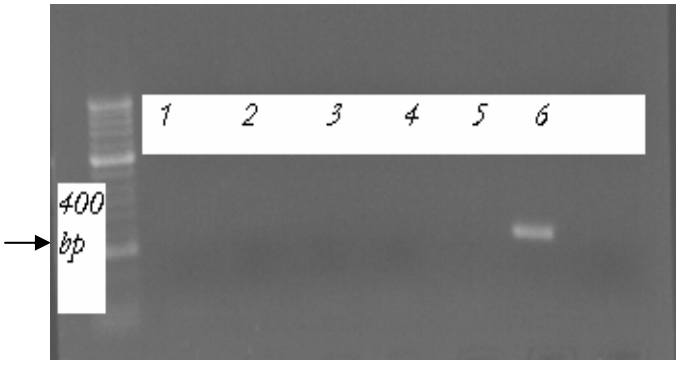

Figure 2.5.3. Amplification of donor parents using the ROC 11 primer: Lane 1 = USWK 6; 2= USCR 9; Lane 3 =TARS VAR 1S; Lane 4 = USCR7; Lane 5 = TARS VAR-1 and lane 6 = CAL96.

Activity 2.6. Improvement of beans (*Phaseolus vulgaris*) for resistance to Fusarium root rot (*Fusarium solani* f.sp. *phaseoli*) in large seed size

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Rationale

Fusarium root rot disease caused by the fungus *Fusarium solani* fsp. *phaseoli* (FSP) is an important soil-borne fungal disease causing Fusarium root rot on common bean (*Phaseolus vulgaris* L.). A number of control measures exist but the use of resistant varieties is considered the most economical method for small-scale bean growers in East Africa. Generally, large seeded bean varieties are preferred in central, south and western Uganda while small seeded bean varieties are more preferred in eastern and northern Uganda. There is also a general preference for large seeded beans at market level. However, large seeded bean varieties generally lack resistance to FSP. Resistance has been reported in small and late maturing varieties having less preferred seed characteristics. The objective of this study is to develop large seeded market class beans in Uganda that are resistant to *Fusarium solani* fsp. *phaseoli.*

Materials and Methods

Characterisation of potential sources of resistance: Germplasm (57 lines) obtained from CIAT Colombia including documented sources of resistance to FSP, Uganda land races (37 lines), Pythium root rot nursery (57 lines), 21 lines from Potchefstroom Agricultural Research Council, South Africa and 10 from the University of Kwa-Zulu Natal were characterized basing on seed color, size, hypocotyl color, flowering and maturity dates, flower color and growth habit.

Isolation and pathogenicity of FSP isolates: A protocol adopted from Burgess et al. (Burgess *et al*., 1994, Lab. manual for Fusarium research, 3rd Edition, pp 133) with several modifications was followed in the isolation of the pathogen from plant tissues. Inoculum was prepared by growing the FSP isolate on sorghum seed in 500ml inoculum bottles for 2-3 weeks. Three isolates, 2 (FSP 1 and FSP 3) which had the characteristic blue centre and white margin and slow growing described for pathogenic FSP and macro-conidia shape (Tusiime, 2003, PhD. Thesis. Makerere University Kampala, Uganda, pp113) and one with white mycelia and fast growing (FSP 2) were tested. The isolates were added to the trays at a rate of 500ml of sorghum inoculum per 74 x 42 x11.5 cm^3 tray of sterilised soil. Seed of three local and popular varieties K20, Kanyebwa and K132 that are susceptible to FSP were planted in the infected soil. Two documented sources of resistance G4830/ Rio Tibagi, and G 4495/ Porrillo Sintetico varieties and MLB-49-89A (partially resistant), were also planted as checks. The trays were watered every two days to provide ideal environment for disease development.

Screening potential sources of resistance: Local and introduced potential sources of resistance were evaluated at Kawanda Agricultural Research Institute as described above and were assessed on the basis of incidence and disease severity (percentage of plant tissue showing symptoms of FSP; where 0% = no visible symptoms and 100% approximately the whole of the hypocotyl and root tissues have lesions). Plants with severity scores of 0-15% were regarded resistant; 15-25% as moderately resistant; 26-49% as moderately susceptible, and ∅50% as very susceptible.

Development of crossing blocks: A recurrent selection with backcrossing program with the aim of introgressing FSP resistance in some popular but susceptible varieties, i.e., K20, Kanyebwa and K132 was developed. Possible sources of resistance RWR 719, NABE 7C and Umgeni were used in a North Carollina Mating Design II. Genetic parameters, i.e., inheritance of FSP resistance (hf^2), inheritance of

seed size (hs^2) will be determined. Mechanism of resistance to FSP will also be investigated as well as effect of some cultural practices, i.e., inoculation techniques, depth of seed sowing, level of soil compaction, level of soil moisture, presence of nematodes and time of planting, to the expression of resistance to FSP by different cultivars. The study will also shade more light on the genetic relationship between seed size and FSP resistance.

Results and Discussion

Pathogenicity Testing: Among the three isolates, FSP-3 was the most virulent with severities ranging from 27.5% (MLB-49-89A) to 85% on K132 and was hence selected for use in screening for resistance and subsequent activities (Table 2.6.1).

Table 2.6.1. Reaction of 5 common bean lines to 3 FSP isolates (% of root and hypocotyls tissues affected)

FSP Isolates	MLB-49-89A	K132	K20	G4830/ Rio Tibagi	G 4495/ Porrillo Sintetico
FSP-1	32.5	65.0	63.5	76.0	85.0
FSP 2	0.0	5.0	5.0	0.0	0.0
FSP 3	25.7	85	78.1	All dead/rotten	All dead/ rotten

Screening for resistance to FSP: Seventy lines have been screened so far for resistance to FSP which include 49 from the CIAT Pythium root rot nursery, and 21 from Potchefstroom. Of these 18 lines gave severities levels of less than 40%. The line MLB-49-89A from the CIAT Pythium root rot nursery showed the highest degree of tolerance to FSP and will be used as a source of resistance. Thirty-five accession from Cali, Colombia and 10 lines from the University of Kwa-Zulu-Natal gave susceptible reactions (severities Ó50%).

Development of FSP crossing block: Blind crosses were made between CAL 96, Kanyebwa, RWR 719, Vuninkingi and Umgeni using a North Carolina Mating Design II. Reciprocal crosses were also made. F1's (18 crosses) from the crossing block have been planted to obtain F2 seed for screening against FSP. F1 seed will also be screened for resistance to FSP before backcrossing to the large seeded parents.

Actiivity 2.7. Pathogenicity of *Pythium spp* and effects of management options for root rots on crops grown in association with beans in southwest Uganda

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

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. 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. *Pythium* is the major pathogen causing severe root rot on beans (Mukalazi, 2004, Pathogen variation and quantification of *Pythium* species in bean fields in Uganda, PhD dissertation, pp 59-60, Makerere University, Kampala, Uganda) and can result in severe losses up to 100 % in some seasons. Other pathogens, which also display root rot symptoms, include *Fusarium spp.* and *Rhizoctonia solani,* which occur singly, or in complexes with other pathogens including *Pythium spp. (*Tusiime, 2004, Variation and detection of *Fusarium solani* f.sp. *phaseoli* and quantification of soil inoculum in common bean fields, PhD. Thesis, Makerere University, Kampala, Uganda, pp 113). *Pythium spp* are known to have a wide host range. This study is part of broader investigations to determine the role of other crops in a bean-based cropping system to the overall root rot problem in southwestern Uganda. Its focus is to determine the pathogenicity of some *Pythium* species associated with major crops found in the bean based systems.

Materials and Methods

Last year we reported initial results of pathogenicity studies which we continued with this year. Four *Pythium* species pathogenic to beans (*P. ultimum, P. chamaehyphon, P. pachycaule and KAK 5B)* were artificially inoculated on four crops commonly associated with beans namely: sorghum, millet, maize and peas. Autoclaved millet (100 g) was mixed with 200 ml of water in a 500-ml bottle which was used to raise the fungi. After two weeks of incubation, the infested millet was mixed with pre-sterilised soil at a ratio 1:8 v/v in wooden trays. Maize, sorghum, millet and peas 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 symptoms that may be associated with *Pythium* infection using the CIAT 1-9
scale.

Results and Discussion

Emergence of various crops determined one week after planting was not significantly different from the control in all the three trials. However, the Pythium isolates significantly affected the level of disease on CAL 96, sorghum and peas (Tables 2.7.1 & 2.7.2). Maize and millet were not significantly affected by the *Pythium* isolates and gave disease scores of less than 5.0. Sorghum and peas were significantly affected by bean pathogenic *Pythium spp* thus suggesting that these two crops could be playing a role in the root rot problem in southwestern Uganda where they are major intercrops or rotation crops. The root dry matter yield of the various crops did not show any significant difference from the control.

Table 2.7.1. Pathogenicity of bean pathogenic *Pythium* species on CAL 96, maize, sorghum, millet and RWR 719.

Treatment	Trial one					Trial two				
	CAL96	Maize	Sorghum	Millet	RWR 719	CAL96	Maize	Sorghum	Millet	RWR 719
Control	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MS61	6.59***	2.77***	5.67***	1.67ns	1.1ns	6.89***	3.75***	8.03**	1.0ns	2.86***
VIH 2A	4.55***	4.33***	4.11***	5.16***	1.0ns	7.41***	4.61**	8.00**	1.0ns	2.5***
KAK 5B	3.97***	1.89ns	6.44***	1.89ns	1.05ns	8.92***	2.83**	7.39**	1.0ns	2.75***
JM 29 A	5.92***	2.00***	6.00***	3.22***	1.13ns	5.92***	3.5**	6.91**	1.0ns	2.97**

Table 2.7.2. Pathogenicity of bean pathogenic *Pythium* species on CAL 96, peas and RWR 719

Treatment	Trial one			Trial two		
	Disease severity			Disease severity		
	CAL96	Peas	RWR 719	CAL96	Peas	RWR 719
Control	2.2	2.2	2.3	1.0	1.0	1.0
MS61	5.1***	5.4***	2.8ns	4.86***	8.36***	2.97***
VIH 2A	5.7***	5.9***	2.6ns	5.44***	7.72***	2.75***
KAK 5B	4.4***	4.5***	2.7ns	4.72***	5.75***	2.64***
JM 29 A	6.1***	7.6***	2.9***	5.39***	7.31***	4.33***

ns - not significantly different (P>0.05)
***-significantly different (P<0.05) by Dunnett test

Activity 2.8. Effects of management options for bean root rots on crops grown in association with beans in southwest Uganda

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Rationale

In southwestern Uganda beans are often grown in association with other crops, i.e., maize (79%), sorghum (52%), peas (46%), Irish potatoes, sweet potatoes and yams. At any one time there are at least three crops grown together. Some *Pythium* spp have been isolated from the roots of some of these crops (CIAT 2004, Africa: Bean Pathology. CIAT annual report 2004, pp 347 – 350). Besides, *Pythium* spp pathogenic to beans have been observed to cause disease on some crops grown in association with beans. To understand if 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 we continued with some of the studies we initiated last year to document the level of root rot in the major intercrops of beans in this region and to determine the effects of management options for bean root rots on crops grown in association with beans.

Materials and methods

The study was done on farmer fields in Rubaya sub-county, Kabale District in southwestern Uganda, a hotspot for bean root rots. Eight farmer fields with a history of bean root rot incidence and severity were selected on the basis of discussions, interviews and experiences of farmers.

Effect of root rots on major bean intercrops: Root rots were examined on three major intercrops of beans i.e. maize, sorghum and peas grown in a trial that also assessed different management options that are known to reduce root rots in beans. CAL 96, a susceptible bean cultivar was grown as a check.

Effect of management practices: The effects of four root rot management options i.e. farmyard manure, green manure (*Crotalaria)*, NPK fertilizer and a fungicide (Ridomil) previously known to have useful effects against bean root rots were evaluated on sorghum, maize, peas and beans. Farmyard manure and green manure (Crotalaria) were applied on a dry weight basis at a rate of 5t/ha. NPK fertilizer was applied at a rate of 50KgN/ha. Fungicide (Ridomil) was applied as slurry at a rate of 2.5 kg/ha. A plot with no soil amendment was used as a control. Qualitative data was obtained through field observations and photography. Quantitative data included plant stand and disease incidence at 18, 36, 54 and 72 days after planting (DAP) and disease severity at 36, 54 and 72 DAP. Disease severity was evaluated according to the CIAT nine-point scale where 1 is resistant and 9 susceptible. Other parameters included plant vigor and dry matter yield at 36 and 54 days respectively.

Results and Discussion

Effect of root rots on major bean intercrops: Root rot lesions were observed on all the crops indicating the presence of infection by root rot pathogens. Above ground symptoms could be seen on beans and sorghum at 18 days after planting. In beans, damping off at seedling stage, yellowing of leaves, stunted growth and, in severe cases, deaths of plants was observed. Infected roots had brown water soaked lesions and in the severe cases were rotten. Plants that survived to late stages were stunted, yellow and produced a few flowers and pods. Severely infected plots had a poor plant establishment. Sorghum plants exhibited stunted growth, purpling of leaves (Figure 2.8.1), shoot death, extensive tillering, prop root development, dark-red to black root lesions and ultimately a poor plant establishment. Purpling and yellowing seems to be due to nutrient deficiency probably excerberated by reduced root surface as a result of root rot damage. Tillering and prop root development are mechanisms developed by the plant for more water and nutrient uptake, thus its survival.

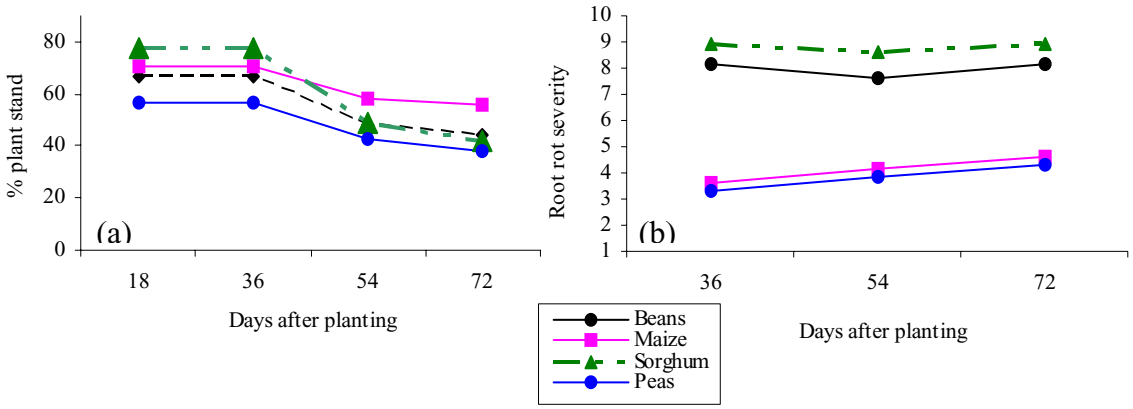

Figure 2.8.2. Plant stand during 2004B season (a), and root rot severities during 2004B season (b).

Plant death was observed on sorghum and beans during the season (Figure 2.8.2a). Similarly, higher lesion severity was recorded in sorghum (> 8) and beans (check) (> 7) than in maize (> 4) and peas (> 3) (Figure 2.8.2b).

Figure 2.8.1. Purpling in severely affected sorghum plants

The above results indicate that sorghum and beans were most affected and are hence more susceptible to root rots than maize and peas. In southwest Uganda, sorghum and beans are the major crops grown (in large acreage) in rotation with each and their susceptibility may indicate that they may be contributing to the root rot pathogen inoculum build-up in soil. However, the high level of susceptibility of sorghum to root rot was unexpected and these results imply that the effects of root rots on sorghum and its role in inoculum build-up may have been underestimated in the past.

Effects of management options on incidence and severity of root rots: The amendments improved crop tolerance to root rots early in the season. They improved crop survival, reduced root rot severity and increased dry matter relative to the control (Tables 2.8.1 & 2.8.2). Improved dry matter production and vigor under Ridomil is probably due to its protective effect against *Pythium* species. Plant recovery was evident in plots amended with GM, FYM and NPK. In addition, FYM, GM and NPK, enhanced root (mass) growth. FYM and GM improve soil physical properties, which probably enhance plant tolerance, and create conditions unsuitable for the root rot pathogens. Ridomil and farmyard manure were more effective resulting in lower incidence and severity in all seasons compared to other treatments. Though GM improved crop tolerance (increase in DM & vigor) it increased root rot incidence and severity early in the season (Table 2.8.1). This necessitates delaying planting to allow decomposition. Effectiveness of the management options varied with time in the season with Ridomil and FYM being more effective early in the season i.e. up to 36 days after planting, while GM from mid to late in the season (Table 2.8.1). From 54 days after planting no significant difference ($P>0.05$) in severity was observd in all the treatments.

Enhancing nutrient availability by addition of NPK also improved crop tolerance as it increased vigor and dry matter yield. NPK resulted in the highest vigor and dry matter yield (Table 2.8.2). This demonstrates the importance of adequate soil nutrient supply in enhancement of crop tolerance to root rot. Manipulation of the variation in effectiveness of the amendments can therefore be exploited for developing a management strategy combining two or more compatible control methods.

Thus using these options does not only contribute to the management of bean root rots, but is also beneficial to other crops. Similar amendment effects were observed in all crops except for GM and FYM in peas. FYM and green manure increased root rot incidence and severity in peas (Table 2.8.1). However, Farmyard manure (FYM) compensated for this effect as shown by the pea dry matter yield (Table 2.8.2).

Conclusion: The major intercrops/ rotational crops of beans in southwestern i.e. sorghum, maize and peas are affected by root rots. Sorghum like beans in this region is susceptible to root rots while maize and peas are tolerant. This suggests that these crops may be contributing to the root rot inoculum load in beans and focus needs to be given to root rot control in these other crops as well. More still, rotations involving beans and some crops such as sorghum may not be helpful in reducing bean root rots. The bean root rot management practices are equally useful to other crops in the system. Delayed planting after addition of organic amendments especially green manures improves their effectiveness against bean root rot pathogens. Therefore in developing management strategies for root rots, it would be advantageous to consider a systems' approach rather than a commodity. However, there is need to investigate the root rot organisms responsible for root rots of the major crops in this region. It is also important to investigate the role of other possible alternative hosts of root rot pathogens of beans apart from maize, sorghum and peas in this region; the effect of a wide range root rot management options; effects of serial application of green manures and compost manures and the socio economic aspects of using these management options in this region.

Table 2.8.1. Effect of soil amendments on root rot severity in beans, maize sorghum and peas from field trials in Rubaya, 2004B season.

Crop	Soil amendment	Days after planting (DAP)		
		Root rot Severity		
		36	54	72
CAL 96	Control	5.987	7.937	8.388
	Farmyard manure	5.750	7.350	8.150
	Green manure *(Clotalaria)*	5.699	7.455	8.186
	Ridomil	5.900	8.000	8.000
	NPK (17;17;17)	6.275	7.475	8.075
Maize	Control	3.913	4.750	5.737
	Farmyard manure	3.137	3.863	4.413
	Green manure *(Clotalaria)*	4.080	4.519	4.293
	Ridomil	3.038	3.625	4.425
	NPK (17;17;17)	3.775	4.050	4.338
Sorghum	Control	8.563	8.750	8.750
	Farmyard manure	7.050	8.625	9.000
	Green manure *(Clotalaria)*	8.042	8.693	8.954
	Ridomil	7.025	8.488	9.000
	NPK (17;17;17)	6.667	8.622	8.924
Peas	Control	3.100	3.737	4.450
	Farmyard manure	3.450	4.188	4.400
	Green manure *(Clotalaria)*	4.046	4.304	4.759
	Ridomil	2.550	3.588	4.175
	NPK (17;17;17)	3.212	3.475	3.825
CV%		**23.300**	**13.000**	**12.000**
LSD (0.05)		**0.526**	**0.788**	**0.778**

[1]Within columns, means with the same letter (s) and no letters do not differ significantly at P>0.05
[2]Season 2004B (September to January)
[3] Disease scale (1-9) 1 = no root symptoms; 3 = 10% of the hypocotyl and root tissues have lesions; 5= 25% of the hypocotyl and root tissues lesions 7= 50% the hypocotyl and root tissues have lesions and the root system suffers a considerable decay; 9= 75% or more of the hypocotyl and root tissues have lesions and the root system suffers advanced stages of decay and considerable reduction.

Table 2.8.2. Effect of bean root rot management practices on dry matter production (54 days after planting) for beans, maize, sorghum and peas from Rubaya, Kabale District, 2004 B season' g/ 5 plants for beans, sorghum and peas; g/3 plants for Maite.

Treatment	Beans	Maize	Sorghum	Peas
Control	9.71b	12.71c	2.81b	15.04 c
Farm Yard Manure	12.81b	28.65 a	6.24a	25.30 a
Green Manure	14.85a	20.19 b	3.44a	14.66 c
Ridomil	12.20b	14.65 c	2.70b	16.15 bc
NPK	13.21a	23.08 b	3.29a	19.76 b

[1]Means of the same letter(s) and no letters within columns do not differ significantly at P≤0.05
[2]Season 2004B (September to January)
Adaptation scale (Abawi and Pastor-Corrales, 1990, CIAT publication, 114 pp), 1- most vigorous; 9- least vigorous.

Actiivity 2.9. Assessment of the potential of candidate organism as a biocontrol agent against Pythium root rot

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Rationale

Root rots of beans have become increasingly important in several areas of eastern and central Africa. Occurrence and severity are associated with high intensity of bean production, and where intensification of land use has resulted in reduced crop rotation and fallow periods, leading to decline in soil fertility and a build up of soil pathogen. Management strategies considered mainly include use of resistant varieties, crop rotation, cultural practices and the use organic and inorganic amendments. Biocontrol agents offer an option that could contribute in strategies to manage *Pythium* root rot. The objective of these studies is to identify and evaluate the interactions between naturally occurring soil-borne disease-moderating organisms that have potential in the management of *Pythium* root rot.

Materials and Methods

Laboratory screening: Seventeen isolates (MS46, MS49, MS11, MS47, MS34, DFD47, KIS4, MS15, MS27, MS6, MS66, KLE3A, MS61 (*P. ultimum*), VIH4, KB4, MS10 (*Mortierella*) and KB14) were screened against each other for their potential use as biocontrol agents. The antagonistic activity of the isolates were determined in a dual culture assay in which opposite ends of the potato dextrose agar (PDA) plate was inoculated with an isolate and incubated at 24°C. Qualitative data of interactions (inhibition or lack of it) were recorded after 48 h of incubation. Four isolates (KB 14, KB 4, VIH 4 and MS 61) showed mutual inhibition on contact with MS10 whereby the space between the two organisms was small but clearly marked (Figure 2.9.1).

Screen house evaluation: Inoculum of each isolate was raised independently on millet grains. Pythium isolates were evaluated alone and in combination with the biocontrol agent. In one treatment the isolates were each mixed with pre-sterilized soil in a ratio of 1:8 v/v inoculum / soil in a wooden flat tray of 42 cm x 72 cm and left to stabilize for one week before antagonist MS10 was added in the same ratio and mixed with the soil. Planting was done after one week.

In another treatment inoculum of each of the five isolates (KB 14, KB 4, VIH 4, MS 61, and MS10) were each mixed with pre-sterilized soil in a ratio of 1:8 v/v inoculum to soil in wooden flat trays after which MS10 was added and planting done immediately. This was aimed at assessing the appropriate planting time after inoculation.

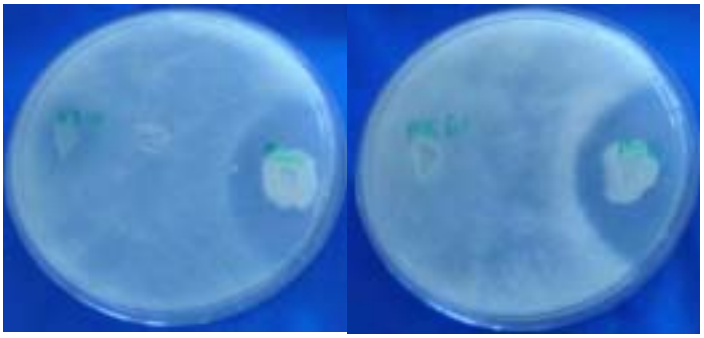

Figure 2.9.1. Level 3 of KB14 and MS61 vs MS10 on culture.

Twenty seeds of susceptible bean varieties CAL96 and K20 as well as resistant bean variety (RWR 719) were planted in two rows, each row consisting of 10 plants. A germination count was taken after full emergence. Disease evaluation was done 21 days after planting by uprooting the plants (Figures 2.9.2 & 2.9.3), rinsing the roots and scoring them according to the CIAT 1-9 scale where 1 = no visible symptoms, and 9 = complete discoloration of the tap root.

Figure 2.9.2. KB4 alone

Figure 2.9.3. KB4 vs MS10

Results and Discussion

Isolates KB14 (*P. ultimum*), KB4 (*P. vexans*) and MS61 (*P. ultimum* var. *ultimum*) applied singly showed severe root rot disease on CAL 96 confirming that these isolates are pathogenic to beans VIH4 and MS10 (*Mortierella*) isolates were non pathogenic to both susceptible bean varieties. Marked reduction in disease severity was observed in treatments where MS10 was added as antagonist to the pathogenic Pythium isolates KB14, KB4 and MS61. The highest disease severity was recorded in susceptible variety CAL 96 followed by K20. RWR 719 which is a resistant variety.

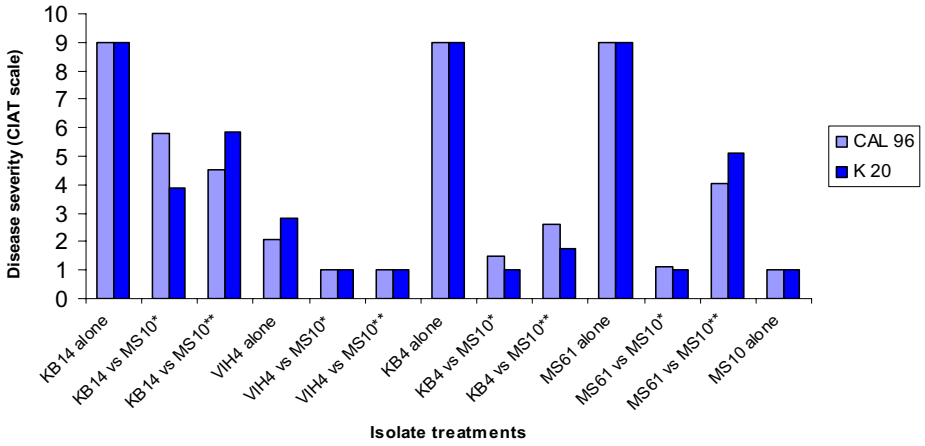

Figure 2.9.4. The effect of MS10 on pathogenic *Pythium* isolates: * = planting done one week after addition of antagonist MS10: ** = planting done immediately after addition of MS10

The results further indicate that application of MS10 one week prior to planting reduced disease severity better than applying followed by immediate planting. The one week interval before planting probably allowed the MS10 to colonize the soil and limit the effects of the pathogens. Application of MS10 also resulted in significant increase in root and shoot dry weights of susceptible varieties CAL 96 and K20 (Figures 2.9.4 & 2.9.5). It is logical to expect reduced root and shoot dry weight on plants with high disease severities as shown by isolate KB4 (highest disease severity and lowest root dry weight). It is not yet known how MS10 works but it was evident that its effects were influenced by the method and time of application. Microbial parasites and antagonists need time to have an effect.

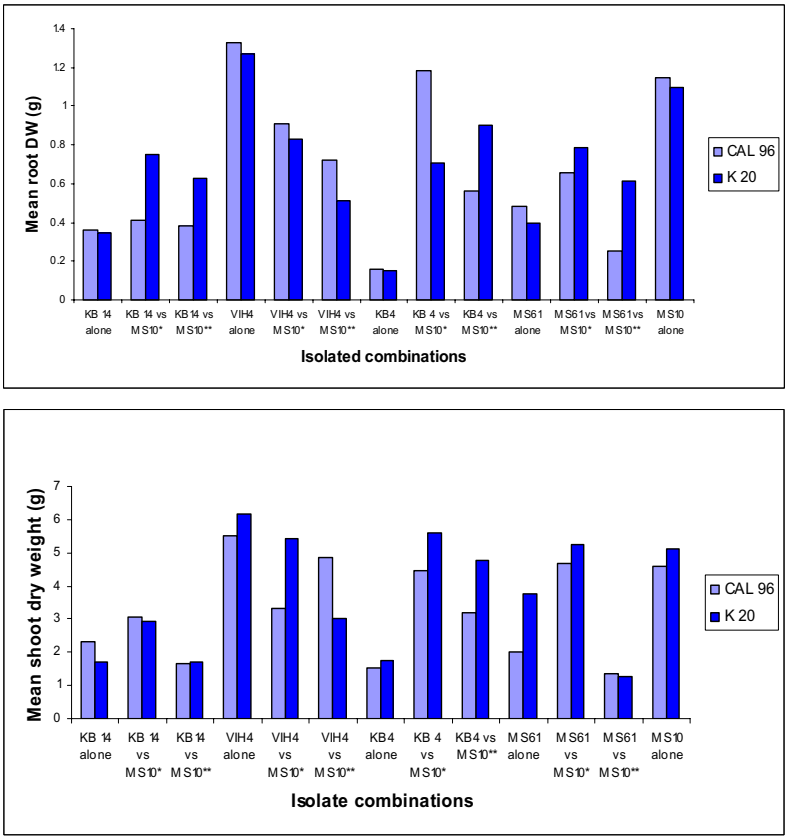

Figure 2.9.5. The effect of MS10 on mean root (top) and shoot (bottom) dry weight per plant of susceptible bean varieties CAL 96 and K20. * = Planting done one week after addition of antagonist MS10; ** = Planting done immediately after addition of MS10.

These results demonstrate the useful interactions mediated by MS10 in suppressing root rots and increasing root dry weight. However, the inoculum carrier used (millet grain, at a ratio of 1:8) may not be sustainable under field condition. These screenhouse results need to be verified under field conditions.

Activity 2.10. Farmers perception of Bean root rots and relationship to variety preference in Uganda

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Rationale

Despite beans being a major source of protein for most people in Uganda, there has been a great reduction in bean production. Bean root rots have been cited as one of the major cause of low bean yields in south western Uganda (CIAT, 1995, Strengthening collaborative bean research in sub-Saharan Africa, (PABRA), 61pp; Opio *et al*., 2001, Agriculture in Uganda, Vol. II CROPS: 162-191). Over time, this has resulted in a shift such that farmers have been forced to grow less preferred bean varieties due to the susceptibility of major preferred market class mainly the large seeded varieties. However, it has also been documented that large-seeded varieties lack resistance to Fusarium root rot (*Fusarium* f.sp. *phaseoli* - FSP). A study was therefore initiated to identify major bean varieties grown by farmers and their preferences; assess farmers perceptions on Fusarium root rot and its relationship to varietal preferences; the influence of root rot on varietal preferences; factors affecting bean yields that may or may not be related to Fusarium root rots the incidence and severity of Fusarium root rot in farmer's fields and farmers practices in managing root rots.

Materials and Methods

A participatory rural appraisal was carried out in two major bean producing and root rot affected areas in Uganda; i.e. south western Uganda; Kabale and Kisoro districts and eastern Uganda; Sironko and Mbale districts. Both interviews and focus group discussions were used to obtain both descriptive and numerical data. A questionnaire was designed, pre-tested and used to obtain information. Fifteen bean farmers per village were interviewed making a total of 120 farmers. The farmers interviewed were selected in a random and non-random manner (Systematic technique and accidental sampling). Data from the survey was analyzed using SPSS and Genstat computer programs. Focus group discussions were carried out in 2 villages per sub-county per district, with one group comprising of at least 15 people.

Results and Discussion

Farmer's perceptions of constraints to bean production: Farmers consider diseases, pests, excessive rainfall or too little rainfall, poor soil, soil erosion, lack of staking materials and drought as the major factors that constrain bean production and leading to the poor bean yields (Table 2.10.1). Diseases were the most mentioned factor in Kabale, Kisoro and Mbale while Sironko bean growers mentioned pests (aphids, bean stem maggot and storage pests) as the most important constraints. Too much rain was considered a constraint in Kabale, Kisoro and Sironko while drought was a major constraint in Mbale compared to other districts. This is because generally, Mbale receives less rainfall compared to other districts. Soil erosion was considered a problem in Kabale and Kisoro where beans are grown on mountain slopes due to lack of enough land. In addition, lack of staking material is a problem in southwestern Uganda, where climbing beans are popular.

It was clear that root rot is considered the most important disease of beans in south western Uganda and in Sironko, while not being as important in Mbale. This is likely to be related to the amount of rain received in each region. Other constraints mentioned included aphids, burning like disease, rats and bird. It was apparent that farmers could not differentiate between diseases and pests.

Table 2.10.1. Farmer's perceptions of constraints to bean production.

Constraint	Kabale	Kisoro	Mbale	Sironko
Diseases	93.3	95.0	80	73.3
Pests	30	24.6	77.1	93.3
Excessive	66.7	62.5	22.9	63.3
Poor soil	16.7	22.1	2.9	6.7
Soil erosion	36.7	26.7	0	0
Lack of staking material	26.7	30.5	0	0
Drought/a lot of sunshine	30	25.5	74	8.3

A pair wise ranking (Table 2.10.2) done in Kisoro clearly indicated that root rot was the most important biotic constraints, followed by burning and finally aphids. Rats are not considered very important to bean production though they cause some damage. Other constraints were birds and bean stem maggot. Bean root rots were ranked highest in south western Uganda and Sironko while in Mbale drought was ranked as the major constraint.

Table 2.10.2. A pair wise ranking of farmer's perceptions of bean diseases and pest in Kisoro, Nyarusiza, Village.

	Root rot	Rats and moles	Burning	Cut worm	Bean fly
Rats and moles	Root rot				
Burning	Root rot	Burning			
Cutworm	Root rot	Rats	Burning		
Bean fly	Root rot	Rats	Burning	Cut worm	
Aphids	Root rot	Aphids	Aphids	Aphids	Aphids

Root rot= 5, Aphids=4, Burning= 3, Rats=2, Cutworm=1

Farmer's perceptions on Fusarium root rot: Generally farmers in Kabale and Kisoro (100% of the farmers interviewed) are aware of bean root rots and are aware of its devastating effects on bean yields. In Kabale it is referred to as "*Kiniga*" (Rukiga) and in Kisoro as "*Kirusuka*" (Rufumbira). It is ranked as the highest cause of bean yield losses followed by other factors as shown below. Similarly in Mbale and Sironko farmers did recognize root rot as a disease of beans, i.e., 85.7% in Mbale, and 86.7% in Sironko. In eastern Uganda, the Bagisu refer to it as "*Ukwishikula*", "*Washa*" or "*Kyengu*" depending on what symptoms are seen. However, it is not as an important constraint to bean production as compared to Kabale and Kisoro, i.e., 37.1% in Mbale and 70% in Sironko considered root rot important compared to 93.3% of the farmers in Kabale (Figure 2.10.1).

Causes of root rots: Farmers associate the occurrence and severity of bean root rot to a number of factors including, poor soils, shallow soils caused by soil erosion since most fields are on hill slopes, drought, over cultivation of soil caused by land fragmentation, mist (this settles on plants in the mornings and evenings). Others associate bean root rot to traditional beliefs and witch craft as whole bean fields are lost in some seasons. Cultural rituals are practiced in some homes in case of a root rot epidemic to control it.

Other factors said to be predisposing beans to root rot were; insects in the soil, pests such as bean stem maggot, drought/a lot of sunshine, lack of crop rotation, planting under trees, intercropping especially with maize, lack of resistant varieties and standing water/ stagnation.

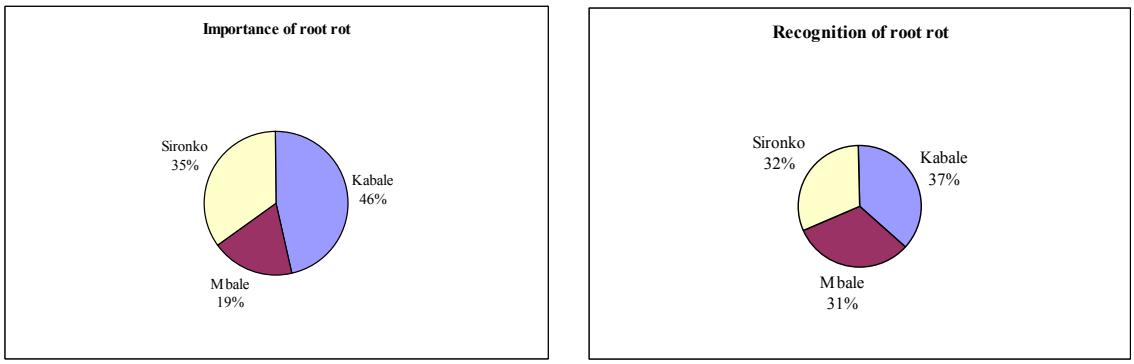

Figure 2.10.1. Recognition and importance of Root rots in Mbale, Sironko and Kabale.

Farmers practices to manage bean root rot: A variety of practices are used in managing root rots and include; addition of fertilizer, spraying with chemicals, timely planting, good bean variety (resistant varieties), good quality seed, soil conservation using drainage trenches, resting of soil, manuring, crop rotation, intercropping planting improved varieties, fallowing, ash, weeding, traditional methods, hilling up, planting mature seed, rouging and burying infected plants, spraying or nothing at all (Figure 2.10.2). The majority of farmers; 49% do nothing to control root rots, while a few practice manuring (mainly in south western Uganda) and crop rotation.

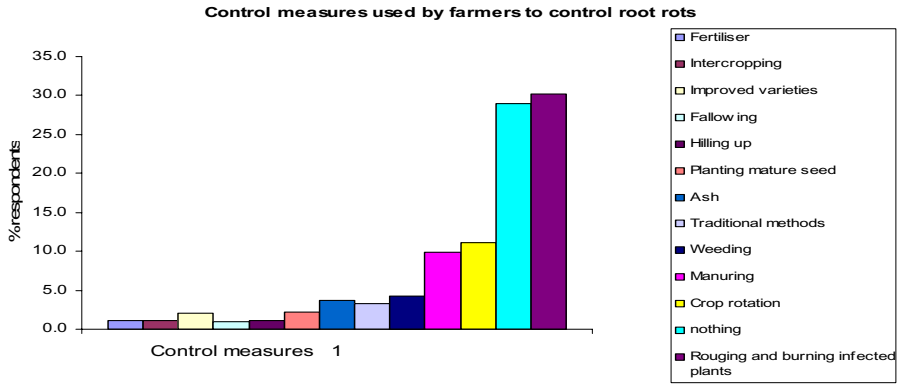

Figure 2.10.2. Management practices used by farmers to manage root rots.

Farmer's perceptions of relationship between Fusarium root rot and variety preference: Bean growers consider taste, marketability, yield, seed size (majority prefer large seeded varieties), seed appearance, soup color/ seed color when considering taking up a new variety. Some farmers have not made any observations on the effect of a variety on the occurrence and severity of root rots but a few especially in

the south western associate the large seeded beans to being susceptible to root rots. A few have observed that small seeded, which are less preferred are generally less affected by diseases including root rots.

Activity 2.11. Developing germplasm with multiple resistance to viral diseases

Contributor: Francisco Morales

A. Bean common mosaic/necrosis viruses

All advanced lines produced by CIAT are expected to possess resistance to bean common mosaic and bean common mosaic necrosis viruses. This activity includes lines improved for their resistance to abiotic stress, such as drought. This year, 305 drought materials were evaluated for their reaction to *Bean common mosaic virus* (BCMV) and *Bean common mosaic necrosis virus* (BCMNV). The first batch of 189 materials showed the presence of recessive genes, probably *bc-3* or protected *I* gene that confer resistance to all virus strains, in approximately 66% of the materials evaluated. Approximately 7% of the materials possessed monogenic dominant resistance (susceptible to necrosis), and only 3.2% of the materials was susceptible to mosaic. In a second trial, 234 materials selected for drought tolerance, architecture and Iron content, the number of lines possessing monogenic dominant resistance to common mosaic increased to 97%.

The incorporation of broad-spectrum recessive resistance to BCMV and BCMNV has been increasingly important in the Bean Project, specially in past years. This type of resistance is stable and prevents undesirable linkage problems associated with certain commercial seed colors and susceptibility to these viruses. This year, a total of 3,012 materials were evaluated for the presence of the responsible gene, *bc*-3. In these inoculations, 20% of the test materials proved immune to mosaic and necrosis induced by BCMNV NL3, the most pathogenic of the BCMV and BCMNV strains found in nature. Over 7% of the materials inoculated had monogenic (*I*) resistance, and 45% were susceptible to mosaic. Approximately 20% of the materials inoculated proved to be segregating for the *bc-3* recessive gene.

One of the most commercially demanded and valuable climbing common bean cultivars in Colombia, is 'Cargamanto'. Unfortunately, this cultivar has been under attack from BCMV in the highlands of Antioquia, the main production area. 'Cargamanto' also presents linkage problems between its commercial seed coat characteristics and susceptibility to the virus, associated with monogenic dominant resistance conferred by the necrosis *I* gene. Consequently, the incorporation of broad-spectrum recessive resistance to BCMV has been the main strategy to improve this valuable variety. This year, we continued the screening of Cargamanto lines produced by CORPOICA. Of a total of 94 materials evaluated this year, 13% proved to be homozygous immune. The *bc-3* gene was present in 58.5% of the materials found to be segregating for this gene. 28% of the materials were devoid of any dominant or specific recessive resistance genes against BCMV/BCMNV.

Additionally, Bean Virology screened 281 parental materials for breeding purposes. 16% of these parental materials were immune to BCMNV-NL3, which indicates the presence of recessive genes, such as *bc-3*, in these parental genotypes. The majority of these genotypes, however, have the necrosis *I* gene. Only 3.5% expressed common mosaic, and the rest of the materials were segregating for all genes. A total of 227 breeding materials were also evaluated for their reaction to BCMV/BCMNV. About 29 lines for the Middle East were totally susceptible (mosaic); whereas 90 RAB and MAB lines showed monogenic dominant resistance to BCMV.

B. Bean golden yellow mosaic virus

Bean golden yellow mosaic virus (BGYMV) is still the number one biotic constraint to bean production in Mesoamerica, including the Caribbean Region. So far, 0ver 25 years of research at CIAT and collaborating national programs has paid off in terms of BGYMV-resistant common bean varieties released, and sources of resistance made available to national and international institutions in this region. International programs, such as USAID's CRSP/TXII and the Panamerican School in Honduras, have been releasing new BGYMV-resistant bean lines based on the available sources of virus resistance.

At CIAT, the BGYMV work continues but it is subordinated to the incorporation of abiotic traits, namely drought tolerance and iron (Fe) content for Central America. In 2005, 407 materials were evaluated for their reaction to BGYMV. A high proportion of F7 genotypes (70%) had an adequate level of BGYMV resistance; as well as 18.5% F8 genotypes from mass selections. The rest of the materials segregated resistant and susceptible. A mass selection of F8 materials for high Fe content, yielded 28 resistant lines (R) out of 71 materials evaluated for their reaction to BGYMV; 9 lines were susceptible (S); and 34 lines were segregating R&S.

The number of resistant materials belonging to different breeding projects were: 8/49 (RCB and NCB); 5/29 (F6 archit/Fe); 5/19 (MIB); 4/28 (MAB-A); and 36/51 (MAB-B).

C. Bean leaf crumple virus

This is the new whitefly-transmitted begomovirus that has practically eradicated snapbean production from the flat lands of the Cauca Valley (*c.* 1000 m above sea level). This virus appears to be a hybrid between the Mesoamerican BGYMV and other begomoviruses that have been introduced in past years in the Cauca Valley, and are currently affecting unrelated crops, such as tomato. In past annual reports, we have reported that Bean Virology, in cooperation with Bean Entomology and Breeding, has identified sources of resistance to this virus in Mesoamerican black-seeded common bean genotypes (Figure 2.11.1).

Although the new biotype (B) of the whitefly species *Bemisia tabaci* is responsible for the outbreaks of this new virus in snap bean plantings in the Cauca Valley of Colombia, the original biotype (A) is still a more efficient vector of this virus. An average disease incidence of 4.9% was obtained for biotype B in six different biological transmission tests, as compared to a 26.6% incidence for the A biotype, using only one viruliferous whitefly per plant. In these tests, the black-seeded parental materials Porrillo Sintetico and BAT 304, registered the lowest disease incidence (0 and 7%, respectively) using up to 25 whitefly individuals per plant. Topcrop and Red Kloud showed incidence of 100% in these tests. A black-seeded commercial cultivar like ICTA-Ligero, suffered a 27% virus incidence.

Resistance to bean viruses, such as BCMV, BCMNV and BGYMV is readily available and highly resistant common bean varieties have been developed from these sources for Central America. In Honduras and El Salvador, two red-seeded common bean varieties have been released in the past year, with excellent adoption thanks to their commercial seed color (Rojo de Seda) and good cooking characteristics. In El Salvador, these varieties are called CENTA-San Andrés (Figure 2.11.2) and CENTA-Pipil, both bred by Dr. Juan Carlos Rosas of El Zamorano, Honduras. In a recent trip to El Salvador, several lines possessing recessive (*bc-3*) resistance to BCMV and BCMNV, were also showing resistance to the main virus problem of Central America, BGYMV. This is a major accomplishment in a region that requires both types of virus resistance without compromising grain quality.

Figure 2.11.1. Broad pathogenicity range of a new whitefly-borne virus in common bean genotypes under field conditions in the Cauca Valle, Colombia.

Figure 2.11.2. Bean golden yellow mosaic-affected cultivar and resistant cultivar developed by El Zamorano, Honduras

Activity 2.12. Efficiency of entomoparasitic nematodes as biopesticides versus *Phyllophaga menetriesi* and *Anomala inconstans* in relation to host age

Contributor: E. L., Melo; C. A. Ortega, A. Gaigl

Rationale

The genera *Phyllophaga*, *Astaena*, *Anomala*, *Isonychus*, and *Macrodactylus* are the economically most important white grubs of the family Melolonthidae in Colombia (more than 600 species) Chemical control of these pests is not feasible due to its costs, environmental risks and the lack of knowledge in fundamental aspects such as population dynamics complicating the development t of control strategies (Falcon & Smith, 1983, El concepto de control integrado de las plagas, UNDP and CIAT, pp 15-20).

The microbial control of white grubs is widely considered as a valuable strategy of a sustainable pest management. Most studies on biological control focus on the third instar larvae (Jackson & Brooks, 1995, J. Nematol 21: 15-20) that cause more damage than those in earlier instars (King, 1984, Tropical Pest Management 30: 36-50.). However, many works with entomoparasitic nematodes (EPN) resented difficulties in controlling this instar (Koppenhöfer & Fuzy, 2003, Biological Control 28: 47 – 59; Simard *et al.*, 2001, Suppl. Journal of Nematology 33: 297-301).

The objective of the present work is to evaluate native strains of EPN on two white grub species at two different stages of development.

Materials and Methods

We conducted this experiment in the laboratory of Cassava Entomology at CIAT, under controlled conditions (25.4ºC and 86% RH) at complete darkness. We tested the two native EPN strains *Steinernema feltiae* (Villapinzón, Cundinamarca) and *Heterorhabditis* sp. (Fresno, Tolima) at a concentration of 10,000 Dauer Juveniles (DJ) per milliliter.

The white grubs (*Anomala inconstans* and *Phyllophaga menetriesi*) were taken from the first generation of CIAT's white grub colony (23 ºC, 70% HR). The first instar larvae were confined in a plastic vessel of 5 liters. The other instars were confined in plastic cups filled with 70 g soil (sand : soil organic matter = 1:1) and fed on roots of young rice plants. We used first, second, and third instar larvae, latter at two and four weeks after molting according to the availability of the different ages of each species during November 2004 and March 2005

We tested both EPN species on *A. inconstans* whereas we applied only *Heterorhabditis* sp. on *P. menetriesi* because *S. feltiae* failed to control larvae in the first instar.

The experimental unit was defined by 12 plastic cups (21 g) filled with humid soil (field capacity). Each cup harbored one white grub. The checked the larvae on mortality after 10 and 20 days after application (daa) of the nematodes. Since the data were not normally distributed after applying Abbott's formula (1925) we transformed them by means of the square root $\sqrt{x\,2\,1}$ prior to ANOVA. Normal distribution of the transformed data was corroborated by using the Shapiro–Wilks Normality Test (1965). The differences of means were tested on significance by the Tukey–Test (PÒ0.05) (Infostat 2004, Grupo INFOSTAT, FCA., Universidad Nacional de Córdoba, p318).

Results and Discussion

Survival of white grubs in the control treatment depended on the age. L1 showed with 16% the highest mortality whereas three percent larvae of L2 died without being effected by any pathogen . Interestingly, more larvae as young L3 instar died than as L2; however, differences were not significant. All advanced L3 instar and pupae survived in the control treatment. In general mortality of untreated white grubs was 11%.

Larvae 2 (L2) of *A. inconstans* was the most susceptible instar to *H. bacteriophora* whereas larvae 3 (L3) was the less affected stage by *S. feltiae*. *H. bacteriophora* was more pathogenic to all instars than *S. feltiae* and we didn't find any differences between the two evaluation times (10 and 20 dda).

The second instar of *P. menetriesi* was the most susceptible stage to EPN. This susceptibility was reduced with increasing age of the insect. We observed differences between evaluation times: mortality was significantly greater after 20 dda. According to these results we suggest to apply EPN as biopesticide when larvae of *P. menetriesi* are in the second instar.

Comparing the two white grub species we observed that *A. inconstans* is more susceptible to an EPN attack than *P. menetriesi*, our results corroborate earlier studies where only 10% of L3 were killed by EPN (CIAT, PE-1 Annual Report 2004). Both soil pests presented highest mortality in the stage of L2

Several reasons may explain the variation in susceptibility between stages of development: (i) immune response associated with the age; (ii) size and behavior of the host; (iii) the smaller diameter of the spiracles of young larvae (Jackson & Brooks, 1995, J. Nematol 21: 15-20); (iv) reduced production of CO_2 and less kairomones of L1 and L2 (Kaya,1985, J. Invertebr. Pathol. 46: 58-62); (v) thickness of cuticle of L3 increases the difficulties of penetration (Koppenhöfer & Fuzy, 2004, Ento.Soc.of America. 97:1842-1849); (vi) the fact that larvae reduce and stop feeding activities when they are approaching the pre-pupal phase lowers the possibility of being penetrated by the nematode via the mouth. Clearly, the developmental stage of the host insect has an important impact on the efficiency of EPN. Moreover, the extent of this effect depends on the species of host and nematode (Koppenhöfer & Fuzy, 2004, Ento. Soc.of America. 97:1842-1849)

Conclusions

- *A. inconstans* presents greatest mortality when *Heterorhabditis* sp. penetrates the second instar (98.3%).
- The most susceptible stage of *P. menetriesi* is the second instar infected by the species *Heterorhabditis* sp. (mortality of 81.1%).
- *P. menetriesi* is more resistant to EPN than *A. inconstans*. Both white grub species are most susceptible during the phase of second instar.
- All developmental stages of *A. inconstans* didn't show any mortality in terms of natural mortality. In contrast, the first instar of *P. menetriesi* showed the greatest mortality, followed by the second. Latter observation is expected since younger organisms are more susceptible to parasitic organisms due to the morphological, physiological, and behavioral characteristics.
- We recommend applying entomoparasitic nematodes as biopesticide versus the second instar of white grubs.

Activity 2.13. Evaluation of different concentrations of the entomoparasitic nematode *Heterorhabditis bacteriophora* (Italia) versus second instar larvae of *Phyllophaga menetriesi* (Coleoptera: Melolonthidae)

Contributors: Elsa Liliana Melo, Carlos Alberto Ortega, Andreas Gaigl

Highlight:

∉ New promising natural enemy of *Phyllophaga menetriesi* identified

Rationale

The larvae of scarabs (Coleoptera: Melolonthidae) are considered as some of the most rhizophagous pests on a wide range of crops such as pasture, cassava, maize or ornamental flowers and many others. *Phyllophaga menetriesi* is the most aggressive and noxious soil pest in the climatic range between 1000 and 1600 m.a.s.l. (CIAT, Annual Report 2004, Integrated Pest and Disease Management in Major Agroecosystems, 417 p). For this reason this species was object for experiments on the efficiency of entomoparasitic nematodes (or entomonematodes) as agents of biological control.

The biological control by nematodes was already suggested in the 1930's (Jackson, 1993, Diversidad y Manejo de Plagas Subterráneas, Soc. Mex. Ent, 261 pp). Bacteria, fungi, virus, and entomonematodes have been used until the present for programs of biological control; however, the use of them against scarabs is limited due to the absence of proper filters, methods of mass production, and inappropriate application. The most successful programs of microbial biocontrol are based on pathogens that are highly adapted to a well defined range of pests. Even the age of the target organism has an important impact on the efficiency of biological control agents such as entomoparasitic nematodes (Melo *et al*., 2005, Resúmenes del XXXII Congreso de SOCOLEN, Ibague, Colombia, p 80).

In the present study we wanted to analyze the potential of the introduced entomoparasitic nematode *Heterorhabditis bacteriophora* as bioinsecticide against the second instar of *Phyllophaga menetriesi* under controlled conditions applying in different concentrations.

Materials and Methods

We conducted this experiment in the laboratory (23 ± 2ºC, R.H. 70±5%, and complete darkness.

Target Insect: We received larvae of *Phyllophaga menetriesi* in the second instar from our lab colony. The larvae were confined in transparent plastic cups of 150 ml with lid. The cups were filled with organic soil (one part organic matter and two parts sand). The white grubs fed on young rice plants (grubs were introduced when plants passed 20 days after germination.

Entomoparasitic nematodes: We used the Italian strain CTN003 of the nematode *H. bacteriophora* that was one of the most pathogenic strains in previous testes (CIAT, Annual Report 2004, Integrated Pest and Disease Management in Major Agroecosystems, 417 p). Nematodes were applied in seven doses: 32, 100, 307, 960, 2000, and 3000 Dauer Juveniles (DJ) per milliliter. These numbers were obtained using arbitrary scale of $\log_{10}$. We applied distilled water as control (no nematodes). We evaluated the experiment 20 days after application (daa) counting the killed larvae. The dead insects were transferred to a White trap (White, 1927, Science 66: 302-303) in order to verify and recover the nematodes.

Experimental Design: The experimental design was at random. All treatments were repeated four times. Each experimental unit harbored 13 larvae. Prior to ANOVA data were transformed by using Abbott's formula (1925) and then by the square root $\sqrt{x\,2\,1}$. Normal distribution of the transformed data was corroborated by using the Shapiro–Wilks Normality Test (1965). The Tukey-Test (PÒ0.05) was used to analyze statistical differences of means (Infostat 2004, Grupo INFOSTAT, FCA., Universidad Nacional de Córdoba, p318).

Results and Discussion

A relative low concentration of only 307 DJ/ml caused a mortality of more than 80%, a concentration of 960 DJ/ml killed more than 90% of the grubs (Figure 2.13.1) the best result of all applied concentrations; more DJ/ml maintained this level of mortality. This indicates that this strain can be applied at low concentrations. This mortality is striking compared to the results obtained applying nematodes on the third instar where 10,000 DJ/ml obtained mortality of only 12% (CIAT, Annual Report 2004, Integrated Pest and Disease Management in Major Agroecosystems, 417 p).

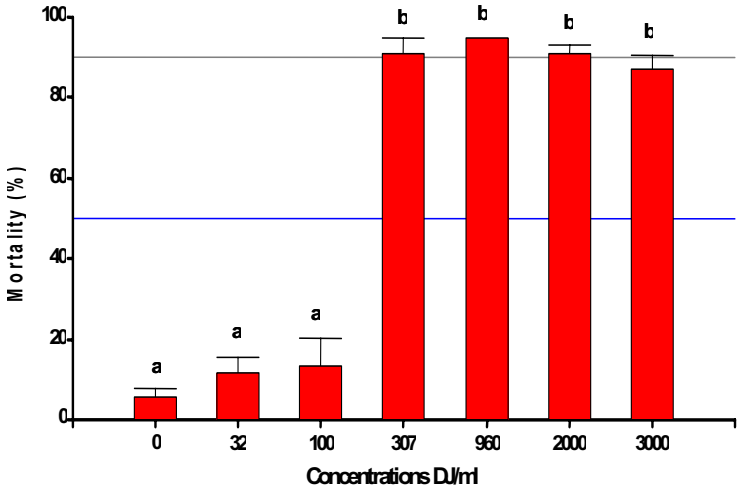

Figure 2.13.1. Mortality of *Phyllophaga menetriesi* (larvae in second instar) in relation to different concentrations of Dauer Juveniles (DJ) of *Heterorhabditis bacteriophora* (Italian strain CTN003).

P. menetriesi is a white grub species that presents a strong resistance to nematodes (Koppenhöfer & Fuzy, 2004, Entomological Soc. of America 97: 1842-1849 ; Simard *et al*., 2001, Suppl. J. of Nematology 33: 297-301) that is indeed an important limitation for the use of entomopathogenic organisms (Cappaert & Koppenhöfer, 2003, Biological Control 28: 379– 386). However, in previous experiments other EPN strains such as *Heterorhabditis* sp., *S. carpocapsae*, *S. glaseri*, and *S. longicaudum* showed promising results versus the second instar of *Anomala orientalis* (Lee *et al.,* 2002, J. of Economic Entomology 95: 918 – 926). These data show that the probability of a successful control is much better when the second larval instar is targeted (Melo *et al*., 2005, Resúmenes del XXXII Congreso de SOCOLEN, Ibague, Colombia, 80 p) suggesting including native strains in the tests of biological control determining the most efficient concentration that are commercially feasible.

When the phenology of the pest insect is known the best time of applying this bioinsecticide can be easily defined, for example *P. menetriesi* is present in Northern Cauca during the months November and

December. Moreover, we suggest conducting experiments combining nematodes with other microbial control organisms and other IPM strategies.

Koppenhöfer & Fuzy (2004, Entomological Soc. of America. 97: 1842-1849), suggest that the efficiency of the EPN depends on the host's age; however, this effect depends on the host species and the applied EPN. Moreover, we find that the commercial dose of 500 DJ/ml indicated by "E-nema" for the hybrid species *H. bacteriophora* is even greater than the optimal concentration of the Italian strain.

Although our Italian strain of *H. bacteriophora* showed highly promising results we recommend for future research continuing the search for native entomoparasitic nematodes due to two reasons: Firstly, in this experiment we couldn't include more nematodes for direct comparison and secondly, the release of exotic organisms in the field may raise legal conflicts due to the national policy of environmental protection.

Knowing that the second instar the larvae of *P. menetriesi* is the most susceptible stage to entomoparasitic nematodes (Melo *et al*., 2005, Resúmenes del XXXII Congreso de SOCOLEN, Ibague, Colombia, 80 p) we suggest continuing research defining best concentration for other strains of nematodes. Our results also help to determine the optimal moment for application of this bioinsecticide according to the pest's phenology.

Many aspects remain for further evaluation in order to respond to farmers' inquiry how and when to control this pest. Since the studies on entomopathogenic organisms against *P. menetriesi* are still in the beginnings this would be the first step for a sustainable biological control of this pest species.

Activity 2.14. Lethal density of *Phyllophaga menetriesi* (Coleoptera: Melolonthidae) associated with maize, beans and cassava plants

Contributors: A. Ortega-Ojeda, E. L. Melo-Molina, A. Gaigl

Highlights:

- ∉ Action threshold of white grub species *Phyllophaga menetriesi* on three crops defined
- ∉ Agricultural methods as strategies of integrated pest management described

Rationale

White grubs (Coleoptera: Melolonthidae) belong globally to the soil biota. The major part of them plays an important role in the recycling of soil organic matter; however, some of them are important pests on a wide range of crops. Especially in the tropics the diversity of this order is impressing. In Colombia more than 600 species of the family Melolonthidae are known. In Pescador Caldono (Cauca) we know 44 species; some of them are of major economic importance such as *Phyllophaga menetriesi*, *Phyllophaga* spp., *Plectris fassli*, *Pl. pavida*, *Ceraspis innotata*, *Astaena valida*, *Anomala inconstans* and *A. cincta* (Pardo, 2002, MSc Thesis, Universidad del Valle, Cali, Colombia, p 33-82). All of these species *P. menetriesi* is the most voracious one on a wide range of tropical crops. The most aggressive phase is the third larval instar and with a length of four centimeters is one of the biggest rhizophagous white grubs (*ibid*.). The third instar is not unique only in terms of aggressive behavior with tendencies to cannibalism but also in terms of resistance to synthetic and biological insecticides (CIAT, PE-1 Annual Reports 2003 and 2004)

White grubs attack crops such as maize or beans at the succulent parts below the soil surface feeding on roots and germs. Damage on cassava is characterized by damage both of bark and internal cambium as well as perforation and tunnels in swollen tubers.

Farmers often do not understand the relation between damage and pest due to its cryptic habitat. Frequently they replace in vain lost plants with new seed only to continue alimenting the grubs and increasing production costs. At present there exist only a few reports on the threshold for white grubs that state that three larvae associated with maize or cassava and coffee, respectively, are necessary to destroy the host. Farmers often see in the use of synthetic insecticides the only possible strategy to minimize losses. Mostly, this strategy only increases the production costs and threatens farmers' and environmental health. Moreover, the development of resistance in these insects is another secondary result.

The objective of this experiment was to establish a threshold of white grub density and develop a tool that helps farmers in decision making when they have to apply control activities against this pest associated with maize, cassava, and beans.

Materials and Methods

This experiment was conducted on the farm "Bellavista" in Pescador (Cauca, Colombia) at an altitude of 1580 m.a.s.l. We selected as host plants maize (variety ICA-305, hard endosperm), red beans (variety "Toné"), and stems of cassava (variety SM 707-17). 15 days old plantlets of maize and beans and the cassava stems were placed individually in plastic buckets (height: 40 cm, diameter: 30 cm, volume: 28,000 cm^3) filled with soil taken from the farm. At the bottom the buckets had drainage of a 6 cm diameter. The treatments consisted of five white grub densities (0, 1, 3, 5, and 7) per bucket. Every treatment was repeated six times.

We chose a randomized complete block design (CRD) for a two-factors experiment where beans and maize were the host plants. The two factors were three evaluation periods (nine days for each period) and the white grub densities. In the case of cassava we only considered the factor white grub densities due to the long growing time of this crop.

Only larvae in the third instar of *P. menetriesi* were used. The insects were taken from the rearing colony at CIAT and confined in the buckets according to the defined densities. The larvae that did not dig into the soil within a lapse of ten minutes were replaced.

We tried to simulate natural conditions for the white grubs as good as possible. We inserted the buckets by 95% of their height into the soil in order to level the experimental unit with the surrounding field and to avoid direct sunshine on the borders and avoid fluctuation of temperature. In each of these buckets we planted one cassava stem or a 15 days old maize or bean plant. One day later we released larvae in the third instar according to the assigned density. When beans and maize were the hosts we started the daily evaluations one day later. These observations lasted nine days and were repeated three times over time. New plants were inserted when a new repetition over time started; however, the grubs remained in the buckets. When cassava was the host we started evaluations after 17 days after planting the stem and collected data of the experiment every fourth days during a lapse of 60 days. During this period we observed plant mortality, estimated plant vigor, development, and color of the foliage. The scale for the estimated plant vigor levels ranged from 1 to 9, where 1 corresponded to a plant in ideal shape and 9 to irrecoverable. We adopted this scale from Schoonhoven & Pastor-Corrales, 1987, CIAT who applied this methodology for evaluating germplasm of beans. Once a plant was dead we extracted the stake and analyzed internal and external damage due to white grub attacks.

Before we run the statistical analysis we checked the data on normal distribution by means of the Shapiro-Wilks-Test (1965). Since the data from the experiment with cassava showed an elevated variation and values of zero we transformed the data applying the formula $\sqrt{x\,2\,1}$. Data were analyzed by means of two-ways ANOVA (beans and maize) and one-way ANOVA (cassava) using software INFOSTAT (2005) to determine single or interaction effects of factors. In case of significant F-Values we compared means using Tukey's test.

Results and Discussion

Maize: The plants of the control treatment reached a height of 23.32 cm nine days after infestation (dai). All plants of the other treatments died before this period. Plants associated with one grub showed the longest agony (3.5 dai) reaching a height of 17.55 cm. In contrast, plants associated with five or more grubs died within 2.4 dai (Figure 2.14.1a) (pÒ0.0007). According to these results we ruled out the conclusions by Ayala & Monterroso, 1998, Manual para Técnicos 2, Costa Rica, p. 7-8 and Pardo *et al.,* 2003 (In: Estudios sobre Coleópteros del suelo en América, Aragon *et al*. (eds), U. Autónoma de Puebla: 283-297) that at least three larvae of *P. menetriesi* are necessary to destroy one maize plant. We recommend not tolerating losses more than 5% of 50 randomly selected plants per hectare.

The white grubs eliminated the plants faster in the first repetition (2.2 dai) than in the other two (2.8 dai) (Figure 2.14.1b) (pÒ0.05). We conclude that the larvae of *P. menetriesi* reduce feeding activities while approaching the end of the third instar giving the farmer the chance to escape the white grub attack by sawing the crops when the larvae are older or have completed the larval stage

Beans: Only the plants in the control treatment survived this experiment. They reached a height of 27.92 cm after 5.2 days when the last plant of treatment 3 (one grub per plant) died having reached an altitude of 24.13 cm. Three and more larvae needed 3.1 days for destroying one plant (Figure 2.14.2a). This corroborates Arguello *et al.* (1999, PROMIPAC-Nicaragua, 18 p), who observed that white grubs frequently destroy 100% of a plot within seven or ten days. Likewise as in the case of maize we conclude that the threshold for beans is only one larva per plant and recommend not permitting plant losses greater than 5%.

In the first repetition larvae of *P. menetriesi* needed 3.21 days for elimination of the plants and in the third 4.5 days (Figure 2.14.2b). This confirms our observations in earlier field and greenhouse experiments that this white grub species reduces its feeding activity when it completes the larval stage. As in the section before we recommend to elude the presence of *P. menetriesi* by altering the date of planting.

Cassava: Only one larva is able to cause an external damage of 53% of the stake feeding on bark and 6.7% on the internal cambium (Figure 2.14.3). Five or seven grubs eliminate almost completely the bark of a stem and eliminate approximately 30% of the cambium. Three larvae reduce the bark by 80%. Damage 24 dai was clearly proportional to white grub density (pÒ0.05) (Figures 2.14. 4 and 2.14.5).

After 49 days one grub damaged a plant by 30% (Figure 2.14. 6). Observing the vigor of the plant and according to earlier observations (Ortega *et al*., 2005, Memorias XXXII Congreso SOCOLEN, Ibague, Colombia, 89 p) we conclude that the attacked plants may continue their development; however, their yield can be heavily reduced.

These results confirm that it is possible to identify levels of damage estimating the vigor of the plant. However, these indicators have to be detected very of the crop because the threshold for cassava is below one white grub per plant. If the visible plant damage is below level 3 (= slight damage) it is possible to keep yield reduction below a range 5%.

We concluded that:

- ∉ One white grub destroyed a maize plant within 3.3 days; three or more larva needed 2.4 days.
- ∉ One larva needed 5.2 days to destroy the one bean plant; three or more larvae needed 3.1 days.
- ∉ The probability was 70% that a cassava stake survives the attack of one white grub; however, we estimated that this damage could reduce yield by 53%.
- ∉ The probability that three white grubs eliminate completely one cassava stem in 56 days is 50%. Seven grubs reduced this period down to 24 days. The probability that seven grubs destroy one cassava stem within 35 days was 100%.
- ∉ The threshold for cassava, beans, and maize is below one white grub associated with a plant.
- ∉ We recommend varying the date of sawing beans or maize in order to elude the presence of the larval instar III.
- ∉ In order to keep plant losses in maize and beans below 5% we recommend revising randomly selected 50 plants per hectare. When three plants of those are associated with three white grubs farmers should get active against this pest.

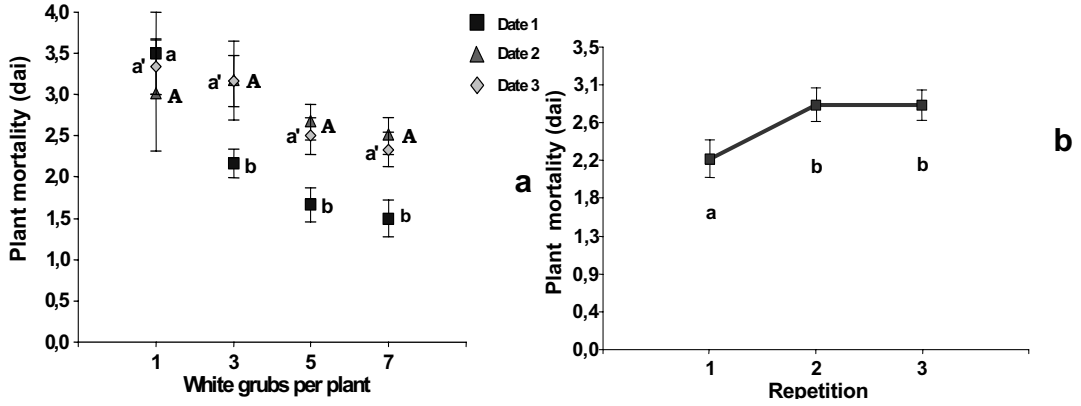

Figure 2.14.1. Mortality of maize plants. **a**) mortality in each repetition **b**) means of each repetition (Tukey pÒ0.05).

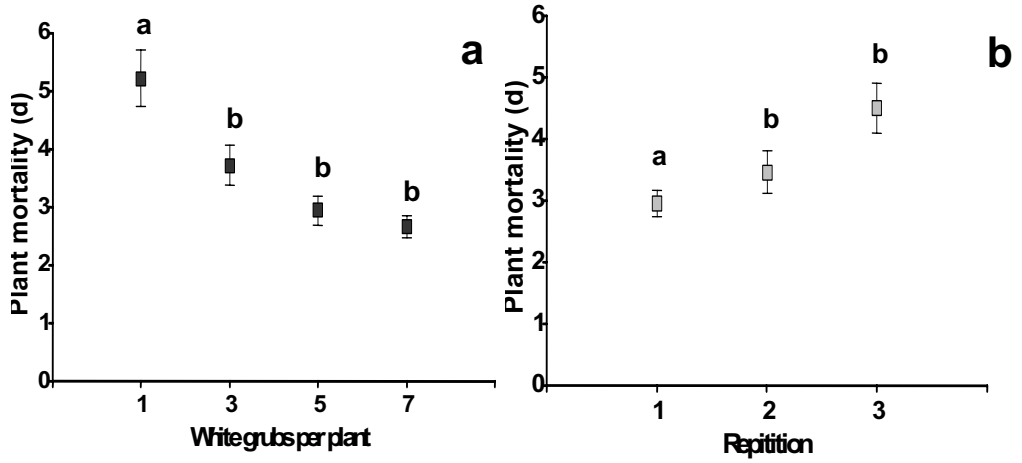

Figure 2.14.2. Mortalityof bean plants. **a**) means of treatments per repetition; **b**) means per treatments; y, c. all means per repetition (Tukey pÒ0.05).

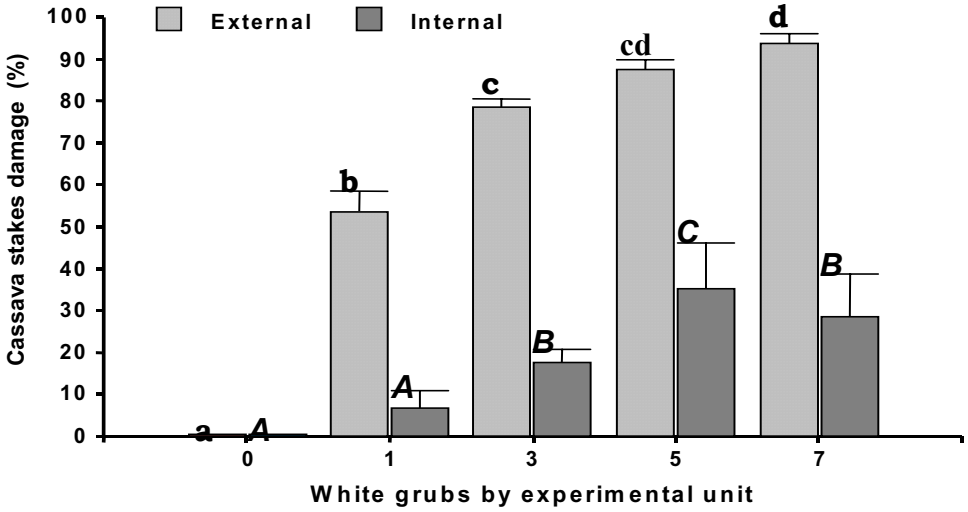

Figure 2.14.3. External and internal feeding damage on cassava stakes in relation to 0, 1, 3, 5 y 7 larvae (Tukey pÒ0.05).

Figure 2.14.4. Differences in vigor (left) and external and internal damage (right) due to white grub attacks on cassava stakes during the first two months (Photos by Ortega)

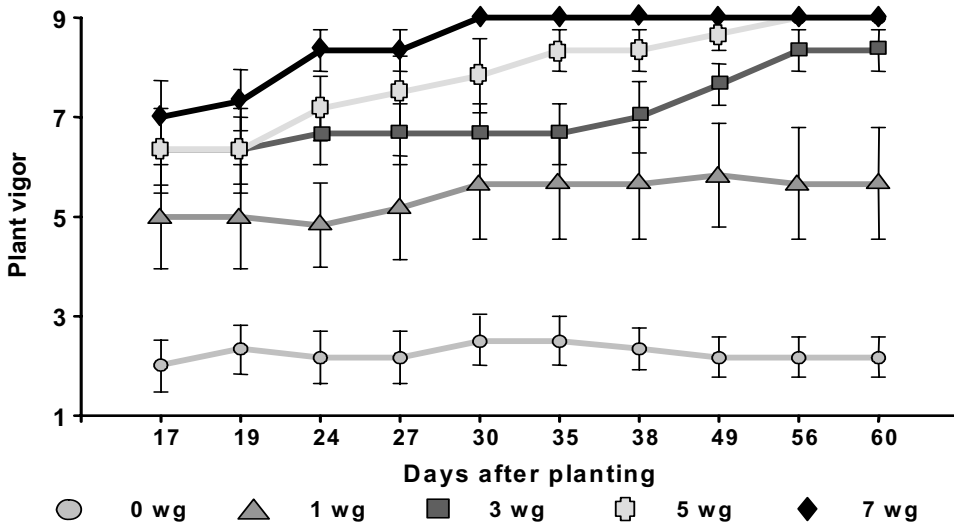

Figure 2.14.5. Vigor of cassava plants in relation to white grub (wg) density (Tukey pÒ0.05).

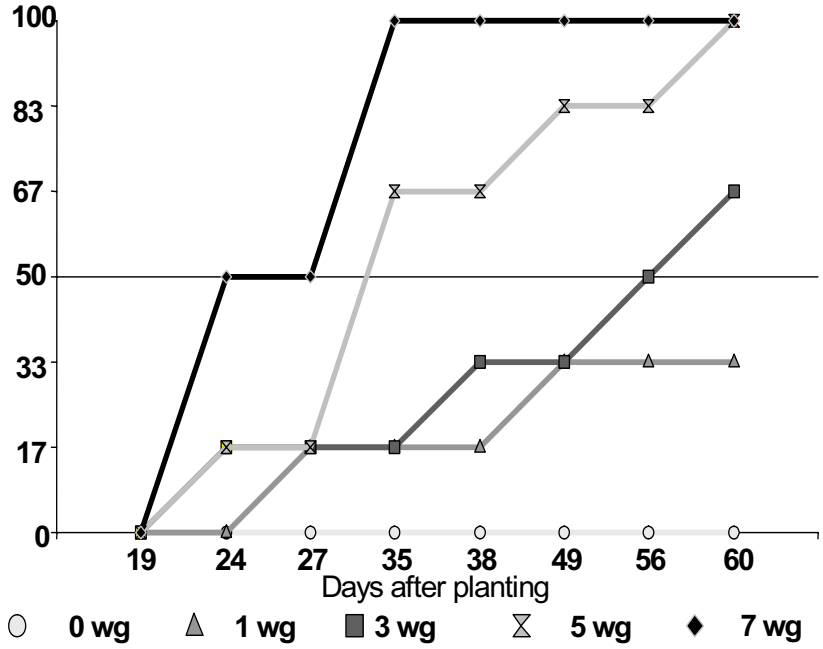

Figure 2.14.6. Probability of elimination of cassava plants in relation to duration of feeding (in days) and white grub density (Tukey pÒ0.05).

Activity 2.15. Pathogenicity of the bacteria *Paenibacillus popilliae* on larvae of *Phyllophaga menetriesi* (Coleoptera: Melolonthidae)

Contributors: Carolina Buitrago, James Montoya, Andreas Gaigl, Martha Londoño

Highlight:

- Highly virulent bacteria for the control of the white grub *Phyllophaga menetriesi* identified

Rationale

Bacteria traditionally play an important role as natural antagonists of insects, and particularly of white grubs. *Paenibacillus popilliae* is the pathogenic agent of milky disease and has been the subject of many studies. Its wide host range of about 70 white grub species (Tanada and Kaya, 1993, Insect Pathology, Academic Press Inc, California, USA) indicates this bacterium as an important component of biological control. In Colombia, the national agricultural research institution, Corporación Colombiana de Investigación Agropecuária (CORPOICA) in Rionegro, Antioquia, developed a methodology for artisanal on-farm rearing of *P. popilliae* in order to control *Phyllophaga obsoleta*, a key pest on potatoes in the agroecological cold zones of Antioquia between 1800 and 3000 m. However, no studies have been conducted on this bacterium as control agent of *P. menetriesi*, a key pest on cassava, maize, coffee, pasture, etc. in the 1000 to 1500 m.a.s.l. zone.

P. popilliae is also one of the most frequently identified microbial pathogens of *Phyllophaga menetriesi.* However, no experimental studies have been conducted of this bacterium as control agent of this grub. This present work aimed to test the pathogenicity of various strains of *P. popilliae* on *Phyllophaga menetriesi* and *P. bicolor*.

Materials and Methods

From the white grub-rearing colony at CIAT and CORPOICA in Rionegro, we selected six strains of *Paenibacillus popilliae* for the experiments. These bacteria were previously isolated from white grubs mainly collected in Pescador (Cauca, Colombia) (Table 2.15.1). Before initiating the experiments, we reactivated the isolates on agar L.

Table 2.15.1. Origin of the six *Paenibacillus popilliae* strains deployed in this experiment.

Strain	Site	Host
CIAT-LF24	Pescador (Cauca)	*Phyllophaga menetriesi*
CIAT-BP1	Pescador (Cauca)	*P. menetriesi*
CIAT-BP4	Pescador (Cauca)	*P. menetriesi*
CIAT-LG	Pescador (Cauca)	*P. menetriesi*
CIAT-381	Pereira (Risaralda)	*P. menetriesi*
Corpoica-B386	Rionegro (Antioquia)	*Ancognatha* sp.

Insects: For the assays for spore reproduction we used larvae of *Phyllophaga menetriesi* and *P. bicolor* in the third instar. We collected the insects on cassava and pasture fields in Pescador (Cauca, Colombia) and

quarantined for 8 weeks. For the pathogenicity bioassays we used larvae in the second instar taken from the white grub-rearing colony at CIAT.

Description of bioassays: We conducted four bioassays where we produced the inoculums, and one bioassay where we evaluated pathogenicity and virulence of the six strains in soil. We used two methodologies for the reproduction of inoculums: injection or forced feeding. After each bioassay, we verified the species of the bacteria according to Koch's postulates.

Reproduction of inoculums by injection and forced feeding: The experimental unit consisted of one larva of three white grub species—*Phyllophaga menetriesi*, *P. bicolor*, and *Anomala cincta*—confined in a 2-oz plastic cup, which was filled with sterilized soil and organic matter (1:1) at a humidity of 7% of field capacity. The grubs were fed with three maize grains. We used 50 larvae for each bacterial strain. The bacteria were introduced by injection into the third segment of the abdomen of the larva (10 µL suspension with 10^6 spores/ml). As control treatment we injected 10 µL distilled water into the insect. The treated larvae were checked every second day during 2 weeks. The insects were disinfected with alcohol and hypochlorite after each revision.

For the forced feeding of the grubs we developed an artificial diet that consisted of a mixture of healthy hemolymph, yeast extracts, beans, agar, NaCl, and inoculums. Prior to feeding, the larvae were starved for 48 hours. We revised the larvae in the third and sixth week after infestation.

After the extraction of hemolymph, we quantified spore concentration and validated the morphology of the bacteria (macroscopically and under microscope). This made it necessary to extract 0.1 ml of hemolymph from each larva that was diluted in 0.9 ml of distilled water. We then made serial dilutions until 10^6 spores/ml. These were cultivated in agar and counted using the colony forming unit (CFU).

Bioassay on pathogenicity of bacteria when applied on the soil surface: For the bioassay on pathogenicity and virulence we modified an experimental design described by Londoño *et al*. (2001, Technical Report, Rionegro, Antioquia, p 78) using 10 plastic cups of 16 oz each, with perforated lids. Each cup was filled with sterile soil and organic matter in a relation of 1:1, and some rice grains as food for the insects. We confined one larva (second instar) of *P. menetriesi* in each cup. The spores were applied in a solution of 0.5 ml and in two concentrations ($1x10^5$ and $1x10^8$) on the soil surface. Each experimental unit was stored in a dark room, at 23 °C and 70% RH. The suspensions were prepared on agar L. After 20 hours we harvested the colonies of each strain in Eppendorf tubes of 1.5 ml. These suspensions were diluted in 1 ml of sterile and distilled water, after which the suspension was ready for counting. Every dilution ($1x10^8$ spores/ml of distilled water, equivalent to 600 lambdas in the spectrophotometer) was poured into the Neubauer camera or hemacytometer to obtain the appropriate dilution of spores. These dilutions were measured with the spectrophotometer.

Results and Discussion

Reproduction of inoculums by injection and forced feeding: All dead insects showed a milky color under the cuticle. Only the larvae infested with BP1 were black; the mortality of the latter strain at 50% was not very high. We observed microscopically all the typical characteristics of bacterial spores. From these observations on BP1 we conclude that while spores penetrated into the gut they propelled the production of spores of other strains of *Paenibacillus popilliae* due to the well-known mechanism of antagonism between microorganisms. However, we also have to consider the possibility that this strain was contaminated with *P. lentimorbus*. Nevertheless, all dead larvae shared some characteristics, such as a soft and swollen body, smell of fermentation, the fact that after 24 hours all larvae turned black, and all responded to Koch's postulates.

When the bacteria were introduced by forced feeding, some diseased larvae showed typical symptoms of agony such as slow movements with white curd coloration. After the sixth week, the bodies turned soft, and then after a few days turned black. Microscopical examination of the hemolymph revealed that the endospores were shaped like a footprint. This is the typical sign for completing the fundamental cycle of infestation (Steinhaus, 1967, Principles of Insect pathology, Hafner Publishing Company, New York-London, 862 pp). In other words, all six strains were able to sporulate until the insect started the "bacteremia" completing the Koch postulates.

Most of the larvae in the second instar (soil experiment) were dead and decomposed after 8 weeks. The presence of bacteria was proven by soil analyses confirming macroscopically and microscopically the morphological characteristics.

The *P. popilliae* strain LF24 killed all larvae of all three white grub species. Strains 381 and LG performed with similar efficiency on *P. bicolor* and *P. menetriesi*, whereas on *A. cincta* these strains were less successful (Figure 2.15.1). Strain B386 was significantly less efficient on *P. menetriesi* than on the other two species. Surprisingly, LG caused a mortality of only 4% on *A. cincta*, whereas both *Phyllophaga* species were efficiently controlled (between 90% and 100%). The high mortality rate of *P. menetriesi* caused by B386 was not expected. This strain was isolated from a white grub of the genus *Ancognatha*. These white grubs are endemic in agroecological zones above 2400 m, a much colder region where *P. menetriesi* is endemic. However, there are several cases where microbial organisms obtained efficient control of white grubs although they do not share the same agroecological zones. Shannon and Carballo (1996, CATIE Informe Tecnico No. 227, Costa Rica) found that the entomopathogenic fungus *Metarhizium anisopliae* was the most efficient antagonist of *Phyllophaga vicina* although both are endemic in different zones. These authors concluded that exotic entomopathogens could develop more efficiency as control agent than endemic ones that are intimately related to both host plant and insect.

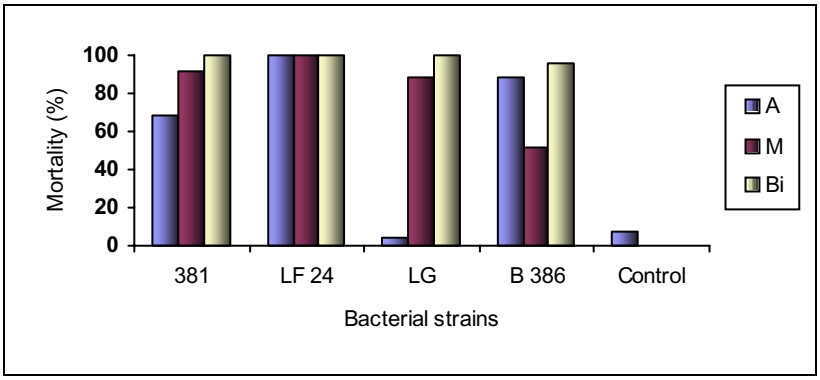

Figure 2.15.1. Mortality of four bacterial strains on three white grub species (second instar): A = *Anomala cincta*; M = *Phyllophaga menetriesi*; Bi = *Phyllophaga bicolor*.

All six bacterial strains caused a mortality of larvae of *P. bicolor* between 90% and 100% (Figure 2.15.2) suggesting that all isolates are a promising tool for the control of this white grub. Interestingly, only the strain BP1 was isolated from *P. bicolor*; nevertheless, all other strains were highly pathogenic on this white grub. These results also corroborate Shannon and Carballo (1996, CATIE Informe Tecnico No. 227, Costa Rica) who observed an increased pathogenicity of exotic entomopathogenic fungi on white grubs.

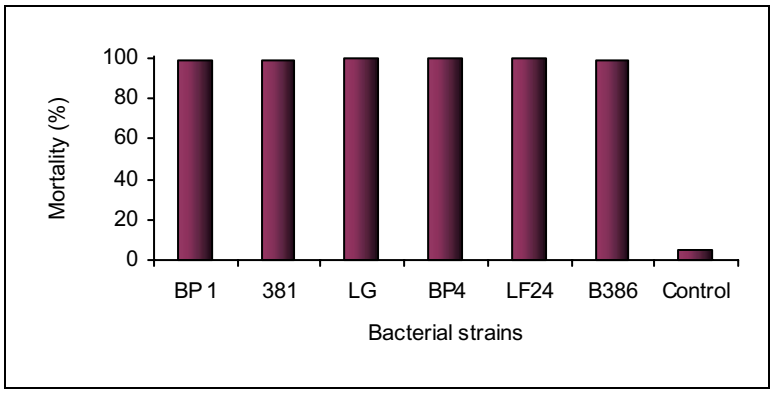

Figure 2.15.2. Mortality of larvae of *Phyllophaga bicolor* after injection of six strains of *Paenibacillus popilliae*.

Pathogenicity of the bacteria on *P. menetriesi* was similar to the experiment with *P. bicolor* (Figure 2.15.3). All strains must be considered as promising control agents of this pest. As expected, we obtained the highest mortality of grubs when the bacteria were injected. After the fourth day we found those larvae infested with LF24, BP1, B386, and 381were considerably diseased. Nevertheless, these larvae continued feeding. Beard (1945, Conn. Agr. Exp. Sta. Bull. 491: 505-83) made a similar observation, showing that white grubs do not die in a defined moment after an attack by entomopathogens, and that the time taken depends on the vigor of each individual. It is even possible that white grubs diseased by *P. popilliae* or *P. lentimorbus* may molt into pupa or adults, although with deformations.

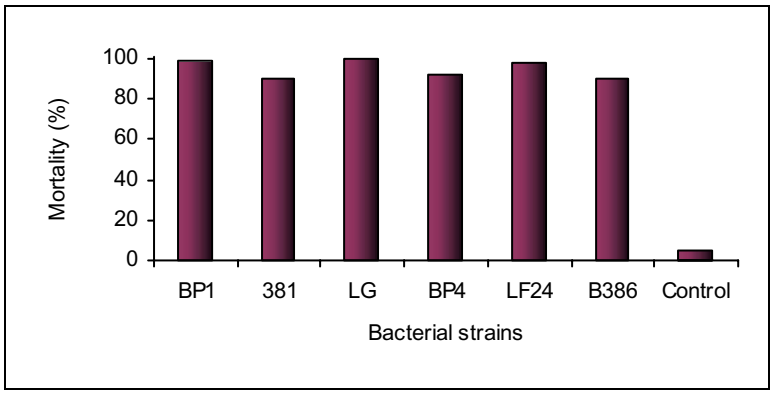

Figure 2.15.3. Mortality of larvae of *Phyllophaga menetriesi* after injection of six strains of *Paenibacillus popilliae*.

When forced feeding was used on the larvae, 50% of them died in the third week, except for BP1 and B386. The range of mortality showed a wide variance (42% to 87%). The most efficient strains were LF24 (87%) and BP4 (79%) (Figure 2.15.4). Comparing the results between forced feeding and injection, we conclude that the behavior of the bacteria varies more when the penetration succeeded in a natural way (by feeding).

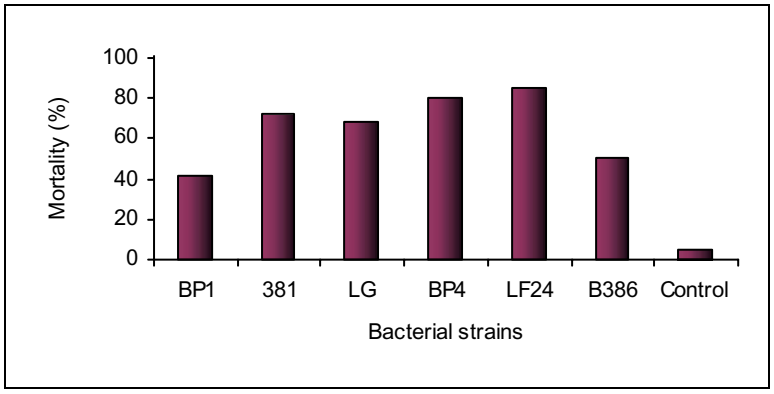

Figure 2.15.4. Mortality of *Phyllophaga menetriesi* when *Paenibacillus popilliae* penetrated the insect by forced feeding.

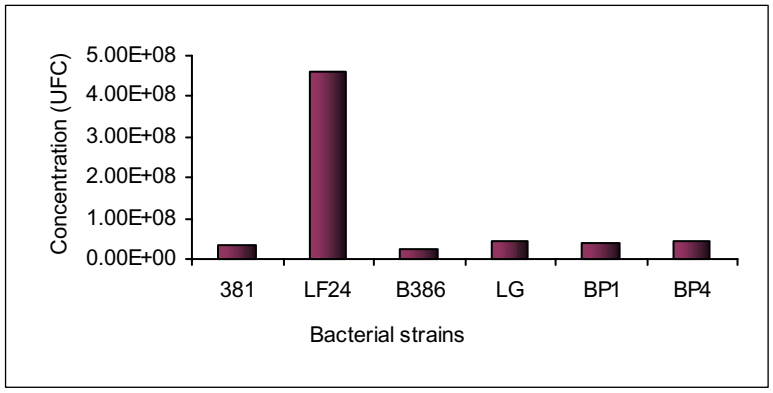

Figure 2.15.5. Cell concentration (UFC) per ml of six strains of *Paenibacillus popilliae* reproduced in *Phyllophaga menetriesi.* Bacteria applied by injection.

In these experiments, we included the commercial strain "Doom Japidemic", which is used in the US against the white grub *Popillia japonica*. This product caused only a poor mortality of *Phyllophaga menetriesi*, corroborating the findings of Diaz (1992, Msc thesis, CATIE, Costa Rica, 67 p) and Londoño (2002, In: Control biológico: Componente fundamental de manejo integrado de plagas en una agricultura sostenible, Lopez-Avila (ed), CORPOICA, Bogota, p 40-48). Following our observations, we share the opinion of Klein and Jackson (1992, In: Use of pathogens in scarab pest management, Jackson and Glare (eds), Intercept, Andover, UK, p 43-62) that tests on cross infectivity between species normally yield negative results. Hence, we suggest not including this strain in further studies on biological control of *P. menetriesi*.

Reproduction of inoculums by injection and forced feeding: As expected, mortality and reproduction rate of inoculums is directly proportional. Both *P. bicolor* and *P. menetriesi* presented high levels of spores in their hemolymph when mortality was high. In general, we obtained greater mortality when spores were injected than by forced feeding. Our results on feeding are similar to studies by Hidalgo *et al*. (1998, Avances en el estudio de la diversidad, importancia y manejo de los coleópteros edafícolas americanos, Universidad Autonoma de Puebla y la Sociedad Mexicana de Entomologia, Mexico, pp165-172) who obtained a 29% mortality of *P. menetriesi* when they applied $1x10^7$ spores per larva.

LF24 performed outstanding cell reproduction when bacteria were applied by injection (Figure 2.15.5). When we introduced the bacteria into the grub by forced feeding, LF24 gave the best reproduction; however, difference to other strains was not as pronounced as in the previous experiment (Figure 2.15.6). Obviously, the bacteria reproduced much better when they were injected. The natural form of penetration can explain this—the bacteria entered with food through the mouth, and the insect ingested them naturally. Subsequently, they penetrated the intestinal wall, germinated in the lumen, and entered the hemolymph where they finally caused the bacteremia. We conclude that the penetration into the intestinal wall is a decisive step in the infection process and recommend applying this methodology for future research on milky disease.

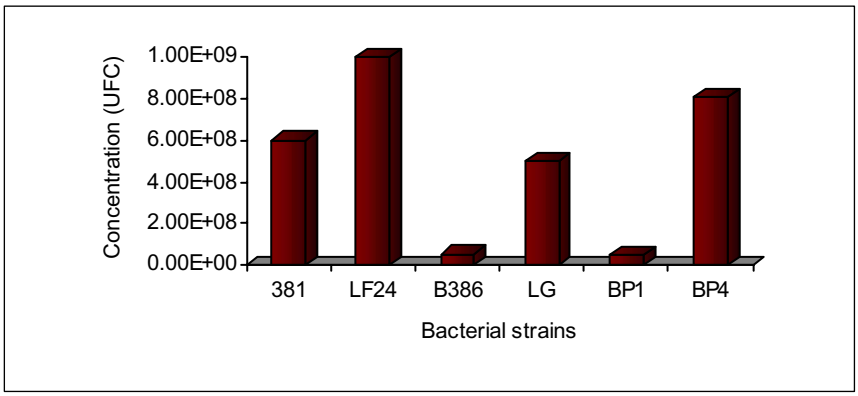

Figure 2.15.6. Cell concentration (UFC) per ml of six strains of *Paenibacillus popilliae* reproduced in *Phyllophaga menetriesi*. Bacteria applied by forced feeding.

Bioassay on pathogenicity of bacteria when applied on the soil surface: Since we did not observe any significant differences between the concentrations of each strain we present all data of this experiment in one chart. LF24 caused a white grub mortality of 83% and was by far the most efficient bacterial strain (Figure 2.15.7); the second strain, BP4, only controlled 51%.

These data corroborate the results of the experiment of reproduction of inoculums by forced feeding and stress the outstanding efficiency of LF24 followed by BP4. Moreover, our experiments suggest that strain LF24 in particular is a highly promising candidate as biopesticide of white grubs and that it is effective against second and third instar of *P. menetriesi*. Other microbial entomopathogens such as nematodes (unpublished data) or fungi did not obtain satisfying results when they were applied on the third instar of this species, even when applied at extremely high dose directly on the insect (10,000 nematodes/ml) As in the case of nematodes, the combinations with other microbial pathogens or insecticides (e.g., imidacloprid or fipronil at sublethal dose) may even improve efficiency of this bacterium.

We hypothesize that two circumstances favored this bacterial strain: First, the inoculums were extracted from the same host (*P. menetriesi*); and second, the spores were recently extracted from white grubs.

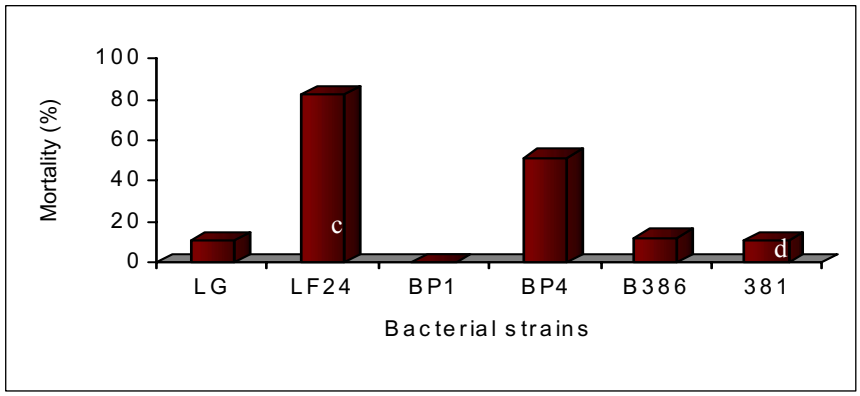

Figure 2.15.7. Mortality of second larval instar of *Phyllophaga menetriesi* when bacteria were applied on the soil surface of the experimental unit.

In the near future, LF24 should be tested in the greenhouse and in field experiments. Moreover, other bacterial strains should be tested on each developmental stage of *P. menetriesi*. The combination with other pathogens such as nematodes or fungi may enhance efficiency of the bacteria.

Activity 2.16. Estimating grade of damage caused by the soil pests *Phyllophaga* spp. (Coleoptera: Melolonthidae) in maize, beans and cassava

Contributors: Alberto Ortega-Ojeda, Elsa Liliana Melo-Molina, Andreas Gaigl

Highlights:

- Economic threshold of white grub species *Phyllophaga menetriesi* on three crops defined
- Tool developed to detect white grub attack by plant phenology.

Rationale

Maize (*Zea mays* L.), beans (*Phaseolus vulgaris* L.), and cassava (*Manihot esculenta* Crantz) are three of the principal tropical crops grown at north of the Department of Cauca in Colombia after sugar cane (*Sacharum officinalis*), according to surveys carried out with farmers of the area (unpublished data). These crops are severely affected by white grubs or *chisas* when these attack during the crops' first month of establishment. These rhizophagous (root-eating) pests from the Melolonthidae family (Coleoptera) have evolved a wide diversity of species associated with different thermal floors and agroecosystems in Colombia. The greatest diversity of white grubs in southwestern Colombia is in Caldono, Department of Cauca, where the white grubs of greatest economic interest include several species of *Phyllophaga*, *Anomala inconstans*, *Cyclocephala lunulata*, *C. fulgurata*, *Plectris fassli*, and *P. pavida*. As many as 5 larvae per square meter were observed in cassava and coffee crops. Among them, *P. menetriesi* is the most important pest because its third-instar larvae are long, reaching 4 cm. They are also voracious, aggressively consuming roots of a large variety of cultivated plants and causing significant crop losses.

Most damage to annual crops such as maize and common bean occurs when the insect attacks the plant, close to where the stem meets the root. The plants present little development with symptoms of wilting

and chlorosis as consequence of root losses, in accordance to the observations by the authors in field and greenhouse experiments between 2003 and 2005 (King, 1984, Tropical Pest Management 30: 36-50).

White grub damage on cassava is verified by consumption of the stake, of its roots, cortex and young outbreaks. Some reports even mention perforated swollen tubers of mature plants in Panama and in the Departments of Quindío and Casanare in Colombia. The harm of the rhizophagous escapes notice, as there has verified it the author in crops of cassava, due to its ground development, which means that the farmer replaces fruitlessly its plants of cassava affected with new stakes, achieving only to continue the feeding of the white grub and increasing an additional cost of seed to the original losses.

Most damage done to annual crops such as maize and beans can be seen as well-defined patches of sick plants, or plants with sick parts, on the farm. The insect attacks the plants close to where the stems and roots join. The plants develop little, show wilting, and change color as they lose roots and so suffer nutrient and water deficiencies. These field observations in Cauca corroborated findings by King (1984, Tropical Pest Management 30: 36-50), and were supported by greenhouse experiments carried out over 2003–2005 in Valle del Cauca.

In cassava, damage by white grubs is confirmed by consumption of stakes, their rootlets and roots, bark, and young shoots. Some reports in Panama even mention attacks on mature plants, where one to three large perforations were found in thickened roots. Perforating galleries were also observed in cassava roots in the Departments of Quindío and Casanare of Colombia.

Damage by rhizophagous escapes notice because of their underground development. The farmer fruitlessly replaces affected cassava plants with new stakes, only to continue feeding the white grubs and add further seed costs to the original losses. In seeking fast solutions, some farmers use synthetic pesticides but with unsatisfactory results and at the cost of their own and the environment's health, as well as the product's own high costs. Such products also tend to generate resistance in the pests, forcing farmers to increase dosage with the consequent increase in the problems just mentioned.

A few authors suggest, based only on their observations, that the possible economic threshold (level of pest infestation at which farmers should take action to prevent significant crop losses) was 3 larvae per plant. However, the monitoring tool used to manage the pest lacks an indicator for deciding when to take control measures over the insect. We therefore proposed to establish, for the three crops mentioned above, a relationship between the degree of visible damage to the plant in the first month of establishment and the presence of *Phyllophaga* larvae.

In contrast to previous studies where the experimental unit for infestation was artificial, we conducted this experiment in the field under natural infestation. This innovative challenge was intended to contribute knowledge on the dynamics of the pest with its host crops. The study aimed to phenotypically establish degrees of damage in the young plant that would tell the farmer when to apply control measures for *Phyllophaga*; quantify potential yield losses in terms of the initial damage done by the rhizophage; and, in the case of cassava, evaluate the damage done by a second generation of larvae that develop during the crop's stage of maturity and thus verify reports of damage in this stage. For the bean and maize crops, whose maturity stage did not coincide with a new generation of *Phyllophaga*, we evaluated the effect of other white-grub genera on the final yield.

Materials and Methods

The experiment was carried out at Bellavista Farm, located in the Village District of Pescador, Municipality of Caldono, Cauca, at 1580 m above sea level (2°49'15.1" N and 76°33'45.6" W). We

followed recommended cultural practices in planting and raising maize, bean, and cassava crops. Maize was planted on 1200 m^2, using variety ICA V-305 at a density of 37,500 plants/ha. Bean variety ICA-Toné was also planted on 1200 m^2 but at a density of 90,000 plants/ha. Cassava variety SM 707-17 was planted on 1300 m^2 at a density of 10,000 plants/ha. The plots lay in an area usually infested by white grubs, as confirmed by a preliminary random sampling of soil. The insects were disturbed as minimally as possible and, after sampling, were left where they were found.

The study aimed to classify the health of young plants, between 0 and 30 days old, according a phenotypical scale based on agronomic parameters to determine damage done by white grubs. Visual criteria were classified into grades 1, 3, 5, 7, and 9, where 1 corresponded to a healthy plant (ideal) and 9 to an agronomically irrecoverable plant (Table 2.16.1).

Table 2.16.1. Phenotypical scale based on the agronomic value for determination of treatments in young plants of maize, to the 30 days from the seeding**.**

Level	Plant	Correspondence
1	Excellent (Control)	Ideal plants, with excellent architecture (stems, petioles and erect leaves); leaves dark green color; good vegetative development (Ó20 cm).
3	Good	Plants with good architecture but smaller height (Ø15 and <20 cm) slightly thinner; smaller number of leaves that the previous.
5	Intermediate	Plants with poor architecture, mild chlorosis; less leaves that the previous; stalk lengthØ10 and <15 cm, thin.
7	Poor	Chlorotic and rachitic plants (poor architecture, weak stalksØ5 and <10 cm; few and small leaves.
9	Very Poor	Irrecoverable plants; without or very limited and small leaves; very chlorotic and/or wilted stalks, with length <5 cm

One plot with 10 planting sites was chosen for each of the 5 grades (i.e., one experimental unit of 1 m^2 x 10 replications × 5 = 50 sites). The total experimental area was therefore 500 m^2. The size of the unit (1 m^2) was chosen according to the recommendation for capturing white grubs. Each site was planted with 3 plants of maize, 9 of beans, and 1 of cassava. A randomized complete block design was used with a unifactorial arrangement. Evaluations were made at 30, 90, and, for cassava, 400 days after planting (DAP). After an analysis of variance (Anova) the Tukey's Multiple Comparisons Test (P Ò0.05) was carried out for the significant variables. Before statistical analysis data were transformed by $\sqrt{x\ 2\ 1}$.

The sale prices used for the economic analyses of the maize and bean crops were provided by the Prices Information Service of the Agricultural and Livestock Sector (SIPSA 2005). The following prices were used (25 June to 1 July 2005): for threshed dry grain of maize, Col$733/kg; and dry grain of beans, $3088/kg. For cassava, two calculations based on sales were made: one for the starch industry, where a sales value of Col$340/kg (wt/kg) was calculated because variety SM 707-17 was produced in a "cold", not "hot", climate, which otherwise would have cost $250/kg. The other calculation was based on the fresh-root market, for which the price $400/kg was calculated.

Results and Discussion

Maize: The height of the plants was strictly related to the density of the white grubs (pÒ0.05) (Figure 2.16.1a). In the absence of the pest the plants reached a height of 15 cm 30 days after planting (DAP) and

275 cm 90 DAP. In contrast the plants that were associated with many grubs reached an altitude of only 125 cm 90 DAP. The typical average height of this variety is 234cm (Navas *et al*., 1993, Fitotecnia Colombiana 4:55-65) indicating that the crop was accurately managed during this experiment. When the damage is of intermediate level or more plant height (181.9 cm) is significantly lower.

The number of maize plants did not show any differences among the treatments (white grub densities) at 30 DAP (pÒ0.05), although differences in agronomic value were present. This is because the white grubs first fed on roots and then on the plant's hypocotyl, which meant that no losses of plants were yet seen (Figures 2.16.1b). At 90 DAP the plants showed advanced damage scoring 5 and 7. Control plants and those with minimal damage (level 3) had an average of 3.3 plants, in contrast to plants with damage at level 5 or more (plants of intermediate appearance up to irrecoverable), which had an average of 1.3 plants per site after initially damaging roots and rootlets, *P. menetriesi* had changed plants, thus significantly reducing (*P* Ò0.009) the severity of damage (Figure 2.16.1b). This contrasts with what was observed at 30 DAP.

Insertion heights for ears reached 145.0 cm in control plants, whereas, in plants with the worst degree of damage, the height was only 32.3 cm. The other treatments had an average of 117.2 cm. The varietal mean was 130 cm (Navas *et al*., 1993, Fitotecnia Colombiana 4:55-65). Our findings therefore suggested that the genotype was influenced by all degrees of damage, except for the control. Moreover, this variable correlated strongly with plant height (Figure 2.16.1a), as was expected from what is known of this crop's genetic patterns.

The major diameters of the stalk (2.63 cm max. and 2.17 cm. min.) corresponded to the scales of damage 1 (control), 3, and 5 of harm (mild and intermediate) (Figure 2.16.1c). There was a clear tendency that the diameter of stalk diminished while damage increased.

The thickest stem diameters (2.17 to 2.63 cm) correlated with scores 1 (control), 3 (minor damage), and 5 (moderate damage) (Figure 2.16.1c). The trend clearly showed that the stems became thinner as damage increased. This variable is important in that it can influence lodging to a lesser or greater extent and, hence, indirectly affect yield.

The heaviest ear corn weight was presented by the control at 358.7 g, in contrast to the average of 78.7g of treatments 2 to 5 (degree of damage 3 to 9) (Figure 2.16.2a). That is, an obvious inversely proportional trend is shown between yield in ear weight per plant and severity of damage.

Results for dry grain yield in maize were similar to those for the ears, with the control distancing itself at a yield of 305.4 g (Figure 2.16.2a) from all the other treatments. The results for this and the previous variables are remarkable, as they are direct indicators of yield, which is affected, even though no drastic impact was observed for such important variables as the number of plants per treatment in the crop's early stages.

Likewise, the 100-seed weight in maize showed a clear trend to drop as plant damage increased (Tukey's; *P* Ò0.05). The best treatments were the control and the least degree of damage, with an average of 38.8 g. These contrasted with the unstable intermediate treatment (degree of damage 5), which had 30.6 g, and the worst degrees of damage, 7 with 23.0 g and 9 with 2.63 g (Figure 2.16.2b). On relating these results with those of the previous two variables, we can infer that, although the first three levels of damage were statistically similar for 100-seed weight, the quantity of grain produced by the control (largest number of ears per plant) sets it at a distance from the other two. Hence, the minimal presence of the rhizophage must clearly be avoided to prevent yield loss. Moreover, to maintain only a 5% loss, measures need to be taken when 3 in 50 sites per hectare (chosen at random) are found to have at least one white grub each (assuming a density of 30,000 plants/ha).

Mature maize plants did not suffer damage. Larvae of *P. menetriesi* had already become pupae by harvest and were located at a depth of 30 cm. Any larvae found corresponded mainly to *Astaena* sp., *Cyclocephala* sp., *Anomala* sp., *Plectris fassli*, and *P. pavida*, as identified according the form of the palidia in their rasters. These larvae were not as aggressive as *P. menetriesi*, which meant that any damage they caused did not reflect in the crop. In addition, it can be stressed that the presence of white grubs was correlated with increasing agronomic values (Figure 2.16.2c) (Tukey pÒ0.05). We hypothesize that female beetles prefer to oviposit near healthy plants to ensure their progeny survival. This population, however, did not cause any significant damage since the crop was going to complete its cycle. This contrasts with Posada's findings (1993, Agricultura Tropical 30: 71-79), where, in their most advanced stages of development, crops would show yellowing and even lodging.

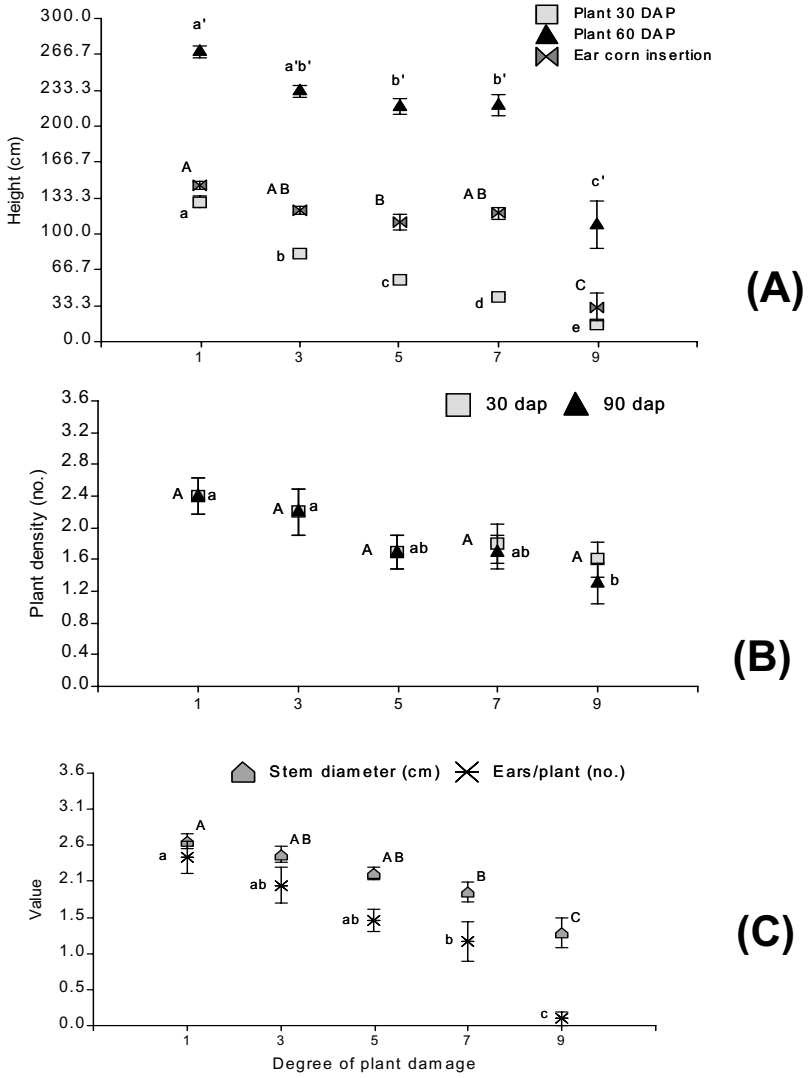

Figure 2.16.1. a) Altitude of plants to the 30 DAP (days after planting) and 90 DAP (physiological maturity) and Altitude of insertion of ear of corn (90 dap); **b**) Number of plants by site to the 30 DAP and to physiological maturity (90 dap); **c**) Diameter of stalk (cm) and number of ear of corns by plant to physiological maturity (u) (Tukey pÒ0,05)

The economic analysis of dry grain yield of maize can be appreciated in Table 2.16.2. Even with the least observed damage, losses were catastrophic (50%). This finding suggests that when farmers detect any damage, they need to eliminate the white grubs, and re-plant the crop.

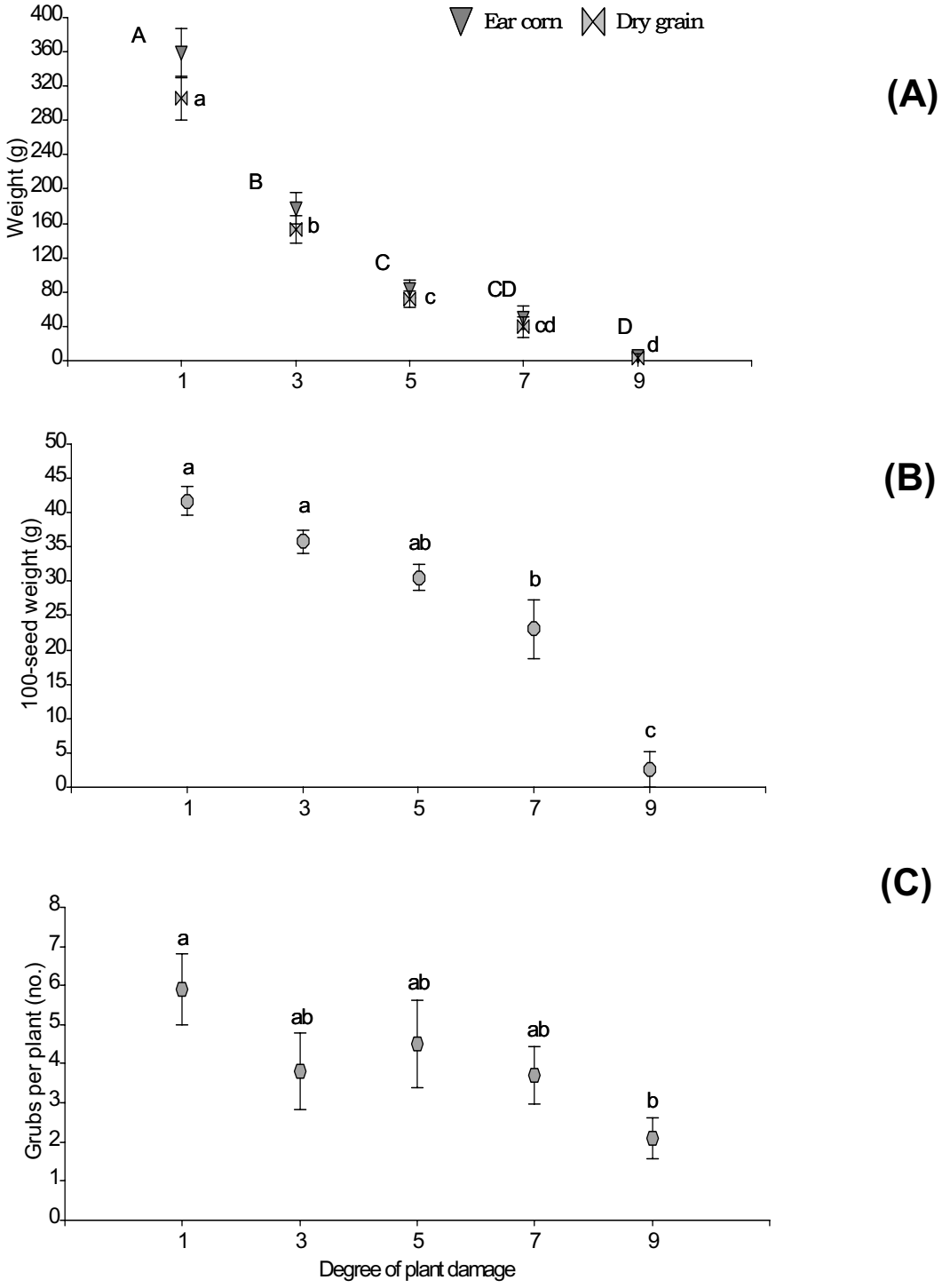

Figure 2.16.2. Evaluated variables: **a.** Yield, in grams, of ear of corns by plant and of dry grain, to the harvest (150 days after planting); **b.** Weight of 100 seeds in dry grain (g); **c.** Number of white grubs of second generation by plant and by treatment (Tukey pÒ0,05).

Beans: None of the affected plants reached the height of the controls (pÒ0.05) indicating that even the least damage (level 3) during the initial phase affects significantly the development of the crop, although the number of plants per experimental unit did not decrease (Figure 2.16.3).

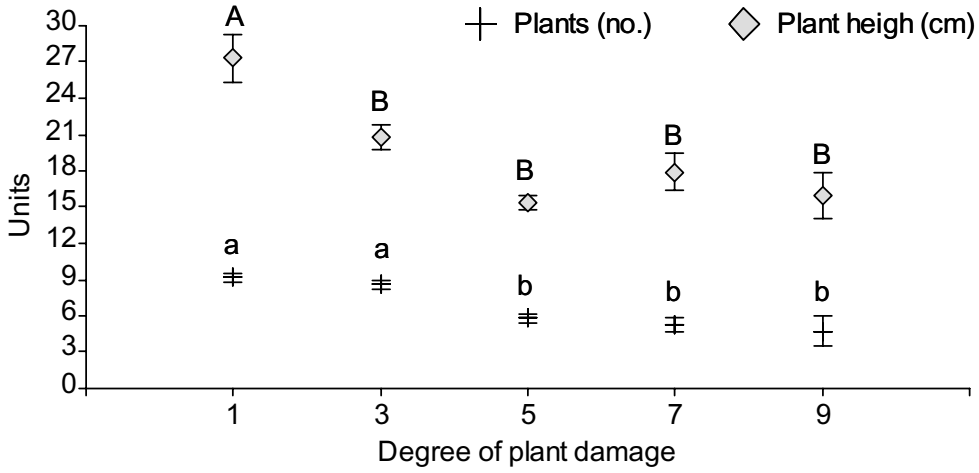

Figure 2.16.3. Number and height of plants of shrub-like beans per square meter (treatment), to the harvest in dry grain (Tukey pÒ0,05).

None of the control plants and of those of with minimal damage suffered any plant losses, unlike the others with damage classified as 5 to 9 (Figure 2.16.3) that were associated with an increased number of white grubs. However, the weakest damage was product of consumption of the roots by white grubs affecting yield, even though the elimination of plants was not notable (pÒ0.05).

The differences in the losses of the dry grain (g) corresponded to the different degrees of damage (Figure 2.16.4). Losses reached 39.8% even when visible damage was minimal, worsening with each higher degree of damage evaluated (leves 5, 7 and 9). From these results and reasoning businesslike, we recommend permitting only a white grub by plant in maximum 5% of the population of the crop of beans.

At the end of May and beginning of June we found a population of white grubs that was composed by the genera and species *Plectris* spp., *Cyclocephala* spp., *Anomala* spp., and *Phyllophaga bicolor*. These species are economically less important than *P. menetriesi*. In spite of the white grub diversity there were not differences between levels of estimated damage, which made it possible to conclude that other white grubs than *Phyllophaga menetriesi* did not affect the yield of beans. Hence, we recommend to plant beans when *P. menetriesi* completes the larval stage in order to elude its attacks.

In bean crops, the economic losses caused by even the least degree of damage can reach 60% (Table 2.16.2). This means that, to reduce losses, damage must be prevented and/or the crop quickly re-planted after measures have been taken against the rhizophagous.

Table 2.16.2. Ratio of gains and losses per kilogram of grains according to different degrees of damage by white grubs to plants growing in a commercial plot of beans.

Damage[a]	Yield (kg)	Income (Col$/kg)	Losses	
			(Col$/kg)	(%)
1	0.131	440.19	0.00	0.00
3	0.052	175.02	265.17	60.24
5	0.037	123.65	316.55	71.91
7	0.034	113.60	326.59	74.19
9	0.015	50.13	390.06	88.61

a. On a scale of 1 to 9, where 1 = no damage (control) and 9 = severe damage (agronomically unrecoverable plant).

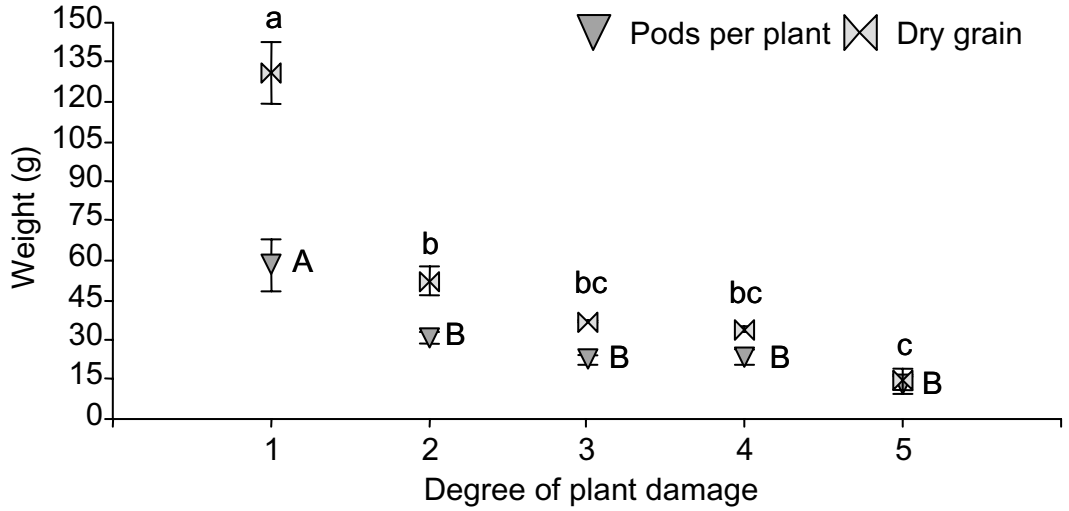

Figure 2.16.4. Number of pods per plant and yield of dry grain (g) in bush beans per square meter (treatment). Bars indicate standard errors (Tukey's; *P* Ò 0.05). The same letters per variable indicate that the values are not statistically different to each other

Cassava: In this long-cycle crop, we observed that plants with the least degree of damage (grade 3) improve over time, whereas those suffering moderate (grade 5) to severe damage (grade 9) tend to worsen (Figure 2.16.5a). In other words, the cassava plant can recover after an initial, temporary, and minimal damage to the propagule (stake and root system). The final yield will have been directly compromised in proportion to the degree of initial damage.

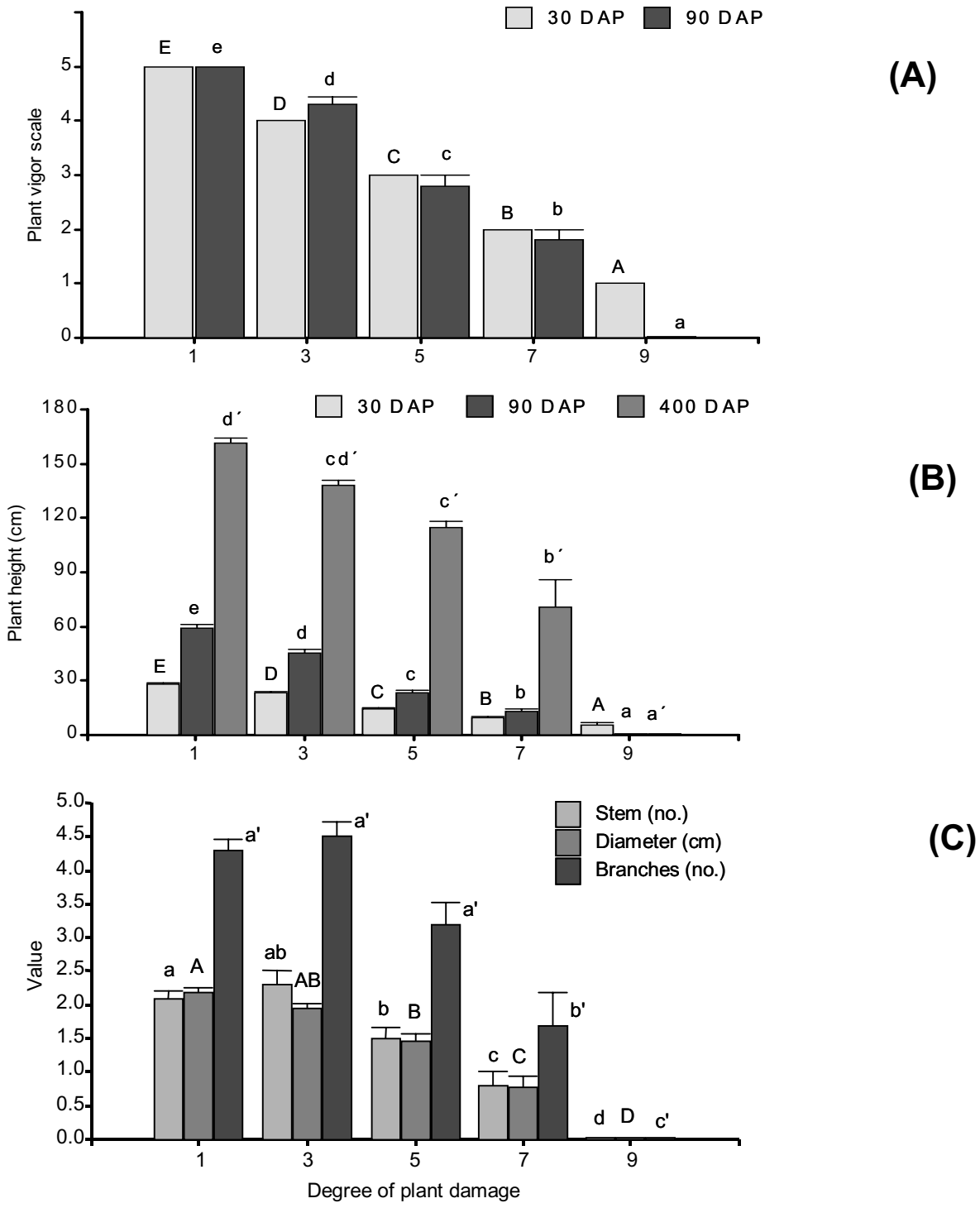

Figure 2.16.5. Evaluated variables for cassava: (A) plant vigor at 30 and 90 days after planting (DAP), on a scale where 0 indicates dead plant and 5, very vigorous plant; (B) plant height at 30, 90, and 400 DAP; and (C) average number of stems and branches per plant at 400 DAP. Bars for standard errors are shown (Tukey's; *P* Ò 0.05). The same letters per variable indicate that the values are not statistically different to each other.

At 90 DAP, no damaged cassava plants (whether scoring 1 or 9) reached the height of the control plants, thus showing that even the least degree of damage during crop establishment significantly affects vegetative development. However, at 400 DAP, plants scoring 3 for damage attained a height of 1.62 cm, like the control plants (*P* Ò 0.0001), with a maximum height of 1.62 cm (control). This finding corroborates the theory that if the plant survives and can recover well, once the larva stops feeding and passes to the prepupal stage (Figure 2.16.5b).

Stem number and diameter are similar until the degree of damage is 3, that is, these genotypic traits are not observed as affected if damage is minimal. For damage grades 1 and 3, the highest averages for diameter and number of stems (Figure 2.16.5c) are 2.1 cm and 2.2 stems, respectively. The other damage grades (5 y 7) produced averages of 1.2 stems and 1.1 cm of diameter. The plants classified as level 9 died (*P* Ò0.0001).

Plants with degrees of damage 1 and 3 were characterized by an average of 4.4 branches (Figure 2.16.5c). However, from 5, the number of branches declined significantly, which would occur if more than one white grub feeds on the propagule.

For the total number of roots, both commercial and noncommercial, we observed significant differences corresponding to the degree of damage (Figure 2.16.6a). The control produced 19.5 roots per plant, whereas plants with minimal damage produced only 13.4 roots, the number dropping to zero as level 9 was reached. If the cassava was destined for the starch market, the economic losses would be significant (*P* Ò0.0001).

In contrast to the previous variable, the number of only commercial roots did not differ significantly between the control and least damage (grade 3), which suggests that if the attack is light, then the plant recovers and its yield is satisfactory for the fresh-root market (Figure 2.16.6b). The maximum number of commercial roots per plant was 7.2 for control plants, followed by 5.3 for plants scoring 3 for damage (Figure 2.16.6a). These values surpass the variety's historical average of 4.0 commercial roots per plant. This indicates that the soil's nutritive conditions and the climate were favorable and did not act as covariables in the results. Where the degree of damage was 9, no commercial roots were produced.

The maximum yield in terms of weight of *all* roots was obtained by the control plants with 4.3 kg/plant, followed by plants scoring 3 for damage with 3.0 kg/plant (*P* Ò 0.0001). These values surpass the variety's historical average of 2.4 kg/plant. Meanwhile, plants scoring 5 and 7 damage produced an average of 0.6 kg of roots. Level 9 did not have plants (Figures 2.16.6a and 2.16.6b).

Unlike the previous variable, yield in terms of weight of *commercial* roots was similar for healthy plants and those suffering minimal damage, averaging 2.6 kg /plant (Figure 2.16.6b). Again, this finding suggests that, if the initial damage is limited, the plant can recover and produce equally to a healthy plant. According to our observation this might happen when:

- the larvae have sufficient space and can continue feeding on other plants
- the stake is planted when the larvae are finalizing instar III
- the plant has surpassed the first month of its development before larvae of *P. menetriesi* in the third instar.

We hypothesize that, where damage is minimal, a given larva had had sufficient space to travel through the soil to feed on more than one stake during its development, or the stake was planted at the end of third instar when the rhizophage could do little damage to the propagule, or the plant was more than 1 month old before the third-instar larva reached it.

As we mentioned with regard to maize and beans, we recommend that losses should not exceed 5% before taking control measures against white grubs, that is, when 3 out of 50 randomly selected planting sites are found with larvae. Also, because the samples of *Phyllophaga* larvae on farms did not show generalized infestations, measures should be applied after sequential samplings to discover where the infested areas are (Velásquez, 1994, CATIE Informe Técnico No. 277, Costa Rica).

*Economic analysis of damage of cassava roots***:** The economic losses incurred for plants with even the least damage reached almost 26% for the fresh-root market, whereas for the starch market, losses reached almost 30% (Table 2.16.3). These losses are sufficiently significant to oblige, as for maize and beans, roguing the crop, eliminating the white grubs, and re-planting the crop immediately. Losses incurred by moderate degrees of damage exceed even these percentages.

The economic losses incurred for plants with even the least damage reached almost 26% for the fresh-root market, whereas for the starch market, losses reached almost 30% (Table 2.16.4). These losses are sufficiently significant to oblige, as for maize and beans, roguing the crop, eliminating the white grubs, and re-planting the crop immediately. Losses incurred by moderate degrees of damage exceed even these percentages.

Table 2.16.3. Relation of gains and losses per kg of root according to each treatment.

Treat.*	Yield (kg)		Entries ($)		Losses			
	Total	Commercial	Sold for starch	Sold in fresh	Sold for starch ($)	Sold in fresh ($)	Sold for starch (%)	Sold in fresh (%)
1	4,28	2,98	1455,20	1192,00	0,00	0,00	0,00	0,00
2	3,02	2,21	1026,80	884,00	428,40	308,00	29,44	25,84
3	1,06	0,73	360,40	292,00	1094,80	900,00	75,23	75,50
4	0,13	0,00	44,20	0,00	1411,00	1192,00	96,96	100,00
5	0,00	0,00	0,00	0,00	1455,20	1192,00	100,00	100,00

* Treat. = Treatment

Table 2.16.4. Relation of gains and losses per kg of root of cassava according to different levels of harm in plants of a commercial lot.

Treat. *	Yield (kg)		Entries ($)		Losses			
	Total	Commercial	Sold for starch	Sold in fresh	Sold for starch ($)	Sold in fresh ($)	Sold for starch (%)	Sold in fresh (%)
1	4,28	2,98	1455,20	1192,00	0,00	0,00	0,00	0,00
2	3,02	2,21	1026,80	884,00	428,40	308,00	29,44	25,84
3	1,06	0,73	360,40	292,00	1094,80	900,00	75,23	75,50
4	0,13	0,00	44,20	0,00	1411,00	1192,00	96,96	100,00
5	0,00	0,00	0,00	0,00	1455,20	1192,00	100,00	100,00

* Treat. = Treatment

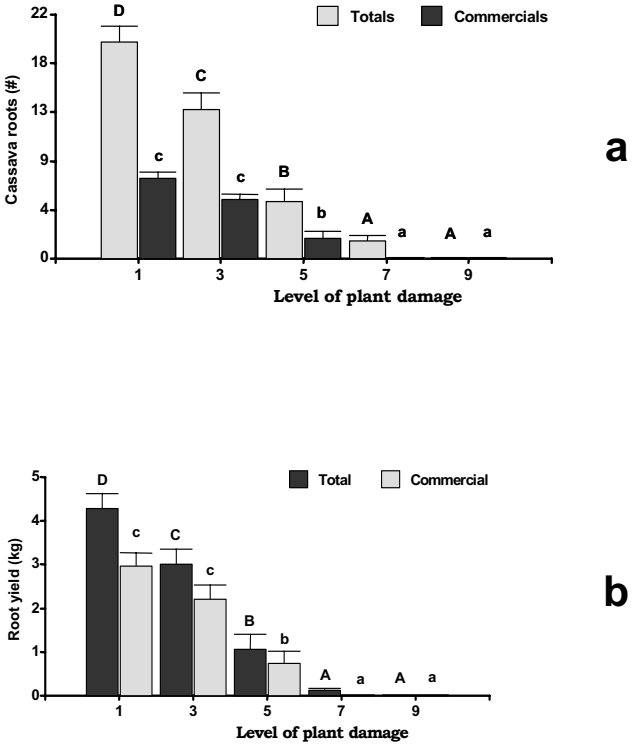

Figure 2.16.6. Evaluations to the harvest (400 dap): **a**) Number of total and commercial roots by plant; **b**) Yield in kg/plant of total and commercial roots; is shown the standard error (Tukey pÒ0,05).

Root damage due to the second white grub generation: The cassava crop's long cycle can expose it to two generations of the pest. We observed that the second-generation rhizophages developing with the maturing cassava did not damage roots, even though as many as 3 third-instar larvae were found in association with more than 100 plants in the plot.

Our findings differed from those of E. L. Melo-Molina (, unpublished data) and C. J. Herrera (unpublished data), who had detected orifices and galleries in cassava roots in Panama and Colombia. The explanation may lie with the experimental site, that is, the larvae found sufficient food in the abundant quantity of succulent rootlets, both of the cassava and neighboring plants that often grow up by harvest time in long-cycle crops. Hence, damage caused before harvest does not produce economic losses nor is it detected in leaves. We therefore recommend conserving live mulch in the crop's furrows, which would distract the insect and prevent its attacking the thickened roots.

Conclusions

- ∉ It is possible to identify levels of damage in cassava, beans, and maize within 30 days after planting and develop strategies for the control of white grubs of *Phyllophaga* spp.
- ∉ The minimal damage identified during the development of maize cause significant yield losses
- ∉ Although white grubs didn't eliminate a significant number of bean plants the minimal visible damage on leaves can diminish yield and needs means of control
- ∉ The relative economic losses are highly significant even when observed plant damage was minimal: they skirt 30% of cassava, going through 50% in maize, until reaching 60% in beans

- Healthy and slightly damaged plants obtained similar levels in terms of plant height, umber of stalks, diameter of stalk and branching of cassava
- Cassava: all levels of damage caused finally irrecoverable yield losses of roots for the fresh market
- Cassava: when damage is slight (level 3) plants continue their development producing a satisfying root weight that can be sold to for the starch market
- White grub species different to *P. menetriesi* that coincide with the last months of maize and beans prior to harvest didn't significantly effect neither vigor of the plants nor yield.
- In contrast to other authors we could not evidence that the second generation of *P. menetriesi* causes significant damage on the swollen cassava roots.
- In order to reduce yield losses in maize, cassava, and beans coincidence of planting and third larval instar of *P. menetriesi* should be avoided.
- Four weeks after planting a survey of 50 randomly selected plants per hectare should be carried out (of a density of 30,000 maize plants, 90,000 bean plants and 10,000 cassava plants per hectare). If three plants or more are associated with only one grub the farmer should initiate activities of control.
- As last strategy plants should be replaced immediately after detecting symptoms of damage and after previous elimination of the grubs. Otherwise, economic losses can oscillate between 25 and 60% even if the visual damage of the plant is minimal.

Activity 2.17. Feeding behavior of three white grub species associated with potato in the Savanna of Bogotá

Contributors: Cesar Zuluaga, Daniel Carrillo, Ilan Garzón, Miguel Serrano, Andreas Gaigl

Highlight:

- Importance of soil organic matter on pest status of three white grub species identified

Rationale

One of the most important pests in the cold zones of the Colombian Andean region are white grubs (Coleoptera: Melolonthidae) (Londoño *et al*., 2002, CORPOICA Boletín Técnico N^{o} 3, Colombia). These insects have been reported as pests on horticulture, ornamental flowers, potatoes, and pasture. The prevailing species on potato and pasture in Cundinamarca are *Clavipalpus ursinus, Heterogomphus dilaticollis, and Ancognatha scarabaeoides* (Ruiz & Posada, 1985, Rev. Colombiana de Entomología 11: 21-26). However, the pest status of these species is not clear. Scholtz (1990, Journal of Natural History 24: 1027-1066) proposed that within the Melolonthinae subfamily (i.e. C. ursinus) larvae usually feed on roots, but also on humus, or rarely, on dung; whereas larvae that belong to the Dynastinae subfamily (i.e. *A. scarabaeoides*, *H. dilaticollis*) feed on plant roots or rotting vegetable matter. In the case of A. scarabaeoides there are contradictory reports. Ruiz & Posada (1985, Revista Colombiana de Entomología 11: 21-26) claimed that this white grub feeds mostly on material in process of decomposition whereas other reports state that this grub is a serious pest on potato. Likewise, the pest status of *C.ursinus* and *H. dilaticollis* is not documented. The objective of this work was to study the feeding behavior of these three white grub species that are abundant in Savannah of Bogotá. We evaluated i) the effect of soils with different portion of SOM on the size and weight these larvae, ii) the effect of different food types on the

development of these grubs, and iii) the damage caused bythese three species on tubers, potato germs, and wood.

Materials and Methods

The complete experiment was conducted on the farm "Potosí" in the municipality of Subachoque (Cundinamarca, Colombia). Approximately 1000 white grubs of each species were collected in the field and confined in groups of 20 individuals in plastic pots (one gallon) filled with soil and germinated wheat during a period of 15 days prior to the experiments establishment. The offered food types were pieces of wood, potato tubers, or no food (control). The experimental unit consisted of one pot (one gallon) filled with sand (0% SOM) or soil (14% SOM), one food type and five larvae in second and third instar of *C. ursinus*, *H. dilaticollis*, and *A. scarabaeoides*, respectively. *A. scarabaeoides* and *A. ustulata* were present at the sites where we collected grubs. However, *A. scarabaeoides* dominated. Since it is very difficult to distinguish these larvae morphologically we included both in our tests. We weighted the white grubs every eight days and measured the diameter of the larvae every 15 days.

We estimated the consumption ("damage") on potatoes and wood using a scale from zero to four, where 0 represents no damage, 1 = 1 – 25%, 2 = 26 – 50%, 3 = 51 – 75%, and 4 = 76 – 100%.

Results and Discussion

The type of nutrition didn't significantly affect the diameter of larvae's bodies when soil was the substrate. Larvae of *Ancognatha* spp. reduced weight in all treatments. Likewise, the weight didn't significantly differ when we offered only soil, potato, or wood. However, *C. ursinus* increased weight on soil without any other food and lost weight feeding on wood and potato. *H. dilaticollis* increased weight when wood was offered and lost weight when the larvae fed only on potato or soil (Table 2.17.1). The good adaptability of *C. ursinus* and *H. dilaticollis* to soil alone and wood, respectively, attracts attention. This finding was corroborated by the low mortality (29 and 25%, respectively) of grubs in these two treatments.

When sand was the substrate none of the three white grub species found their ideal conditions. All grubs lost weight independently of the food type. Nevertheless, *Ancognatha* spp. was the species that less suffered when potatoes were offered (Table 2.17.1). Interestingly, *C. ursinus* lost more weight on pure sand and wood, indicating that this species definitely depends on fresh plant material such as potatoes (Table 2.17.2).

Table 2.17.1. Effect of three food types on weight (g) of *Ancognatha* spp., *Clavipalpus ursinus*, and *Heterogomphus dilaticollis* in soil (14% SOM). Different letters indicate significant differences between means (Tukey, $P<0.05$).

Species	No food	Wood	Potato
Ancognatha spp.	-0.057 ± 0.038 a	-0.005 ± 0.022 a	0.001 ± 0.014 a
Clavipalpus ursinus	0.062 ± 0.077 a	-0.077 ± 0.028 b	-0.034 ± 0.029 ab
Heterogomphus dilaticollis	-0.006 ± 0.003 b	0.052 ± 0.065 a	0.012 ± 0.024 ab

Table 2.17. 2. Effect of three food types on weight (g) of *Ancognatha* spp., *Clavipalpus ursinus*, and *Heterogomphus dilaticollis* in sand. Different letters indicate significant differences between means (Tukey, P<0.05).

Species	No food	Wood	Potato
Ancognatha spp.	-0.026 ± 0.102 b	-0.016 ± 0.048 b	-0.041 ± 0.139 a
Clavipalpus ursinus	-0.354 ± 0.199 a	-0.308 ± 0.222 a	-0.092 ± 0.196 a
Heterogomphus dilaticollis	-0.006 ± 0.022 a	-0.074 ± 0.019 a	-0.003 ± 0.012 a

All three species fed on potato tubers. *H. dilaticollis* caused most damage on potato in sand, whereas *C. ursinus* was the most noxious species on potato in soil with SOM. All three species fed on wood. In sand *H. dilaticollis* consumed more wood than the other species. All white grubs fed more on wood when the environment was soil.

All three species attacked in similar magnitude potato germs, this in spite of the different size of these three species; however, damage was greater in soil as substrate. This can be explained by the fact that potatoes germinated more in soil than in sand and that white grubs developed better when SOM was present. (Figure 2.17.1)

Ancognatha spp. prefers soil organic matter, however, when SOM is absent this species converts into an aggressive pest of potatoes. We conclude that this species has strong capacities to feed on potatoes under normal conditions as well as in soils of low content of SOM. This corroborates Villalobos *et al.*, 1996, Applied Soil Ecology 5: 231-246) who observed that the presence of SOM reduced feeding activity of *Costelytra zealandica* on carrots. Villalobos (1999, Journal of Sustainable Agriculture 14: 5-29) suggested that melolonthids larvae feed on a range of substrates that vary from living and dead roots of plants to other fractions of the complex pool defined as SOM or humus.

It is important to mention that in the savanna of Cundinamarca farmers cultivate intensively potatoes on plots with a slope of more than 50% deploying conventional agricultural methods like tillage promoting erosion. These conditions cause modifications of the physiochemical soil characteristics. These continued alterations are responsible of SOM reduction, forcing *Ancognatha* spp. to attack fresh tubers. Due to this insect's capability to replace easily soil organic matter with fresh tubers as food increases dramatically its pest status in this region. The increasing soil degradation during the last decades due to SOM depletion has increased root herbivory by white grubs and disrupted the presence of entomopathogens.

In spite of being the smallest larva in this experiment *C. ursinus* was the species that most damage caused on potato tubers. Moreover, it is the species that less consumed on wood in both substrates, indicating its high specialization in consumption of potatoes and little acceptance of other food alternatives.

C. ursinus is one of the most abundant species in the Savanna of Bogotá. In spite of having the capability of feeding on organic matter this grub shows a clear preference for fresh material. These results support the observations made in potato, horticulture, pasture and reports of farmers in this region that this species is the economically most important white grub.

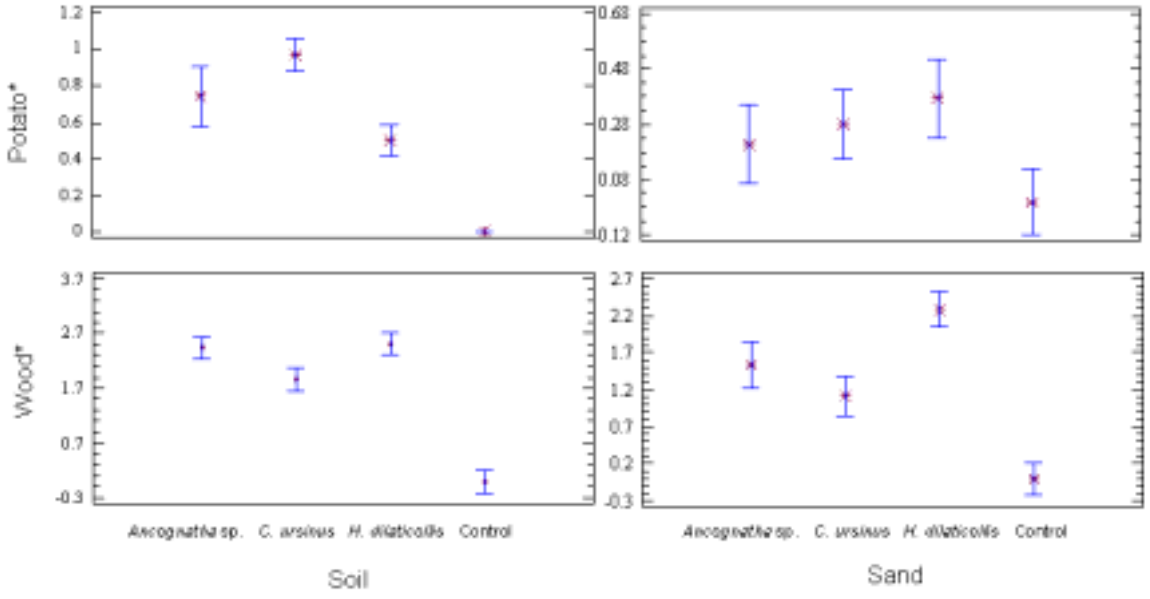

Figure 2.17.1. Feeding behavior of *Ancognatha sp., C. ursinus* and *H. dilaticollis*. Damage on potato tubers and wood in soil with SOM and sand. Means and Standard Errors (P<0.05).*measured using a scale from zero to four, where 0 represents no damage, 1 = 1 – 25%, 2 = 26 – 50%, 3 = 51 – 75%, and 4 = 76 – 100%.

H. dilaticollis was the species that most consumed on wood. This fact is corroborated by our observation that this white grub is mostly associated with trunks in decomposition in the field. We observed a pronounced preference for this type of food in spite of the capability to feed on SOM or potatoes. This explains the observation that *H. dilaticollis* caused less damage on potato than the other two species. However, when no organic matter is available this species may convert into a serious pest.

In general we want to claim that this experiment yielded highly valuable results for example the importance of SOM on the pest status of white grubs. However, other results are very difficult to explain and indicate some errors in the experimental design that should be eliminated in future works. For example, it is against any logic that larvae of *C. ursinus* weight less than they feed on potatoes and more than there is food available. It is possible that 20 larvae in one experimental unit blur the results. The variance of these 20 individuals may vary a lot. Moreover, the parameter "Diameter of Larvae" was not an appropriate indicator of the development and behavior o the grubs. It is possible that the three month lapse of observation was too short. On the other hand, weight was a good indicator of the development of the immature stages.

Conclusions and Recommendations

- ü *Ancognatha* spp. may damage potato tubers when no SOM is available
- ü The same applies to *H. dilaticollis*
- ü *C. ursinus* prefers fresh material and is a principal pest on potato and other crops. It is necessary to develop strategies of integrated control of this insect.
- ü We recommend using in future experiments only one individual per experimental unit.
- ü To evaluate effect of alternative agricultural strategies such as no tillage or crop rotation on the feeding behavior of *Ancognatha* spp. and *H. dilaticollis*.

- ü To generate programs of transferring technology for farmers that allow to identify white grub species in the zones where potatoes are cultivated and develop strategies of control

Activity 2.18. Screening *Brachiaria* genotypes for spittlebug resistance

Contributors: C. Cardona, G. Sotelo, J. W. Miles, and A. Pabón

Highlights:

- ∉ Numerous sexual hybrids (SX03, SX05) with high levels of antibiosis resistance to *Aeneolamia varia*, *A. reducta*, and *Zulia carbonaria* were identified
- ∉ High levels of antibiosis resistance to *A. varia*, *A. reducta* and *Z. carbonaria* were detected in 9 apomictic hybrids (series BR04)
- ∉ Six apomictic hybrids of the MX02 series, selected for resistance to *Prosapia simulans*, also showed resistance to *A. varia*, *A. reducta, Z. carbonaria*, and *Mahanarva trifissa*
- ∉ Six apomictic hybrids of the series BR02 and 11of the series MX02 were identified as resistant to *A. varia*, *Z. carbonaria*, *Z. pubescens*, and *M. trifissa* under field conditions

Activity 2.18.1. Greenhouse screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species

Rationale

Assessment of resistance to spittlebugs is an essential step in the process of breeding superior *Brachiaria* cultivars at CIAT. In 2005, intensive screening of selected hybrids was conducted under greenhouse and field conditions. All available genotypes were evaluated.

Materials and Methods

Screenings for resistance in the greenhouse were conducted with *Aeneolamia varia, A. reducta, Zulia carbonaria, Z. pubescens, Mahanarva trifissa* and *Prosapia simulans*. Using a new methodology (Cardona *et al*., 1999, J. Econ. Entomol. 92:490-496) test materials were usually compared with five checks fully characterized for resistance or susceptibility to *A. varia*. Plants were infested with six eggs per plant of the respective spittlebug species and the infestation was allowed to proceed without interference until all nymphs were mature (fifth instar stage) or adult emergence occurred. Plants (usually 5-10 per genotype) were scored for symptoms using a damage score scale (1, no visible damage; 5, plant dead) developed in previous years. Percentage nymph survival was calculated. Materials were selected on the basis of low damage scores (<2.0 in a 1-5 scale) and reduced percentage nymph survival (<30%). All those rated as resistant or intermediate were reconfirmed. All susceptible hybrids were discarded.

Results and Discussion

In 2005, 119 pre-selected sexual (SX03) hybrids were simultaneously screened for resistance to *A. varia*, *A. reducta*, and *Z. carbonaria*. We used five replications per hybrid per insect species. For comparison, we used five well-known checks replicated 10 times per insect species. All but one of the hybrids were

resistant to all three test species. To the extent that mean percentage nymph survival in the population did not differ from percentage survival in our most resistant check, the hybrid SX01NO/0102 (Table 2.18.1.1). These results clearly indicate that a very significant progress has been made in incorporating antibiosis resistance to all of the three test species in a relatively short period of time.

Table 2.18.1.1. Levels of resistance to three spittlebug species in 119 sexual Brachiaria hybrids and checks.

Genotype	Spittlebug species					
	Aeneolamia varia		*Aeneolamia reducta*		*Zulia carbonaria*	
	Damage scores[1]	Percentage nymph survival	Damage scores	Percentage nymph survival	Damage scores	Percentage nymph survival
BRX-44-02[2]	5.0a	86.6a	4.9a	83.3a	4.5a	79.6a
CIAT 0606[2]	4.8a	93.3a	4.4a	85.2a	4.4a	69.9a
CIAT 6294[3]	1.9bc	36.7bc	3.1b	52.4b	2.4b	50.0b
CIAT 36062[3]	2.2b	26.7bc	1.7cd	18.5c	2.2b	38.3b
CIAT 36087[4]	2.3b	48.1b	2.0c	18.3c	1.2c	1.7c
Mean 119 SX03 hybrids	1.4c	13.1c	1.2d	2.9d	1.3c	6.5c
SX01NO/0102[3]	1.2c	0c	1.1d	3.3d	1.0c	0c

[1] On a 1 – 5 damage score scale (1, no visible damage; 5, severe damage, plant killed)
[2] Susceptible check
[3] Resistant check
[4] 'Mulato 2'; commercial check.
Means of 5 reps per genotype per insect species. Means within a column followed by the same letter are not significantly different at the 5% level according to Scheffe's multiple range test for arbitrary comparisons. Each species analyzed separately.

Further proof of the rapid progress made in incorporating resistance to spittlebug was obtained when 565 new hybrids (SX05 series) were tested for resistance to three spittlebug species. As shown in Figure 2.18.1.1, 96.2%, 94,7% and 93.9% were rated as resistant to *A. varia*, *A. reducta*, and *Z. carbonaria*, respectively. 468 hybrids (82.8%) were classified as highly resistant to all three species tested. Progress was also detected when resistance reactions in two consecutive cycles were compared (Figure 2.18.1.2). It is valid to conclude that there has been a steady increase in the frequency of resistant genotypes as a result of recurrent selection through cycles.

In support of continuous breeding activities we screened a set of 141 apomictic BR04 hybrids. Most were susceptible but a handful of them showed acceptable levels of antibiosis resistance to all three tests species (Table 2.18.1.2). As in previous occasions, correlations between damage scores and percentage nymph survival were highly significant: 0.802** for *A. varia*, 0.924** for *A. reducta* and 0.840** for *Z. carbonaria*.

In 2004 we reported on varying levels of resistance to *Prosapia simulans* (one of the most important species affecting *Brachiaria* in Mexico) in 34 apomictic hybrids (coded MX). These hybrids had been pre-selected in Mexico for good adaptation and desirable agronomic characteristics. In 2005 we conducted a series of replicated tests to evaluate the resistance of these genotypes to four major species present in Colombia. Those showing multiple resistances are listed in Table 2.18.1.3.

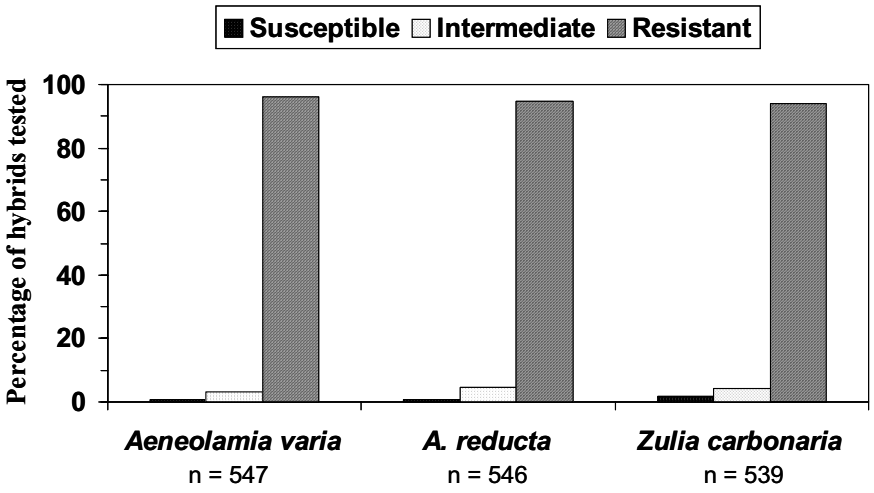

Figure 2.18.1.1. Frequency distribution of resistance reactions in a population of 565 sexual *Brachiaria* hybrids (SX05 series) tested for resistance to three major spittlebug species.

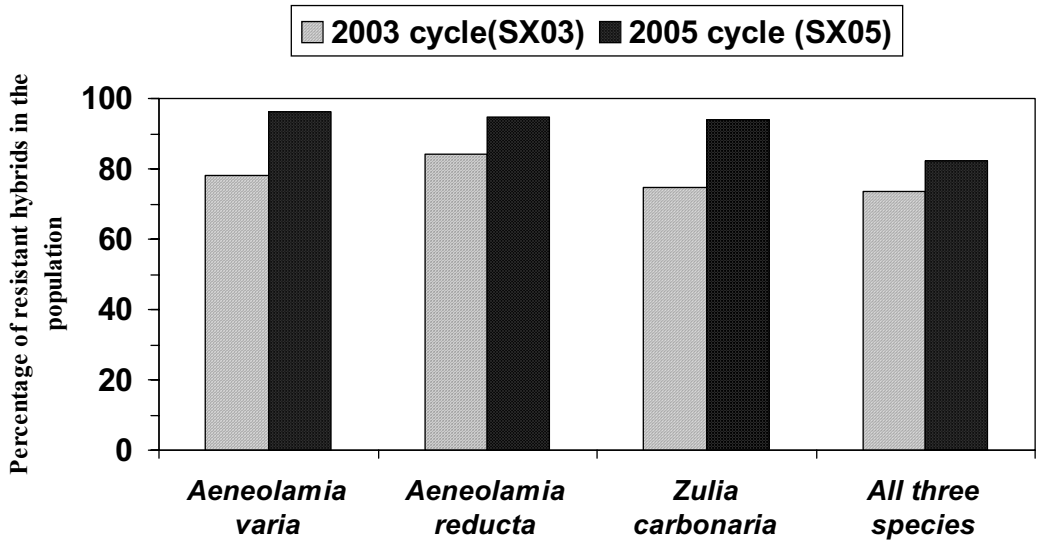

Figure 2.18.1.2. Frequency distribution of resistant reactions in two consecutive cycles of selection in *Brachiaria* for resistance to three major spittlebug species.

Table 2.18.1.2. Percentage nymph survival in selected *Brachiaria* genotypes screened for resistance to three major spittlebug species.

Genotype	Spittlebug species		
	Aeneolamia varia	***Aeneolamia reducta***	***Zulia carbonaria***
BR04NO/1751	6.7	13.3	3.3
BR04NO/1819	13.3	0	0
BR04NO/1889	10.0	-	3.3
BR04NO/2007	26.7	0	13.3
BR04NO/2405	23.3	23.3	16.7
BR04NO/2455	-	0	3.3
BR04NO/2515	33.3	6.7	10.0
BR04NO/2557	33.3	20.0	0
BR04NO/2793	-	0	16.7
BRX-44-02[1]	93.3	91.7	90.0
CIAT 0606[1]	91.7	93.3	81.7
CIAT 6294[2]	58.3	56.7	38.3
CIAT 36062[2]	12.5	13.3	25.0
CIAT 36087[3]	81.7	28.3	20.0
SX01NO/0102[2]	5.0	0	0

[1] Susceptible check
[2] Resistant check
[3] Commercial check
Means of 5 reps per genotype per species.

Table 2.18.1.3. Percentage nymph survival in selected *Brachiaria* apomictic hybrids tested for resistance to five spittlebug species. Means ± SEM of five replications per genotype.

Genotype	Spittlebug species				
	Aeneolamia varia	***Aeneolamia reducta***	***Zulia carbonaria***	***Mahanarva trifissa***	***Prosapia simulans***
MX02NO/1809	41.7 ± 9.7	45.0 ± 9.3	40.0 ± 8.7	0	16.7 ± 1.8
MX02NO/1905	25.0 ± 7.1	23.3 ± 8.3	13.3 ± 6.9	3.3 ± 2.4	3.3 ± 0.7
MX02NO/2273	3.3 ± 3.3	0	1.7 ± 1.7	13.3 ± 5.7	6.2 ± 1.9
MX02NO/2552	30.0 ± 6.5	16.7 ± 7.8	48.3 ± 10.7	0	33.3 ± 2.3
MX02NO/3056	8.3 ± 5.1	1.7 ± 1.7	13.3 ± 6.9	26.7 ± 6.2	1.7 ± 0.5
MX02NO/3213	5.0 ± 2.5	13.3 ± 7.8	43.3 ± 9.4	10.0 ± 7.1	9.2 ± 1.3
BRX-44-02[1]	88.3 ± 2.6	90.4 ± 3.7	68.3 ± 7.2	80.0 ± 5.7	68.3 ± 2.4
CIAT 0606[1]	80.0 ± 9.6	91.7 ± 3.5	75.9 ± 4.6	76.7 ± 4.7	49.9 ± 2.0
CIAT 6294[2]	38.3 ± 8.2	55.0 ± 11.1	57.0 ± 8.8	3.3 ± 2.4	6.7 ± 1.2
CIAT 36062[2]	8.3 ± 3.7	11.1 ± 7.0	30.0 ± 6.2	3.3 ± 2.4	0
SX01NO/0102[2]	5.0 ± 3.5	1.7 ± 1.7	18.3 ± 7.6	0	-
CIAT 36087[3]	63.3 ± 11.0	30.0 ± 7.8	1.7 ± 1.7	30.0 ± 8.7	1.7 ± 0.5

[1] Susceptible check
[2] Resistant check
[3] Commercial check.

Activity 2.18.2. Field screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species

Contributors: C. Cardona, G. Sotelo, and J. W. Miles

Rationale

Assessment of spittlebug resistance under natural levels of infestation in the field is very difficult due to the focal, unpredictable occurrence of the insect. This problem has been overcome since 1998 when we developed a technique that allows us to properly identify resistance under field conditions. Evaluating for resistance under field conditions is important because it allows us to reconfirm levels of resistance identified under greenhouse conditions.

Materials and Methods

Using the experimental unit described in our 1998 Annual Report (Sotelo and Cardona, 2000, Rev. Col. Entomol. 27: 17-20), the genotypes (usually 10 replicates) are initially infested in the greenhouse with an average of 10 eggs per stem. Once the infestation is well established, with all nymphs feeding on the roots, the units are transferred to the field and transplanted 10-15 days after infestation. The infestation is then allowed to proceed without interference until all nymphs have developed and adults emerge some 30-35 days thereafter. The plants are then scored for damage by means of the 1-5 visual scale utilized in greenhouse screenings. The number of stems per clump is counted before and after infestation and a tiller ratio (tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process) is then calculated. Using this methodology, 20 major screening trials (seven with *A. varia*, six with *Zulia carbonaria,* five with *Z. pubescens*, and two with *Mahanarva trifissa*) were conducted in Caquetá in 2005. The main purpose of these trials was to reconfirm resistance in 36 apomictic hybrids (BR02) and 34 apomictic hybrids (MX) that had been previously evaluated in Palmira under greenhouse conditions.

Results and Discussion

Using tiller ratios (the ratio between tillers per plant at the beginning of the infestation process and tillers per plant at the end of the infestation process) as the main selection criterion, we found that most of the BR02 hybrids tested were susceptible to spittlebug (Figure 2.18.2.1). A handful, listed in Table 2.18.2.1 showed a more or less acceptable level of field resistance due to the relatively high levels of antibiosis resistance present in these hybrids. The mechanism protected the plants from intense insect damage, allowing the plants to grow and lose less tillers than the susceptible checks. One of the commercial checks (CIAT 36087, 'Mulato 2') was resistant.

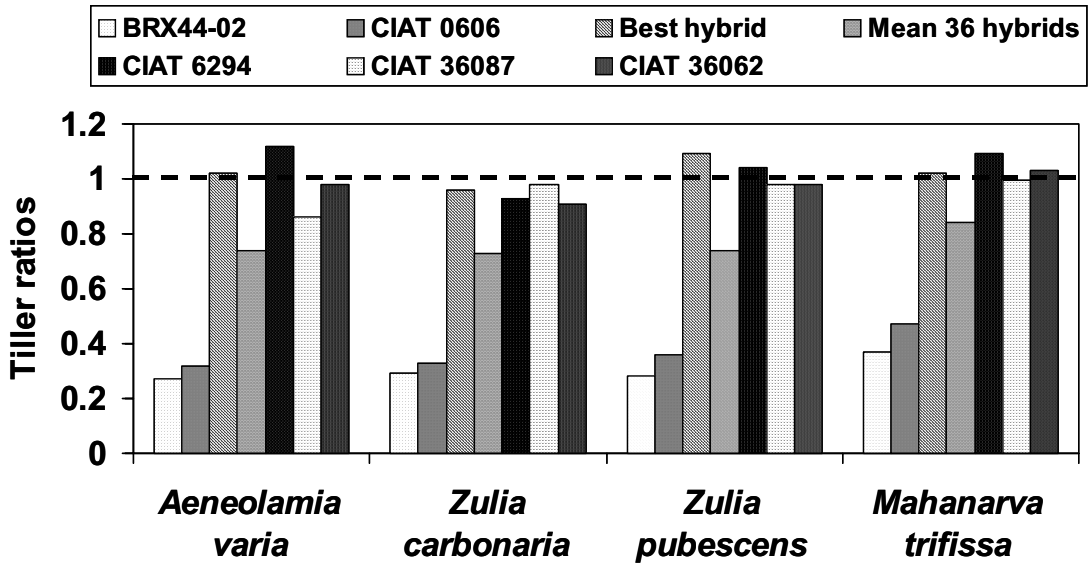

Figure 2.18.2.1. Resistance to four spittlebug species in selected *Brachiaria* apomictic (BR02) hybrids and checks tested under field conditions. The dotted line represents the cut-off point for resistance rating and selection.

Better results were obtained when 33 apomictic hybrids coded MX were tested for resistance to *A. varia*, *Z. carbonaria* and *M. trifissa*. Most of the genotypes were classified as resistant both in terms of damage scores (data not shown) and tiller ratios (Figure 2.18.2.2). The mean of selected genotypes did not differ from the mean of the resistant checks. The two commercial checks, 'Mulato' and 'Mulato 2' showed a high level of field resistance (Table 2.18.2.2).

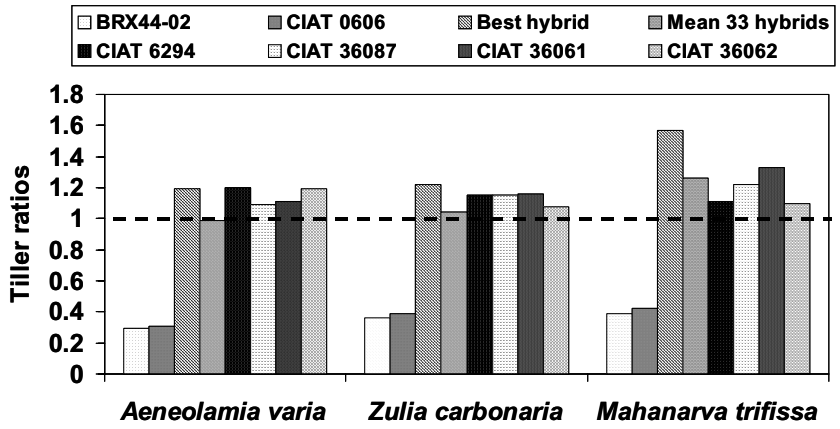

Figure 2.18.2.2. Resistance to four spittlebug species in selected *Brachiaria* apomictic (MX02) hybrids and checks tested under field conditions. The dotted line represents the cut-off point for resistance rating and selection.

Table 2.18.2.1. Tiller ratios (tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process) in selected *Brachiaria* genotypes tested for resistance to four spittlebug species under field conditions in Caquetá, Colombia.

Genotype	Spittlebug species			
	Aeneolamia varia	***Zulia carbonaria***	***Zulia pubescens***	***Mahanarva trifissa***
Selected hybrids				
BR02NO/1487	0.92	0.95	0.90	0.91
BR02NO/1912	0.73	0.96	0.83	0.98
BR02NO/1245	0.80	0.85	0.79	0.91
BR02NO/0638	1.01	0.65	0.69	1.02
BR02NO/0892	0.96	0.83	0.81	0.87
BR02NO/1747	0.72	0.76	1.01	0.88
Mean selected hybrids	0.86b	0.83c	0.84b	0.93b
		Resistant checks		
CIAT 6294	1.12	0.93	1.05	1.09
CIAT 36062	0.98	0.91	0.98	1.03
Mean resistant checks	1.05a	0.92b	1.01a	1.06a
		Commercial check		
CIAT 36087	0.86b	0.98a	0.98a	0.99a
		Susceptible checks		
CIAT 0606	0.32	0.33	0.36	0.47
BRX44-02	0.26	0.29	0.28	0.36
Mean susceptible checks	0.29c	0.31d	0.32c	0.41c

Means of 10 reps per genotype per species per trial; 4 trials in the case of *A. varia*, 3 trials with *Z. carbonaria* and *M. trifissa*, and 2 trials with *Z. pubescens*. Means within a column followed by the same letter are not significantly different at the 5% level according to Scheffe's multiple range test for arbitrary comparisons. Each species analyzed separately.

Table 2.18.2.2. Tiller ratios (tillers per plant at the end of the infestation process/tiller per plant at the beginning of the infestation process) in selected *Brachiaria* genotypes tested for resistance to three spittlebug species under field conditions in Caquetá, Colombia.

Genotype	Spittlebug species		
	Aeneolamia varia	***Zulia carbonaria***	***Mahanarva trifissa***
Selected hybrids			
MX02/2273	1.19	1.22	1.57
MX02/3861	1.14	1.16	1.35
MX02/3056	1.10	1.07	1.64
MX02/3213	1.14	1.10	1.38
MX02/2531	1.09	1.15	1.32
MX02/1809	1.03	1.14	1.40
MX02/1942	1.12	1.06	1.32
MX02/3567	1.06	1.14	1.20
MX02/1769	1.12	1.07	1.13
MX02/1660	1.09	1.14	1.09
MX02/3426	1.03	1.05	1.39
Mean selected hybrids	1.10a	1.11a	1.34a
Resistant checks			
CIAT 6294	1.20	1.15	1.11
CIAT 36062	1.19	1.08	1.10
Mean resistant checks	1.19a	1.11a	1.10c
Commercial checks			
CIAT 36061	1.11	1.16	1.33
CIAT 36087	1.09	1.15	1.22
Mean commercial checks	1.10a	1.15a	1.27b
Susceptible checks			
CIAT 0606	0.31	0.39	0.42
BRX44-02	0.29	0.36	0.39
Mean susceptible checks	0.30b	0.37b	0.40d

Means of 10 reps per genotype per species per trial; 3 trials with *A. varia* and *Z. carbonaria*, 2 trials with *M. trifissa*. Means within a column followed by the same letter are not significantly different at the 5% level according to Scheffe's multiple range test for arbitrary comparisons. Each species analyzed separately.

Activity 2.19. Identify host mechanisms for spittlebug resistance in *Brachiaria*

Contributors: M. F. Miller, C, Cardona, and G. Sotelo

Highlights:

- Finalized studies on the effect of host plant resistance on the demography of *Zulia carbonaria*
- Initiated studies on possible biochemical factors associated with antibiosis resistance to spittlebug
- Initiated studies on tolerance to adult feeding damage as a component of resistance to spittlebug
- Continued studies on mechanisms of resistance to *Deois incompleta, D. schah,* and *Notozulia entreriana* in Brazil and *Prosapia simulans* in Mexico. These will be reported in 2006

Activity 2.19.1. Effect of host plant resistance on the demography of *Zulia carbonaria*

Rationale

Varying levels of antibiosis resistance to nymphs of several spittlebug species have been well characterized in a number of resistant *Brachiaria* genotypes. The effects of antibiosis on the biology of nymphs have also been studied (Cardona *et al*., 2004, J. Econ. Entomol. 97:635-645). Not much was known about possible direct effects of antibiotic genotypes on the biology of adults. Even less was known about sub-lethal effects (i. e., reduced oviposition rates, reduced longevity, prolonged generation times, reduced rates of growth, etc.) on adults resulting from nymphs feeding on antibiotic genotypes. In 2004 we initiated a series of studies aimed at measuring how antibiotic genotypes may directly or indirectly (through sub-lethal effects) affect the biology of adults of *A. varia*. In 2005, similar studies were conducted with another major species, *Z. carbonaria*. We used the life-table technique, which is widely recognized as one of the most effective means of teasing apart the subtle, interrelated aspects of changes in population density. Longevity, age-specific fecundity, sex ratio and generation time can be examined and compared among treatments as they relate to the most important demographic parameter, the intrinsic rate of natural increase.

Materials and Methods

A comprehensive series of experiments aimed at determining whether antibiosis to nymphs has an adverse effect on the demography of *Z. carbonaria* were conducted. For this, 8 life tables (four fecundity, four complete) were constructed. Treatment combinations are shown in Table 2.19.1.1. For each of these treatments we established cohorts of 105 pairs of spittlebug and the fate and reproductive rate of individuals were recorded until death occurred. From these data the following life-table statistics were derived: net reproductive rate (R_o) [net contribution per female to the next generation]; mean generation time (T) [mean time span between the birth of individuals of a generation and that of the next generation]; doubling time (D) [time span necessary to double the initial population]; finite rate of population increase (ə) [multiplication factor of the original population at each time period]; and intrinsic rate of natural increase (r_m) [innate capacity of the population to increase in numbers]. Life-table statistics were analyzed using the SAS program based on jackknife estimates of demographic parameters (Maia *et al*., 2000, J. Econ. Entomol. 93: 511-518). Other variables recorded were sex ratios, percentage egg fertility and adult dry weights. These data were submitted to analysis of variance and when the *F* test was significant, we performed mean separation by LSD.

Results and Discussion

Sub-lethal effects of resistance on the demography of Zulia carbonaria: The resistant genotype SX01NO/0102 caused significant effects on the demography of *Z. carbonaria*. In general, rearing of nymphs of *Z. carbonaria* on the resistant genotype had a deleterious effect on the weight of resulting males and on the number and fertility of eggs laid per female (Table 2.19.1.2).

Table 2.19.1.1. Treatment combinations to study possible sub-lethal effects of high levels of nymphal antibiosis on adults of *Zulia carbonaria*.

Nymphs reared on:	**Resulting adults feeding on:**	**Null hypothesis**
CIAT 0654[a]	CIAT 0654	Absolute check
CIAT 0654	SX01NO/0102	A genotype that is highly antibiotic to nymphs does not affect adults
SX01NO/0102	CIAT 0654	High antibiosis to nymphs does not affect resulting adults
SX01NO/0102	SX01NO/0102	High antibiosis to nymphs does not affect resulting adults even when these are feeding on a highly antibiotic genotype

[a] CIAT 0654 is a highly susceptible accession; SX01NO/0102 (a resistant hybrid) possesses high levels of antibiosis resistance to nymphs of *Z. carbonaria*.

Table 2.19.1.2. Life history parameters of *Zulia carbonaria* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes.

Treatment[a]		**Adult dry weight ($g \times 10^{-3}$)**		**Eggs per female**	**Percentage egg fertility**
Nymphs reared on:	**Resulting adults feeding on:**	Females	Males		
CIAT 0654 (S)	CIAT 0654 (S)	1.52a	0.81a	451.4a	97.4a
CIAT 0654 (S)	SX01NO/0102 (R)	1.48b	0.78ab	440.0a	96.8a
SX01NO/0102 (R)	CIAT 0654 (S)	1.47b	0.75bc	353.7b	88.1b
SX01NO/0102 (R)	SX01NO/0102 (R)	1.43c	0.73c	286.9c	85.2c

[a] S, susceptible; R, resistant.
Within a column, means followed by the same letter are not significantly different at the 5% level by LSD.

Age-specific survival and age-specific fecundity curves for *Z. carbonaria* adults are presented in Figure 2.19.1.1. Mean survival times for the four treatment combinations did not differ at the 5% level, meaning that there was not a major impact of nymphal antibiosis on the survival of resulting males or females. On the contrary, rearing of the insect on the resistant genotype SX01NO/0102 did have a pronounced effect on the ability of resulting females to lay eggs. Independently of the food substrate used to feed the adults, females obtained from rearing the nymphs on the resistant genotype laid fewer eggs, for a slightly shorter period of time, than those obtained from rearing the insect on the susceptible genotype. This can be interpreted as a sub-lethal effect of nymphal antibiosis on the reproductive capacity of the insect.

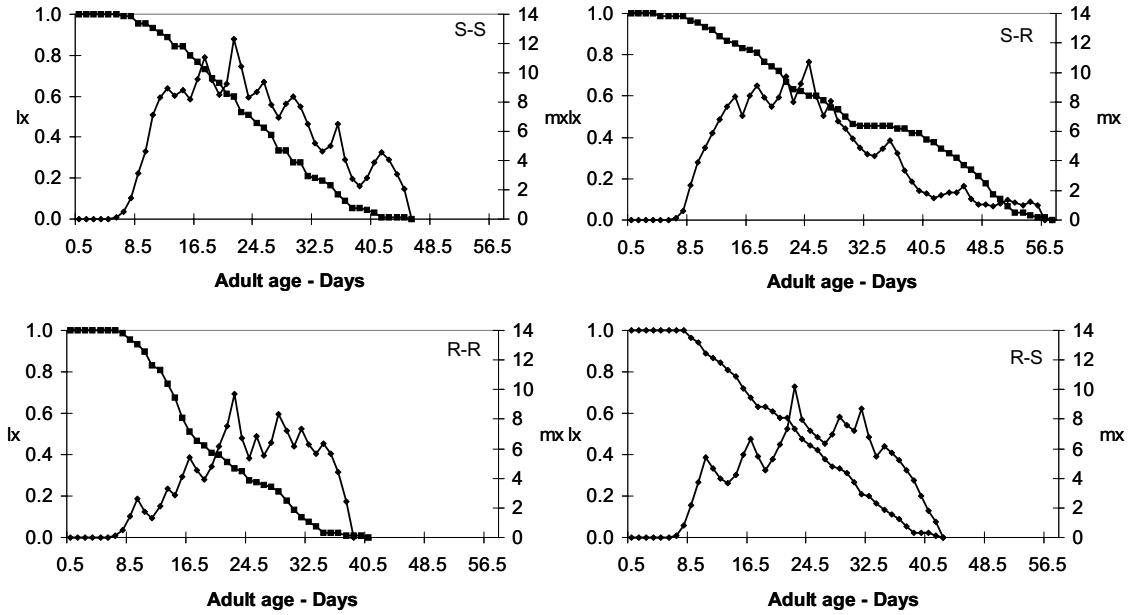

Figure 2.19.1.1. Age-specific survival (l_x) (y) and age-specific fecundity (m_x) (D) curves for adults of *Zulia carbonaria* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (CIAT 0654); R, resistant genotype (SX01NO/0102).

All demographic parameters of *Z. carbonaria* adults were significantly affected by the antibiotic effect of SX01NO/0102 on the nymphs (Table 2.19.1.3). Females originating from nymphs reared on the resistant genotype had lower net reproductive rates, lower intrinsic rates of natural increase, and lower finite rates of increase than those obtained from rearing the insect on the susceptible genotype. We conclude that antibiosis to nymphs in the resistant *Brachiaria* hybrid SX01NO/0102 causes significant sub-lethal effects on the reproductive biology of resulting adults.

Total effects of resistance on the demography of Zulia carbonaria: To measure the total impact of antibiosis resistance on the demography of *Z. carbonaria*, we took into account the rates of immature mortality caused by both the resistant and the susceptible genotypes. Age-specific survival curves for nymphs and adults, as well as age-specific fecundity curves for *Z. carbonaria* adults are presented in Figure 2.19.1.2. The antibiosis to nymphs present in the resistant genotype SX01NO/0102 had a significant deleterious effect on the biology of the insect, which reflected in very high levels of immature mortality. As a result, survival curves were very low as compared to those obtained with the susceptible genotype. Rearing of the insect on the resistant genotype caused a delay in the emergence of adults. Antibiosis also had a significant effect on the ability of resulting females to lay eggs. Independently of the food substrate used to feed the adults, females obtained from rearing the nymphs on the resistant genotype laid less eggs than those obtained from rearing the insect on the susceptible genotype.

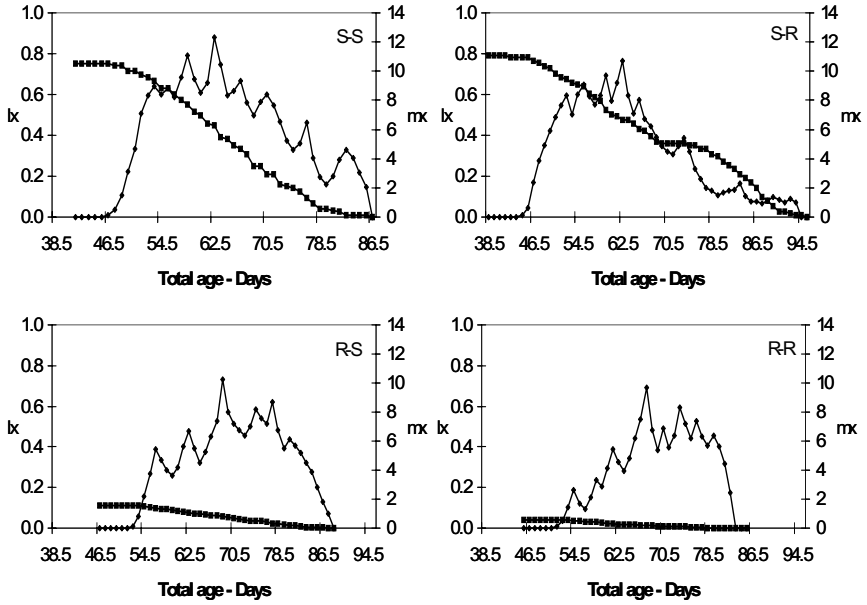

Figure 2.19.1.2 Age-specific survival (l_x) (y′) and age-specific fecundity (m_x) (Dž curves for *Zulia carbonaria* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (CIAT 0654); R, resistant genotype (SX01NO/0102).

As a result of high immature mortality and sub-lethal effects on resulting adults, all demographic statistics of the *Z. carbonaria* population tested were significantly affected by the antibiosis present in SX01NO/0102 . Populations derived from the resistant genotype had lower net reproductive rates, lower intrinsic rates of natural increase, lower finite rates of increase and longer generation times than those obtained from rearing the insect on the susceptible genotype.

Table 2.19.1.3. Fecundity life-table statistics for *Zulia carbonaria* adults as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes.

Treatment[a]		**Demographic parameters**		
Nymphs reared on:	**Resulting adults feeding on:**	**Net reproductive rate (R_o)**	**Intrinsic rate of natural increase (r_m)**	**Finite rate of increase (ə)**
CIAT 0654 (S)	CIAT 0654 (S)	229,8a	0.295a	1.344a
CIAT 0654 (S)	SX01NO/0102 (R)	230.1a	0.267a	1.306b
SX01NO/0102 (R)	CIAT 0654 (S)	184.3b	0.260b	1.297b
SX01NO/0102 (R)	SX01NO/0102 (R)	140.6c	0.248c	1.282c

[a] S, susceptible; R, resistant

Within a column, means followed by the same letter are not significantly different at the 5% level by LSD Jackknife estimates of the intrinsic rate of increase (per capita rate of population growth).

The finite rate of increase is a parameter that describes deleterious effects on a given population. It is defined as a multiplication factor of the original population at each time period. The decimal part of the finite rate of increase corresponds to the daily rate of increase expressed as a percentage. This means that populations reared on the susceptible genotype would grow by 8% whereas those on the resistant genotype would grow by 2.3-4.2% (Table 2.19.1.4). We conclude that high immature mortality caused by the resistant *Brachiaria* hybrid SX01NO/0102 and sub-lethal effects of antibiosis on resulting adults have a very major impact on the demography of *Z. carbonaria*.

Table 2.19.1.4. Life-table statistics for *Zulia carbonaria* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes

Treatment[a]		Demographic parameters				
Nymphs reared on:	**Adults feeding on:**	**Net reproductive rate (R_o)**	**Intrinsic rate of natural increase (r_m)**	**Mean generation time (T)**	**Doubling time (Dt)**	**Finite rate of increase (λ)**
CIAT 0654 (S)	CIAT 0654 (S)	172.3a	0.077a	66.7b	9.0c	1.080a
CIAT 0654 (S)	SX01NO/0102 (R)	181.8a	0.071a	66.5b	8.9c	1.081a
SX01NO/0102 (R)	CIAT 0654 (S)	20.3b	0.041b	73.8a	17.0b	1.042b
SX01NO/0102 (R)	SX01NO/0102 (R)	5.6c	0.023c	74.8a	29.9a	1.023c

[a] S, susceptible; R, resistant

Within a column, means followed by the same letter are not significantly different at the 5% level by LSD Jackknife estimates of the intrinsic rate of increase (per capita rate of population growth).

Activity. 2.19.2. Studies on possible biochemical factors associated with antibiosis resistance to spittlebug

Contributors: C. Cardona, G. Sotelo, J. Miles (CIAT) and Brent Brodbeck (University of Florida)

Rationale

As stated before, high levels of antibiosis resistance to nymphs of several spittlebug species have been well characterized in numerous resistant *Brachiaria* genotypes. Identification of the biochemical basis of spittlebug resistance, and development of rapid and precise biochemical assays for resistance would provide a valuable addition to breeding efforts to introgress spittlebug resistance into adapted *Brachiaria* germplasm. Scientists at the University of Florida have long proposed that changes in xylem-feeders development may be related to differences in xylem nutrient profiles (i. e. subtle differences in xylem nutrients may result in varying developmental success of the insect). To test this possibility we approached Drs. Brent V. Brodbeck and Peter C. Andersen who kindly accepted our request to analyze xylem samples taken from resistant and susceptible *Brachiaria* genotypes.

Materials and Methods

We used an array of genotypes well characterized for resistance or susceptibility to *A. varia*: 18 sexual hybrids (SX03), two susceptible checks (accessions BRX44-02 and CIAT 0606) and three resistant checks (accessions CIAT 36062, CIAT 6294 and the sexual hybrid SX01NO/0102). Plants were grown in large pots in the greenhouse (24^{o} C, 75% R.H.) and infested with 100 mature eggs each. Infestation was then allowed to proceed without interference. When nymphs reached the fourth instar stage, the plants were cut off at approx. 3 cm from the soil surface. Several stems of approx. 4- to 5-mm diameter were wrapped with tape to increase their effective diameter. The 8-mm interior diameter nozzle of a plastic, disposable syringe was fitted over the entire cut end of the stem wrapped with tape to make a tight connection. Taping externally further sealed the union of the nozzle of the syringe and the cut stem. Suction was applied by withdrawing the syringe plunger, which was held in the withdrawn position until the desired volume of liquid accumulated within the syringe. Xylem samples thus obtained were immediately frozen and shipped to the University of Florida where they were analyzed for contents of 19 different amino acids.

Results and Discussion

There was not a significant correlation between amino acid contents and resistance ratings based on percentage nymph survival. In spite of these disappointing preliminary results, we intend to continue this line of research using a small grant from the USAID-University linkage fund.

Activity 2.19.3. Studies on tolerance to adult feeding damage as a component of resistance to spittlebug

Contributors: F. López, C. Cardona, and G. Sotelo

Rationale

Our studies have clearly identified nymphal antibiosis as the main mechanism of resistance to several different species of spittlebug in many different *Brachiaria* genotypes. In fact, we have also been able to document rapid progress in the incorporation of antibiosis resistance to nymphs in sexual and apomictic hybrids developed through a recurrent selection-breeding scheme (Miles et al., 2005, accepted for publication in Crop Science). Given that adults can be as damaging as the nymphs, it is widely accepted that antibiosis to nymphs should be combined with an acceptable level of tolerance to adult feeding damage. However, nothing is known about mechanisms of resistance to adult feeding damage in *Brachiaria*. For this reason, and for the first time, in 2005 we initiated a series of studies aimed at characterizing tolerance as a possible component of resistance to spittlebug.

Materials and Methods

To study tolerance to adult feeding we initially compared the response of the susceptible accession CIAT 0654 and the resistant hybrid SX01NO/0102 to increasing levels of infestation with adults of *A. varia*. Thirty-day old plants of CIAT 0654 and SX01NO/0102 were exposed to 0, 2, 3, 5, 7, 9, 12, and 15 adults per plant. The 16 host genotype-infestation level treatment combinations were randomly assigned to single-plant experimental units with 10 replications per treatment combination. Plants were infested with neonate adults and the infestation was allowed to proceed until all adults died. Percentage adult survival was calculated. Damage scores in a 1-5 visual damage score scale were taken 5 and 10 days after

infestation. To measure chlorophyll loss as a result of adult feeding, we used a SPAD-502 chlorophyll meter 5 and 10 days after infestation. Four representative readings per plant were taken and their averages were recorded. SPAD index values were then calculated with respect to the uninfested checks. At the end of the trial, when all insects had died, plants were cut at soil level and dried in an oven at 40^{o} C. Percentage biomass losses were calculated with respect to the uninfested checks. Damage scores and percentage biomass losses were used to calculate functional plan loss indices.

Results and Discussion

Adult survival was not affected by the genotype when plants were infested with 2, 3 or 5 adults per plant. At higher infestation levels (7, 9, 12, and 15 adults per plant) adult survival on the susceptible genotype was significantly lower possibly due to depletion of food and increased competition among insects. This means that a 5-6 level of infestation could be used in future studies. SX01NO/0102 plants suffered significantly less damage than susceptible (CIAT 0654) plants at all levels of infestation (Figure 2.19.3.1).

At all levels of infestation, SX01NO/0102 plants suffered significantly less damage (expressed as percentage chlorophyll loss and percentage biomass loss) than susceptible CIAT 0654 plants (Figure 2.19.3.2). Significant correlations were found between damage scores and percentage chlorophyll losses ($r = 0.858$; $P < 0.001$), between damage scores and percentage biomass losses (0.473; $P < 0.001$) and between percentage chlorophyll losses and percentage biomass losses ($r = 0.891$; $P < 0.001$) indicating that damage scores are useful in predicting losses and that SPAD units are useful in measuring insect damage.

Furthermore, when a Functional Plant Loss Index (combining damage scores and percentage biomass losses) was calculated, we found that at all levels of infestation losses were highest for the susceptible genotype CIAT 0654 (Figure 2.19.3.3) Since no obvious signs of antibiosis to adults were found in this experiment, we interpret lower damage scores, lower chlorophyll and biomass losses, and lower functional plant losses as the expression of tolerance to adult feeding damage in the resistant genotype. Further results on this line of research, aimed at developing a mass screening procedure for adult spittlebug damage, will be reported in 2006.

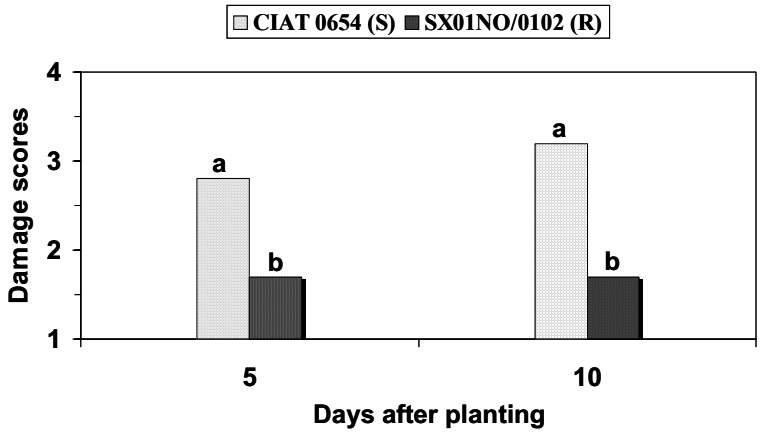

Figure 2.19.3.1. Response of susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes to attack by adults of *Aeneolamia varia*. Means of eight levels of infestation. For each scoring date, bars with the same letter do not differ at the 5% level of significance by LSD.

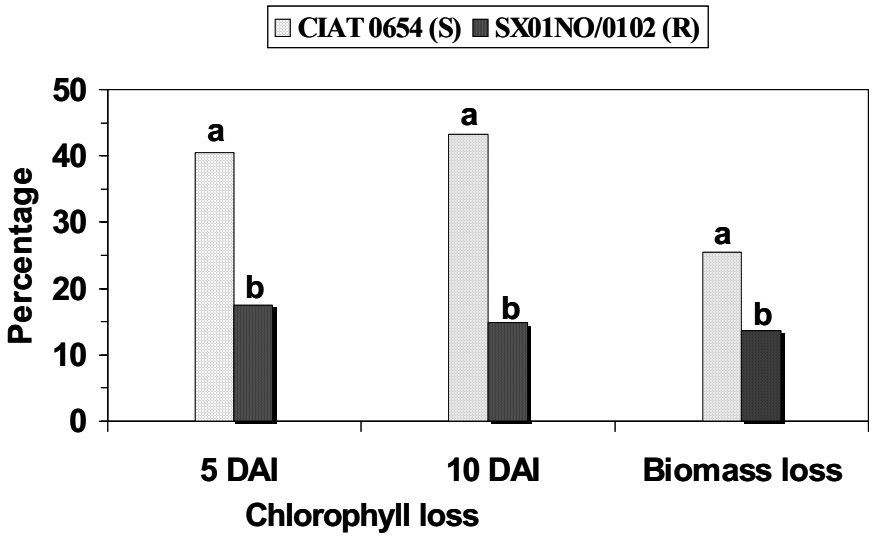

Figure 2.19.3.2. Chlorophyll and biomass losses due to adult *Aeneolamia varia* feeding on susceptible (CIAT 0654) or resistant SX01NO/0102 *Brachiaria* genotypes. DAI, days after infestation. Means of eight levels of infestation. For each variable, bars with the same letter do not differ at the 5% level of significance by LSD.

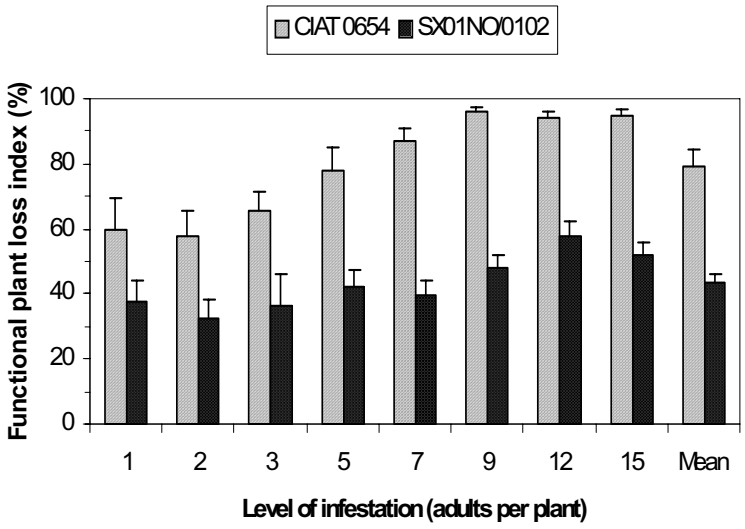

Figure 2.19.3.3. Functional plant loss indices (percentage) for susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes exposed to eight levels of infestation with adults of *Aeneolamia varia*.

Activity 2.20. Validating thermotherapy of stem cuttings, and use of Lonlife® and *Trichoderma* for managing cassava diseases in the Eastern Plains and Cauca, Colombia

Contributors: Elizabeth Álvarez, Germán Llano, John Loke, Juan Fernando Mejía,Víctor Montaña, Wilson Gaitán, and Bolívar Muñoz

Highlight:

- Cassava bacterial blight was successfully controlled under field conditions by treating stem cuttings with hot water, Meta, Colombia.

Rationale

Bacterial blight, *Phytophthora* root rots, and superelongation disease are widespread and cause high losses in important cassava-producing regions in Colombia. Several ecological control practices, like thermotherapy and the use of biocontrol agents, have been evaluated recently for managing root rots in cassava. In this report, we discuss the progress made for each of the project's objectives, which are as follows: (1) with farmer participation, to adjust and validate strategies of integrated management of the constraining diseases found in each region; (2) to improve, through training, farmers' knowledge of integrated disease management; (3) to contribute to the technical and commercial development of the biological inputs needed to implement integrated management of the economically most important diseases of cassava; (4) to evaluate the economic sustainability of the proposal in terms of its effects on competitiveness in employment, income, and internal rate of return; and (5) to evaluate the financial sustainability of the proposal in terms of financing this project's products for introduction into the production sector.

Materials and Methods

Six commercial plots of cassava were established in five municipalities, two in each of the departments of Cauca and Meta, and one in Casanare. The aims were:

- To evaluate the performance of several promising cassava varieties under the conditions of two agroecological areas: the Eastern Plains and Andean Region (Department of Cauca; Figure 2.20.1)

- To validate the effect of treating stakes with Lonlife®, a product of low toxicity and derived from seeds of citrus fruits

- To validate the performance of the fungi *Trichoderma viride* Persoon and *T. harzianum*, which attack soil pathogens and have shown to control several species of *Phytophthora*, causal agents of root rots

Figure 2.20.1. Planting cassava plots in the Department of Cauca, Colombia.
Eastern Plains: Cantaclaro (Puerto López).

Three semicommercial plots were established on the farms "La Vega" (Aguazul, Casanare) and "Cantaclaro" (Puerto López, Meta), and CORPOICA's research farm "La Libertad", located in Villavicencio (Meta), to evaluate the performance of four promising cassava varieties and the effect of treating stakes with Lonlife® and of inoculating them with *T. viride* and *T. harzianum.*

We planted 0.5 ha with the varieties La Reina, Vergara, and CM 4574-7, and treated the stakes and soil as described below. For comparison, 9 ha were also planted with the same varieties under farmer management. Planting was on the furrow ridges.

Treatments. Good quality stakes were selected from productive healthy plants. They were treated as follows:

a Stakes were immersed for 10 min in a solution with Lonlife® and the insecticide Roxion® (dimethoate), each at 2 cc/L.

b Farmers immersed stakes for 10 min in a solution of copper oxychloride (at 3 g/L) and Roxion® (at 2 cc/L). This treatment was used as check.

The fungus *T. viride* strain CIAT-14PDA-4 -an antagonist and plant growth stimulator- was applied directly to the soil around planted cassava stakes, once at 1 month after planting and again at 3 months. The product AgroGuard® (containing *T. harzianum*) were added, each at 0.5 g/L. For the fungus, this was the equivalent of 2.5×10^8 spores/L. The farmer also used the product Bioderma® (containing *T. harzianum*).

Results and Discussion

Evaluations of germination, vigor, and incidence of disease were conducted by the technicians handling the crop. These evaluations will serve to define crop management practices, which are urgently needed as the area planted to the crop expands in response to demand for fuel-alcohol production from cassava.

The variety La Reina had a higher rate of germination than had either Vergara or CM 4574-7. Vergara showed improved germination with Lonlife®. Although CM 4574-7 had the lowest rate, it was similar to the two evaluated stake treatments (Table 2.20.1).

'CM 4574-7' showed no symptoms of either superelongation disease (SED) or cassava bacterial blight (CBB), while 'La Reina' was the most affected by both diseases. The technicians regard CM 4574-7 as the variety that so far shows the best performance.

Although it is too early to observe significant differences of effect between the two *Trichoderma* strains, CM 4574-7 plants were more vigorous under the AgroGuard® treatment, whereas the other two varieties showed no change in vigor between the strains.

La Vega (Aguazul): Two cassava varieties, La Reina and ICA Catumare, were planted on 0.35 ha and the following treatments were carried out for the stakes and soil:

Treatments: Good quality stakes were selected from productive and healthy plants.

a. Stakes were immersed in a solution of Lonlife® at 2 cc/L for 10 min.

b. Stakes were immersed for 10 min in a solution of copper oxychloride (at 3 g/L) and Lorsban® (at 3 cc/L).

c. Stakes received no treatment.

d. Two applications of each fungus strain were used to inoculate the soil around the plants at 1 and 3 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (*T. harzianum*), each at 0.5 g/L, which was equivalent to 2.5×10^8 spores/L.

As shown in Table 2.20.2, the germination rate of the two varieties was more than 96%, except for La Reina without treatment, when germination was 84.19%. Some of the seed treated with Lonlife® (no *Trichoderma*) germinated at a rate of 88.36% because the soil had not been adequately prepared. Inoculation with *Trichoderma* had no relationship with germination because it was applied 30 days after planting.

Table 2.20.1. Rates of germination (G) of cassava stakes, vigor, and incidence of superelongation disease (SED) and cassava bacterial blight (CBB) according to different treatments of stakes and inoculations of the soil with the fungi *Trichoderma viride* and *T. harzianum*, Farm "Cantaclaro", Puerto López, Department of Meta, Colombia.

Variety and treatment	G (%)	Vigor	Incidence (%) SED	Incidence (%) CBB
CM 4574-7				
Lonlife®/*Trichoderma* strain, AgroGuard®	37	Excellent	0	0
Lonlife®/*Trichoderma* strain, Bioderma®	48	Good	0	0
Lonlife®/*Trichoderma* strain, CIAT	51	Good	0	0
Chemical treatment	46	Excellent	0	0
Average germination rate	45.5			
La Reina				
Lonlife®/*Trichoderma* strain, AgroGuard®	58	Average	10	20
Lonlife®/*Trichoderma* strain, Bioderma®	49	Average	15	40
Lonlife®/*Trichoderma* strain, CIAT	61	Average	10	50
Chemical treatment	60	Average	10	25
Average germination rate	57.0			
Vergara				
Lonlife®/*Trichoderma* strain, AgroGuard®	46	Good	3	0
Lonlife®/*Trichoderma* strain, Bioderma®	71	Good	5	0
Lonlife®/*Trichoderma* strain, CIAT	50	Good	5	0
Chemical treatment	40	Good	10	15
Average germination rate	51.8			

La Libertad (Villavicencio): Three cassava varieties, La Reina, Vergara, and CM 4574-7 were planted on 0.5 ha and the following treatments were carried out:

Treatments:

a. Healthy stakes were immersed for 10 min in a solution of Lonlife® at 2 cc/L.

b. Healthy stakes were immersed for 10 min in a solution of copper oxychloride (at 3 g/L) and malathion (at 2 cc/L).

c. Stakes infected with the bacterium *Xanthomonas axonopodis* pv. *manihotis* (*Xam*) were placed in hot water (49 °C) for 49 min.

d. Stakes infected with *Xam* and given no treatment.

e. Two applications of each fungus strain were used to inoculate the soil around the plants at 1 and 3 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (*T. harzianum*), each at 0.5 g/L, which was equivalent to 2.5×10^8 spores/L.

The germination rate for all treatments was very low, with a maximum of 57.5%. Using healthy plant stakes, the variety with the lowest rate was Vergara (36.3%). Treatment with Lonlife® slightly improved germination rates for varieties La Reina and CM 4574-7 (Table 2.20.3).

Table 2.20.2. Germination rate of two cassava varieties receiving different stake treatments, Farm "La Vega", Aguazul, Department of Casanare, Colombia.

Variety and treatment	Germination rate (%)
La Reina	
Lonlife®–no *Trichoderma*	88.36
Lonlife®–*Trichoderma* strain CIAT	96.86
Lonlife®–*Trichoderma* strain AgroGuard®	96.86
Chemical–no *Trichoderma*	97.47
Chemical–*Trichoderma* strain CIAT	99.24
Chemical–*Trichoderma* strain AgroGuard®	98.99
No treatment	84.19
ICA Catumare	
Lonlife®–no *Trichoderma*	99.02
Lonlife®–*Trichoderma* strain CIAT	98.62
Lonlife®–*Trichoderma* strain AgroGuard®	96.66
Chemical–*Trichoderma* strain CIAT	98.74
Chemical–*Trichoderma* strain AgroGuard®	98.99
No treatment	96.19

Table 2.20.3. Germination of stakes and dead plants from stakes infected with *Xanthomonas axonopodis* pv. *manihotis* receiving different stake treatments, Farm "La Libertad", Villavicencio, Department of Meta, Colombia.

Variety and treatment[a]	Germination (%)	Dead plants (%)[b]
La Reina		
Healthy stakes with Lonlife®	57.5	0.5
Healthy stakes with chemical treatment	54.3	0.2
Stakes with CBB, no treatment	35.4	22.9
Thick stakes with CBB, heat therapy	39.1	20.3
Thin stakes with CBB, heat therapy	23.1	3.1
CM 4574-7		
Healthy stakes with Lonlife®	54.6	0.0
Healthy stakes with chemical treatment	49.6	0.2
Vergara		
Healthy stakes with Lonlife®	29.6	0.6
Healthy stakes with chemical treatment	36.3	0.2

a. Healthy stakes were selected from plants with no symptoms of cassava bacterial blight (CBB).
b. From CBB-infected stakes.

For stakes from plants infected with CBB, the heat treatment reduced disease incidence to 3.1% in plants that developed from thin stakes, whereas incidence for thick stakes was 20.3% and the check (no treatment) 22.9%. Normally, germination is reduced when thin stakes are used. These results suggest that thick stakes should receive heat treatment over a longer period to guarantee cleaning of the material. However, farmers should be recommended to select more strictly for healthy plant stakes.

Department of Cauca: La María: Together with the Local Agricultural Research Committee (CIAL, its Spanish acronym) "La María", in the Municipality of Piendamó, we established two plots on the farms of Lilia Rojas and Arbey Agredo. The goal was to evaluate the performance of three promising cassava varieties and the effect of treating stakes with Lonlife® and of inoculating the soil with *T. viride* and *T. harzianum.* Evaluations were made with the active participation of the farmers forming the CIAL.

Varieties: These were SM 707-17, SM 1498-4, and SM 1495-5.
Treatments: Good quality stakes were selected from productive and healthy plants.

a. Stakes were immersed for 10 min in a solution of Lonlife® at 2 cc/L.

b. Stakes were immersed for 10 min in a solution of *Trichoderma* strain AgroGuard® (Live Systems Technology S.A.) or of strain CIAT (0.5 g/L, equivalent to 2.5×10^8 spores/L).

c. Stakes received no treatment.

d. One application of each fungus strain was used to inoculate the soil around the plants at 2 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (containing *T. harzianum*) at 0.5 g/L each.

The farmers evaluated the cassava according to their own criteria, indicating good, regular, or bad results in terms of germination, vigor, and vegetative development. They also ranked the varieties according to their preference (Table 2.20.4). On day 25 after planting, germination was evaluated. The rate for variety SM 1495-5 was lower than for the other two varieties. The farmers did not observe differences among treated and untreated stakes with regard to germination rate, but observed better development of plants from treated stakes.

In an evaluation made jointly with the farmers, when the crop was 55 days old, the best variety was SM 707-17, followed by SM 1498-4, and then SM 1495-5. The plot at Lilia Rojas' farm showed better development. According to the farmers, the cassava crop in Arbey Agredo's plot did not develop well, produced less, and had more rot. Stakes treated with Lonlife® had higher germination rates and greater vigor. Differences were yet to be observed between the two *Trichoderma* treatments.

San Bosco: We established a plot together with the CIAL "San Bosco", in the Village District of Mondomo, Municipality of Santander de Quilichao. The goal was to evaluate the performance of two promising cassava varieties and the effect of treating stakes with Lonlife® or of inoculating the soil with *T. viride* and *T. harzianum.* The evaluations were made with the active participation of the farmers forming the CIAL.

Varieties: These were SM 707-17 and CM 7436-7.
Treatments: Good quality stakes were selected from productive and healthy plants.

a. Stakes were immersed for 10 min in a solution of Lonlife® at 2 cc/L.

b. Stakes were immersed for 10 min in a solution containing *Trichoderma* strain AgroGuard® (Live Systems Technology S.A.) or strain CIAT at 0.5 g/L each, the equivalent of 2.5×10^8 spores/L).

c. Stakes received no treatment.

d. Two applications of each fungus strain were used to inoculate the soil around the plants at 1 and 3 months after planting. The inoculum was either the fungus *T. viride* strain CIAT-14PDA-4 or the product AgroGuard® (containing *T. harzianum*) at 0.5 g/L each.

The farmers evaluated the cassava according to their own criteria, indicating good, regular, or bad results in terms of germination, vigor, and vegetative development (Table 2.20.5). On day 35 after planting, germination was evaluated together with the farmers from the CIAL. Variety SM 707-17 was observed as having the best development. At that time, clear differences could not yet be observed among stake treatments. The farmers used the criteria germination, development, plant vigor, and quantity and uniformity of foliage. No differences were yet observed between the two strains of *Trichoderma* or with respect to the check, which received no treatment.

Table 2.20.4. Participatory evaluation of varieties and treatment of cassava stakes, CIAL "La María", Piendamó, Department of Cauca, Colombia.

		Development evaluation[a]			Preference rank of variety[b]		
Treatment	**Variety**	**Mario**	**Jeimer**	**Gerardo**	**Mario**	**Jeimer**	**Gerardo**
Arbey Agredo's plot							
Lonlife®	SM 1495-5	R	R	R	3	3	3
Lonlife®	SM 1498-4	G	G	G	2	2	2
Lonlife®	SM 707-17	G	R	G	1	1	1
Check (no treatment)	SM 1495-5	R	R	R	3	3	3
Check (no treatment)	SM 1498-4	R	G	R	2	2	2
Check (no treatment)	SM 707-17	R	G	G	1	1	1
Lilia Rojas' plot							
Lonlife®	SM 1495-5	G	VG	VG	3	2	3
Lonlife®	SM 1498-4	G	G	G	2	3	2
Lonlife®	SM 707-17	VG	VG	VG	1	1	1
Check (no treatment)	SM 1495-5	R	G	G	3	2	3
Check (no treatment)	SM 1498-4	R	R	R	2	3	2
Check (no treatment)	SM 707-17	G	G	G	1	1	1

a. Three farmers, whose names appear in the column headings, evaluated the plots. VG = very good germination and development; G = good germination, development, and architecture; R = regular germination and development.

b. The three farmers ranked the cassava varieties according to their germination, development, and vigor, where 1 = the best and 3 = the poorest variety.

Table 2.20.5. Participatory evaluation of treated cassava stakes, CIAL "San Bosco", Santander de Quilichao, Department of Cauca, Colombia.

	Development evaluation[a]	
Variety and treatment	**Bernardino**	**Jobel**
CM 7436-7		
Lonlife®	G	G
Lonlife® + *Trichoderma* strain AgroGuard®	G	G
Lonlife® + *Trichoderma* strain CIAT	G	G
Trichoderma strain AgroGuard®	G	VG
Trichoderma strain CIAT	G	VG
No treatment	G	G
SM 707-17		
Lonlife®	VG	VG
Lonlife® + *Trichoderma* cepa AgroGuard®	G	VG
Lonlife® + *Trichoderma* strain CIAT	VG	G
Trichoderma strain AgroGuard®	G	G
Trichoderma strain CIAT	G	G
No treatment	G	G

a. Two farmers, whose names appear in the column headings, evaluated the plots. VG = very good germination and development; G = good germination, development, and architecture.

Activity 2.21. Evaluation of *Brachiaria* hybrids for resistance to *Rhizoctonia solani* under field conditions in Caqueta

Contributors: Gustavo Segura, William Mera, Ximena Bonilla, John Miles, Segenet Kelemu

Highlight:

- The resistant accession (*B. brizantha* 16320) and four Brachiaria hybrids showed high levels of resistance to Rhizoctonia foliar blight under field conditions.

Rationale

Rhizoctonia foliar blight, caused by *Rhizoctonia solani* Kühn, is a disease of increasing importance on a number of crops. The disease can be very destructive when environmental conditions are particularly conducive (high relative humidity, dense foliar growth, high nitrogen fertilization, and extended wet periods).

Rhizoctonia solani is the most widely known species of *Rhizoctonia* with a wide host range. In nature *R. solani* reproduces mainly asexually and exists as vegetative mycelia and/or dense sclerotia. These sclerotia can survive in soil and on plant debris for several years, and can germinate and produce hyphae that can infect a wide range of host plants. The pathogen primarily infects below ground plant parts in a number of plant species, but can also infect above ground plant parts such as pods, fruits, and leaves and stems as is the case with *Brachiaria*. In *Brachiaria*, infected leaves first appear water-soaked, then

darken, and finally turn to a light brown color. Lesions may coalesce quickly during periods of prolonged leaf wetness and temperatures between 21 and 32 ∀C.

Disease management through the use of host resistance, when available, remains to be the most practical and environmentally friendly strategy. Differences in reaction to *R. solani* exist in genotypes of *Brachiaria.* The ability to uniformly induce disease and measure resistance accurately is crucial in a breeding program for developing resistant cultivars. The objectives of this study are to: 1) artificially inoculate and induce uniform disease development in selected *Brachiaria* genotypes generated by CIAT's tropical forages project, 2) accurately measure resistance and identify resistant materials among these *Brachiaria* genotypes.

Materials and Methods

Plant materials: 137 *Brachiaria* genotypes with BR04 series and provided by the breeding program were planted in the field at Macagual ICA/CORPOICA Research Station in Florencia, Caquetá. CIAT 16320, CIAT 36061 and CIAT 36087 were included as controls. The field location is highly conducive to the development of the disease, with mean annual relative humidity of 84 %, an average temperature of 25.5^{o}C and an annual rainfall of 3793 mm.

Field layout, artificial inoculations and disease evaluations: Six plants (that were generated from the same mother plant) of each of the *Brachiaria* genotypes were transplanted from a CIAT glasshouse to the field site in Caquetá. The space between plants was 80 cm, and 1 m between blocks. The entries were replicated 3 times in a randomized complete block design. Plants were inoculated one month after transplanting by placing 0.7 g dry sclerotia of *R. solani* isolate 36061 on the soil surface at the base of each plant. Plants were evaluated for disease reaction 15, 20, 34 and 38 days after inoculations, using the 0 – 5 (0 = no visible infection; 5 = 20 -100% of the aerial portion of the plant infected) scale that we developed earlier and reported in the 2004 Annual Report.

Results and Discussion

Disease symptoms developed fully in susceptible genotypes 10-15 days after inoculations. Plants were evaluated for disease reaction 15, 20, 34 and 38 days after inoculations. There was a high degree of correlation in disease evaluation data among the various evaluation dates (Table 2.21.1).

Table 2.21.1. Correlations between disease reaction data collected at various days after inoculations using Pearson's Correlation

Days	15	20	34	38
15	1.00	0.82	0.69	0.60
20	0.82	1.00	0.85	0.78
34	0.69	0.85	1.00	0.94
38	0.60	0.78	0.94	1.00

The resistant control CIAT 16320 was consistently evaluated at scale 2. Four genotypes, BR04-2577, BR04-2557, BR04-2983, and BR04-1214 were evaluated at an average between 2.0 and 2.5. Twenty-four others, 1685, 1950, 1963, 3077, 1119, 1252, 1347, 1349, 1824, 1886, 1896, 2060, 2200, 2201, 2265, 3025, 3207, CIAT 36087, 1928, 1941, 2040, 2069, 3066, 3217, scored with an average rating scale of 3.0-3.3 (in the rating scale, this corresponds to a 6% - 9% overall plant tissue damage). The remaining 111 materials, 2429, 2518, 2539, 3051, 3175, 1061, 1219, 1796, 1845, 2774, 2793, 2874, 3214, 1021, 1073,

1141, 1819, 2404, 2405, 2457, 2475, 2515, 2532, 2841, 2940, 3056, 222, 1265, 1311, 1358, 1377, 1592, 1648, 1956, 2110, 2118, 2128, 2179, 2208, 2275, 2403, 2938, 2987, 3069, 3119, 3221, 36061, 1081, 1097, 1113, 1197, 1281, 1296, 1503, 1633, 1697, 2007, 2226, 2235, 2389, 2414, 2969, 1018, 1026, 1060, 1273, 1309, 1360, 1374, 1570, 1629, 1883, 1889, 2093, 2163, 2290, 2338, 2346, 2670, 2681, 2792, 2833, 2849, 2872, 2954, 3068, 3128, 1003, 1058, 1494, 1754, 2109, 2285, 2344, 1428, 1596, 1601, 1751, 1846, 1900, 2156, 2166, 2455, 2863, 2871, 3058, 3134, 2360, 2396, 1552, 3130, scored between 3.5- 5.0. Figure 2.21.1 shows a graphical representation of the results using data from representative genotypes from each of these three groups.

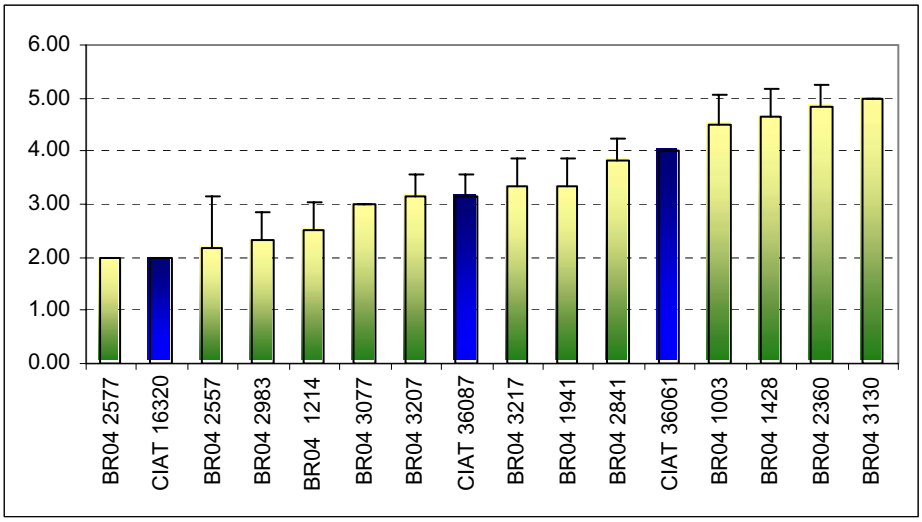

Figure 2.21.1. Ratings of *Brachiaria* genotypes for foliar blight disease reaction on a 1-5 scale 38 days after inoculations with sclerotia of *Rhizoctonia solani* under field conditions, Caquetá, Colombia. Bars indicate standard deviation.

The disease evaluation data taken 38 days after inoculations represented well-developed disease symptoms. The resistant control CIAT 16320 and the four genotypes BR04- 2577, BR04-2557, BR04-2983, BR04-1214 showed less than 6% overall plant tissue damage, and thus, a high-level of resistance (Figure 2.21.2). The second group of 24 genotypes including CIAT 36087 listed above still had an acceptable level of resistance. All the plants in this trial will be maintained in the field to further observe the level of disease at an extended period of time.

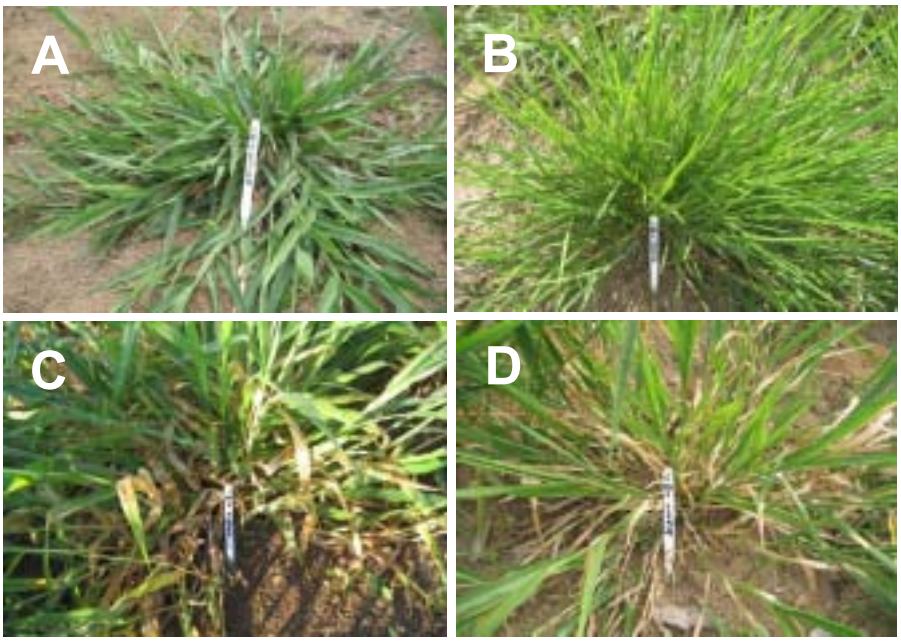

Figure 2.21.2. Rhizoctonia foliar blight disease symptoms 34 days after inoculations under field conditions in Caquetá, Colombia. **A**: BR04-1214; **B**: BR04-2577: **C**: CIAT 36061; **D**: BR04-1754

Activity 2.22. Endophyte transformation and use as gene delivery system

Contributors: Javier Abello, Celsa Garcia (Univ. Nacional, Bogota) and Segenet Kelemu

Rationale

Acremonium implicatum is an endophytic fungus that forms symbiotic association with species of *Brachiaria*. The green fluorescent protein (GFP) gene, isolated from the jellyfish *Aequorea Victoria*, or its derivatives have been expressed in a wide array of organisms including plants and microbes. The practical implication of seed transmission of endophytes in *Brachiaria* is significant: once associated with the plant, the fungus can perpetuate itself through seed, especially in apomictic genotypes of *Brachiaria*, for as long as seed storage conditions do not diminish the survival of the fungus. Several *Brachiaria* hybrids obtained from CIAT's forage breeding program were shown to harbor *A. implicatum*. Therefore, we may be able to exploit this association and its high seed transmission (Dongyi and Kelemu, 2004, Plant Disease 88:1252-1254) by using a transgenic *A. implicatum* as a vehicle for production and delivery of gene products of agronomic interest into the host plant to enhance protective benefits and other traits, and thus improve livestock production. In addition, we want to exploit the qualities of GFP as a reporter and study the interactions between *A. implicatum* and its host *Brachiaria*.

This work describes the establishment of a transformation protocol and expression of the green fluorescent protein (GFP) gene in an isolate of *Acremonium implicatum*. In this study, we used a GFP expression vector, pSK1019, to transform *A. implicatum*.

Materials and Methods

Plasmid: Plasmid pSK1019 kindly provided by Dr. Seogchan Kang of the Department of Plant Pathology, University of Pennsylvania, was used. The plasmid contains the *egfp* gene under the promoter of a gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from *Cochliobolus heterostrophus*. It also contains a hygromycin B resistance gene hph, controlled by the *Aspergillus nidulans* trpC promoter, as well as the Kan gene for kanamycin resistance. Hygromycin B, is an aminoglycosidic antibiotic produced by *Streptomyces hygroscopicus*, and is used for the selection and maintenance of prokaryotic and eukaryotic cells transformed with the hph gene. Vector pCAMBIA 1300 that has CaMV 35S promoter, Kan gene and hph gene was used as control.

Preparation of A. implicatum cells: A. implicatum isolate 6780-201v isolated from *Brachiaria brizantha* CIAT 6780 was used for transformation of its conidia or mycelia. The fungus was grown on YMG agar (D- glucose 4,0g; malt extract 10,0g; yeast extract 4,0g; agar 10,0g; 1L distilled water) medium for 8 days and incubated at 28ºC. Conidia were collected in a solution of 0.15M NaCl and cleaned by passing through a Whatman #1 filter paper. The conidia were then suspended in YMG liquid medium and incubated with shaking (250 rpm) for 4 hours at 28ºC, in order to induce conidial germination. Subsequently, the conidia were collected by filtration and re-suspended in an induction medium IM+AS, (in 1 litre of distilled water: 2.05g K_2HPO_4; 1.45g KH_2PO_4; 0.15g NaCl; 0.5g Mg_2SO_4 .7H_2O; 0.07g $CaCl_2$ .2H_2O; 0.0025g $Fe_2SO4.7H_20$; 0.5g $(NH_4)_2SO_4$; 10 mM D-glucose; 0.5%glycerol; 40mM MES [2-n-morppholino ethanesulfonic acid]; 200σM acetosyringone at a concentration of $1x10^6$ conidia/ml. To obtain mycelia for transformation, the protocol describe above was used, but the incubation was extended to 48 hours instead of only 4 hours, and the concentration was adjusted to OD_{600}= 0.35 in IM+AS.

Transformation of A. implicatum: The transformation protocol was based on the methods described by Mullins *et al.* (2001, Phytopathology 91:173-180) for transformation of the pathogenic fungus *Fusarium oxysporum*. Some modifications were introduced. *A. tumefaciens* strains AGL1 and LBA4404 were transformed with vectors pSK1019 or pCAMBIA 1300 using methods described by Den Dulk-Ras and Hooykaas (1995, Methods Mol Biol. 55: 63- 72.). The transformed bacteria were grown in TYNG medium (for 1L medium: 10.0 g tryptone, 5.0g NaCl, 5.0 g yeast extract, 0.5g $MgSO_4 \notin 7H_2O$, pH 7.5) supplemented with kanamycin [100 σg/ml] and incubated at 28°C in the dark for 16 hours to optical density (OD_{600}) of 0.75. This bacterial cell concentration was subsequently diluted with the induction medium IM+AS to OD_{600}= 0.1 and further incubated for 4 hours to induce virulence genes. Once the incubations was completed, the bacterial cell concentration was adjusted to OD_{600}= 0.2. The *A. implicatum* preparations described above and these *A. tumefaciens* transformant cells were mixed together in equal volumes. Two hundred-σl of each mixture was placed on a 0.45-σm-pore-size, 45-mm diameter nitrocellulose membrane (Whatman, Hillsboro, OR), and plated on IM+AS agar medium (glucose content reduced to 5mM). These mixtures were incubated for 48, 60 and 72 hours. The membranes were subsequently transferred to Petri plates containing YMG agar media containing hygromycin B (100 σg/ml), and cefotaxime (500 σM), and incubated further at 28 °C. Putative transformant *A. implicatum* cells became apparent on the selection media after 5 days of incubation. Control *A. implicatum* cells were treated the same way except that they were co-cultivated with strains of *A. tumefaciens* that were not transformed with the plasmid vectors.

PCR amplifications: DNA isolated (Kelemu *et al*., 2003, Molecular Plant Pathology 4:115-118) from putative transformant bacteria and fungus as well as control ones was analyzed using the polymerase chain reaction (PCR) using primers with sequences of *egfp* and/or *hph*.
[primers glGFP3 (5`-GCCGAGCTCAGATCTCACTTGTACAGCT CGT-3`) and glGFP5 (5`-GCCGGAATTCATGAACAAGGG CGAGGAACTG-3`)]and hph122U (5`-TTCGATGTAGGAGGGCGTGGAT-3`) and hph725L (5`-CGCGTCTGCTGCTCCATACAAG-3`)].

Amplifications were carried out in a Programable Thermal Controller (MJ Research, Inc) programmed to 35 cycles comprised of 45 seconds denaturation step at 94°C (4 minutes for the first cycle), followed by 1 min at 60°C, and primer extension for 1.5 minute (10 minutes in the final cycle) at 72°C. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad Laboratories), stained with ethidium bromide, and photographed under UV lighting.

Southern blot analysis: The DNA of 19 randomly selected putative *A. implicatum* transformants were analyzed using Southern blot analysis. The hygromycin B resistance gene hph was used as a probe. Southern hybridization was carried out using standard procedures described in Sambrook *et al.,* (1989, Cold spring Harbour Laboratory Press, Cold Spring Harbour, NY). Labeling and detection were carried out using Dig-high prime DNA labeling and detection Kit II (Roche Applied Science)

Microscope examination: The putative GFP-expressing transformants were examined under a LEICA fluorescence microscope fitted with a Leica D filter with an excitation range between 355 and 425 nm, and an H3 filter with an excitation range between 420 and 490 nm.

Plant inoculations: Brachiaria seedlings were inoculated with a few selected *A. implicatum* transformants using the method described earlier (Kelemu *et al.,* 2001, Canadian Journal of Microbiology 47:55-62).

Results and Discusión

The endophytic fungus *A. implicatum* was successfully transformed with e*gfp* (enhanced green fluorescent protein) gene. Enhanced color variants [ECFP (cyan), EGFP (green), EYFP (yellow)] have been generated through mutagenesis and these are some of the most widely used reporters in biological research. They can be used as tags to track proteins in living cells, as reporters to monitor promoter activity, and as labels to visualize specific tissues, whole cells or sub-cellular organelles. They are useful for monitoring gene expression and protein localization.

The GFP protein (27 kDa) is a spontaneously fluorescent protein that absorbs light at maxima of 395 and 475 nm and emits at a maximum of 508 nm. This protein is a success as a reporter because it requires only UV or blue light and oxygen, but requires no cofactors or substrates as many other reporters do for visualization.

In 2004, we reported the successful transformation of *A. implicatum* with GFP gene in vectors pWGFP20 and pCT74, although the green fluorescence emitting appeared to be weak, and thus the need for more work to be done in order to get tranformants with a more pronounced emission. We report this year on work of a successful transformation of the fungus with intense emissions.

The protocol that we developed for the transformation of this endophytic fungus is based on the protocol described by Mullins *et al.* (2001, Phytopathology 91:173-180)) for the pathogenic fungus *Fusarium oxysporum*. However, modifications were needed for a successful transformation of *A. implicatum*. For example, *A. implicatum* is a slow growing fungus, and thus the recommended concentration of cefotaxime (200 µM) to inhibit the growth of *A. tumefaciens*, was not sufficient enough to prevent bacterial growth from impeding the growth of *A. implicatum*. Results from the experiments we conducted indicated that cefotaxime concentrations at 500 σM was sufficient to inhibit the growth of *A. tumefaciens* while allowing *A. implicatum* putative transformants to grow on selection media. Introducing TYNG medium instead of MM [for 1L medium: 2.05g K_2HPO_4, 1.45g KH_2PO_4, 0.15g NaCl, 0.5g $MgSO_4 \notin 7H_2O$, 0.07g $CaCl_2 \notin 2H_2O$, 0.0025g FeSO4 $\notin 7H_20$, 0.5g $(NH_4)_2SO_4$] has reduced the time needed to reach the required bacterial concentration (OD_{600} = 0.75) from 48 hours to only 16. In addition, the TYNG medium eliminated the cell aggregation problem we encountered with the growth of *A. tumefaciens* (particularly with strain LBA4404) in MM and that interfered with the transformation process.

A better transformation efficiency was obtained with *A. tumefaciens* strain AGL-1 (Table 2.22.1; Figure 2.22.1). Although *A. implicatum* transformants containing either pSK1019 (*trpC* promoter) or pCAMBIA 1300 (CaMV35S promoter) were obtained, a significantly higher number of transformants were obtained with pSK1019 (Table 2.22.1). However, this suggests that the CaMV35S promoter can function in *A. implicatum* although at a much lower efficiency. The colony size of transformants in both cases is similar with an average size of 19-mm after 12 days of incubation at 28^{O}C on the selection medium.

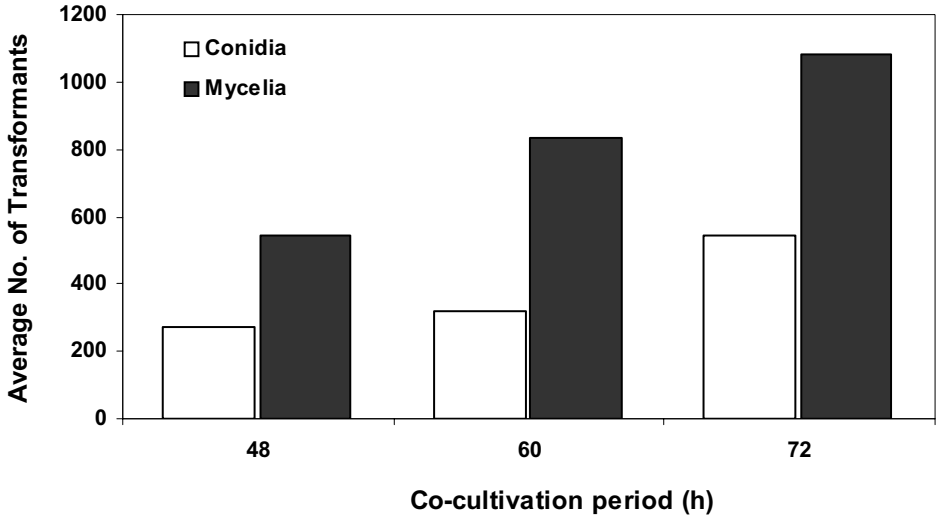

Figure 2.22.1. The effect of *Agrobacterium tumefaciens* strain AGL-1 and *Acremonium implicatum* co-cultivation period on transformation efficiency. The *A. tumefaciens* strain contains plasmid pSK1019 that has enhanced green fluorescent protein *(egfp)* gene under the promoter of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from the fungus *Cochliobolus heterostrophus*. It also contains a hygromycin B resistance gene hph, controlled by the *Aspergillus nidulans trp*C promoter. The data presented are the average of three plates per treatment.

The results indicate that the transformation efficiency is directly influenced by the length of the co-cultivation (*A. tumefaciens* and *A. implicatum*) of period (Table 2.22.1, Figure 2.22.1). As the co-cultivation period increased from 48 h to 72 h, the efficiency increased from 542 transformant colonies to 1084 in the case of mycelial transformation protocol; and from 271 to 542 for conidial transformation (Table 2.22.1, Figure 2.22.1). Similar results have been reported for transformation of *Magnaporte grisea* (Rho *et al.*, 2001, Mol. Cells 3: 407-411) and *F. oxysporum*. The efficiency of transformation also differed depending on whether we used mycelia or conidia for transformation (Figure 2.22.1). The best and optimum transformation results were obtained with *A. tumefaciens* strain AGL-1, plasmid pSK1019 under the control of *trp*C promoter either with mycelial or conidial transformation. However, mycelial transformation consistently generated significantly higher number of transformants than when conidia were used to transform (Figure 2.22.1).

A. tumefaciens-mediated transformation has long been applied to transfer foreign genes to a wide-range of plants. In recent years, this has also been used to transform a wide range of fungi allowing efficient genetic manipulations of the recipient organisms. The presence of acetosyringone is important for successful *A. tumefaciens*-mediated transformation.

Table 2.22.1. Putative *Acremonium implicatum* transformant colonies per Petri dish of selection medium.

***A. tumefaciens* strain**		**AGL-1**				**LBA4404**			
Promoter		***trpC***		**CaMV35S**		***trpC***		**CaMV35S**	
Recipient fungal structure		M*	C	M	C	M	C	M	C
Co-cultivation period (Hours)	48	542	271	1,7	2	0,7	0,3	0	0
	60	836	318	1,3	3,3	1	1,0	0	0
	72	1084	542	0	0	1,3	1,7	0	0

* M = Mycelia, C = Conidia. The values represent the average number of transformants between three plates.

The putative *A. implicatum* transformants selected on hygromycin B containing agar media were further examined using fluorescence microscope, PCR and Southern blot analysis. The PCR method allowed us to quickly examine and further confirm putative transformants that have been selected on antibiotic selection media (Figures 2.22.2 and 2.22.3). To determine the copy number of the transferred T-DNA, genomic DNA from19 randomly picked transformants from each experimental condition was digested with *Hind*III and anlalyzed with Southern blot. The results exhibited genomes with inserts ranging from a single insert to 5 inserts (data not shown), while the negative control, untransformed *A. implicatum*, showed no hybridization. No correlation existed between the average copy number of T-DNA per genome and the co-cultivation period, the mycelial or conidial transformation or other variables introduced in the experiments.

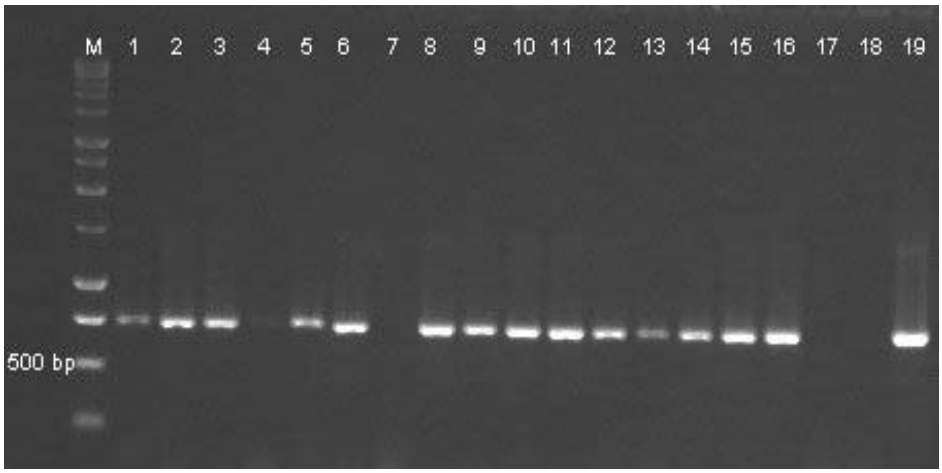

Figure 2.22.2. Polymerase chain reaction (PCR) amplifications, with primer specific for sequences of hygromycin B resistance gene (hph), of template DNA isolated from *Acremonium implicatum* transformants. Lanes M = molecular marker; template DNA from: 1 = conidia transformed with pSK1019 in *A. tumefaciens* strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 2, 3 = conidia transformed with pSK1019 in *A. tumefaciens* strain AGL-1 co-cultivated for 48 hours, 60 hours and maintained without or with antibiotic selection pressure, respectively; 4 = conidia transformed with pCAMBIA1300 in strain AGL-1 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 5 = mycelia transformed with pSK1019 in strain LB4404 co-cultivated for 60 hours, and maintained without antibiotic selection pressure; 6 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 7 = negative control (water); 8-11 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours (lanes 8, 9, and 10) and 72 hours, and maintained with antibiotic selection pressure or without it (lane 10); 12 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 13 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure for 4 generations; 14-16 = conidia or mycelia (lane 16) transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours and 60 hours (lane 16) and maintained with antibiotic selection pressure; 17, 18 = negative controls *Phaeoisariopsis griseola* and *A. implicatum* strain 6780 201V, respectively ; 19, 20 = positive controls pSK1019 and pCAMBIA1300, respectively.

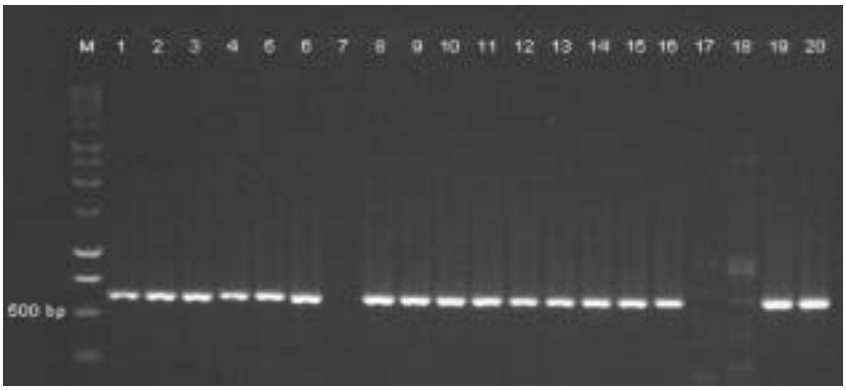

Figure 2.22.3. Polymerase chain reaction (PCR) amplifications, with primer specific for sequences of enhanced green fluorescent protein (*egfp*) gene, of template DNA isolated from *Acremonium implicatum* transformants. Lanes M = molecular marker; 1 = conidia transformed with pSK1019 in *A. tumefaciens* strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 2, 3 = conidia transformed with pSK1019 in *A. tumefaciens* strain AGL-1 co-cultivated for 48 hours, 60 hours and maintained without or with antibiotic selection pressure, respectively; 4 = conidia transformed with pCAMBIA1300 in strain AGL-1 co-cultivated for 72 hours, and maintained without antibiotic selection pressure; 5 = mycelia transformed with pSK1019 in strain LB4404 co-cultivated for 60 hours, and maintained without antibiotic selection pressure; 6 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 7 = negative control (water); 8-11 = mycelia transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours (lanes 8, 9, and 10) and 72 hours (lane 11), and maintained with antibiotic selection pressure or without it (lane 10); 12 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained with antibiotic selection pressure; 13 = conidia transformed with pSK1019 in strain LB4404 co-cultivated for 72 hours, and maintained without antibiotic selection pressure for 4 generations; 14-16 = conidia or mycelia (lane 16) transformed with pSK1019 in strain AGL-1 co-cultivated for 48 hours and 60 hours (lane 16) and maintained with antibiotic selection pressure; 17, 18 = negative controls *Phaeoisariopsis griseola* and *A. implicatum* strain 6780 201V, respectively; 19 = positive control pSK1019.

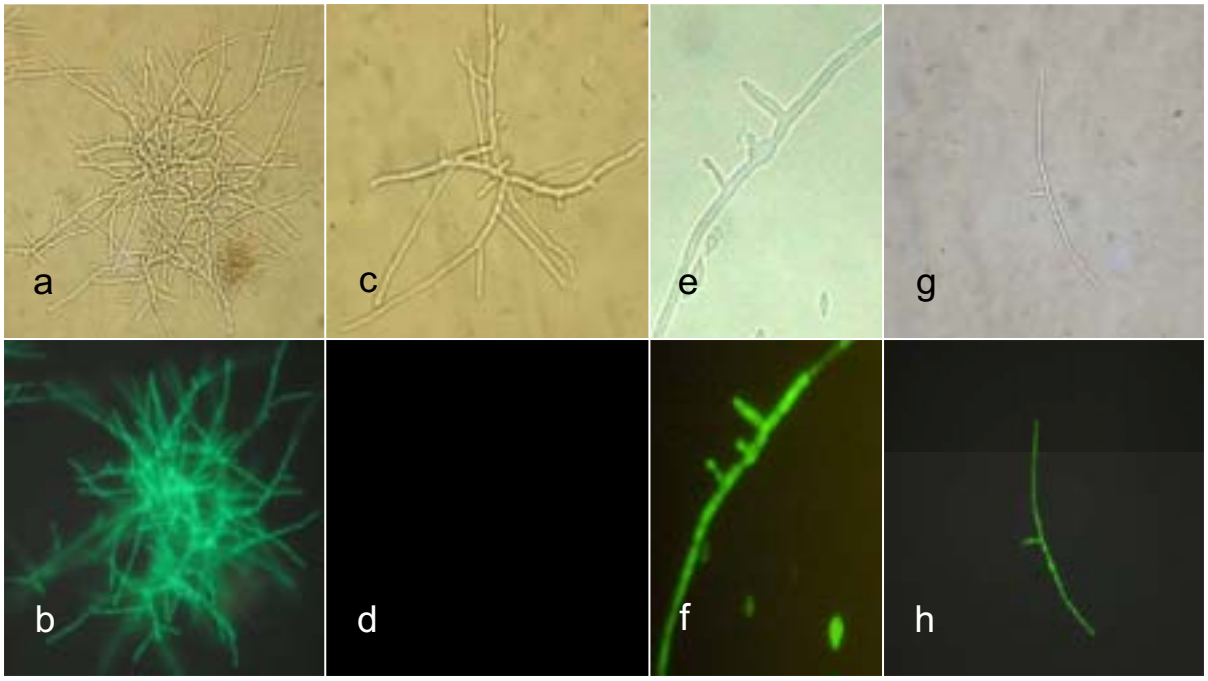

Figure 2.22.4. Structures of *Acremonium implicatum* strain 6780 201v transformed with green fluorescent protein gene (*egfp)* and observed microscopically with UV light. Photos a, c, e and g under normal light; b = fluorescence emission from transformed mycelia under Leica D filter (355 and 425 nm); c and d = control untransformed *A. implicatum* strain 6780 201v without and with UV light, respectively; f and h = transformed structures emitting green fluorescence under UV light with H3 filter (420 and 490 nm).

Microscopic examinations of selected transformants demonstrated strong expression of *egfp* as evidenced by the intense fluorescence emission. All parts of the fungal structure including conidia, mycelia, germinating conidia showed emission. These results demonstrate that the fungal promoter glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from *Cochliobolus heterostrophus* functions well for expression of genes in the endophytic fungus *A. implicatum* (Figures 2.22.4 and 2.22.5).

The mitotic stability of the transferred DNA was examined by growing 10 transformants in liquid and agar media for 6 generations without any selection pressure. In all cases, resistance to hygromycin B was maintained indicating that the transferred DNA was stable. They all retained emission of fluorescence as well. The meiotic stability could not be determined because the fungus cannot be crossed.

Preliminary data showed that *Brachiaria* tissues taken from plants inoculated with GFP-transformed *A. implicatum* expressed fluorescence emission (Figure 2.22.6). Figure 2.22.5 shows the gfp-expressing transgenic *A. implicatum* used to inoculate *Brachiaria* plants. This will allow us to study the endophyte-*Brachiaria* interaction, endophyte distribution within the plant tissue, and stability in the seed. This will in turn allow us to examine the potential use of this endophyte as a gene delivery and expression system in plants.

Although various transformation systems have been developed and reported for many fungi, successful application of the technology is still not routine in many species. Furthermore, developing an efficient transformation system for a previously untransformed fungus can be a technical obstacle. This work

describes the transformation and expression of the GFP-encoding gene in an isolate of *A. implicatum*, an endophyte in species of *Brachiaria*. We have demonstrated that both the mycelia and conidia of *A. implicatum* can efficiently be transformed using *A. tumefaciens*. To the best of our knowledge, this is the first report on transformation of this endophytic fungus.

The stable integration and expression of the introduced gene into the genome of the recipient fungus indicate that the endophyte may be an excellent tool for delivering and expressing genes of agronomic importance such as disease and insect resistance to host plants. For this to be successful, the practical implication of high seed transmission of *A. implicatum* in *Brachiaria* is significant: once associated with the plant, the fungus can perpetuate itself through seed, especially in apomictic genotypes of *Brachiaria*, for as long as seed storage conditions do not diminish the survival of the fungus (Dongyi and Kelemu, 2004, Plant Disease 88:1252-1254) Several *Brachiaria* hybrids obtained from CIAT's forage breeding program were shown to harbor *A. implicatum*. In addition, we want to exploit the qualities of GFP as a reporter and study the interactions between *A. implicatum* and its host *Brachiaria*.

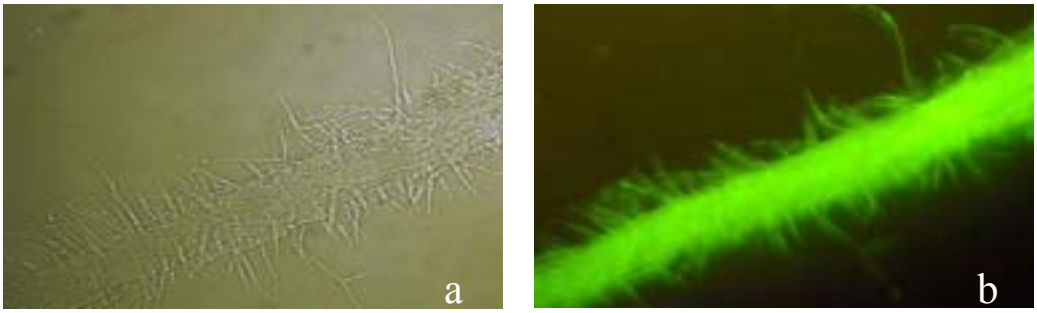

Figure 2.22.5 Mycelium of *Acremonium implicatum* transformed with enhanced green fluorescent protein (*egfp)* encoding gene: a) mycelium observed microscopically (40x) under normal lighting, b) the same mycelium observed under UV lighting, and demonstrating fluorescence emission.

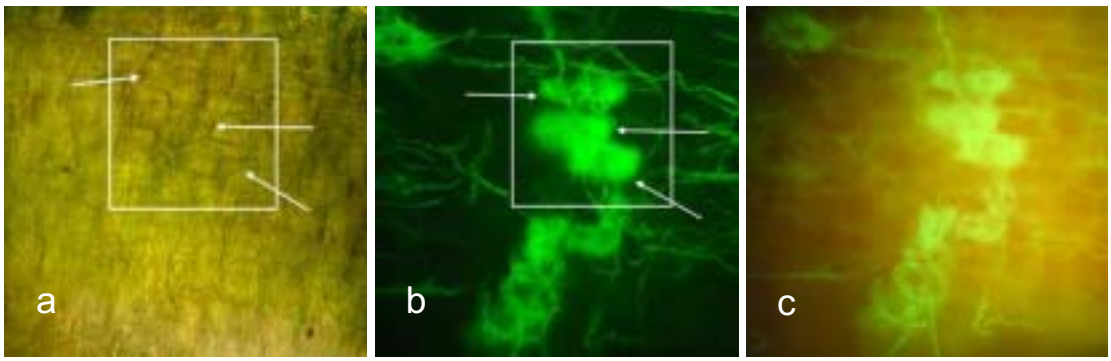

Figure 2.22.6. *Brachiaria* tissues from plants inoculated with *Acremonium implicatum* strain 6780 201v transformed with green fluorescent protein gene (*egfp*) [transformed strain shown in Figure 4). a) under normal lighting, b) fluorescence emission under UV light with Leica D filter, c) fluorescence emission under UV light with Leica H3 filter

Activity 2.23. An antifungal protein isolated from seeds of the tropical forage legume *Clitoria ternatea* controls diseases under field and greenhouse conditions

Contributors: G. Segura, S. Kelemu, and G. Mahuku

Rationale

Seeds use strategies such as production of antimicrobial and/or insecticidal proteins to germinate and survive in soils that are densely inhabited by a wide range of microfauna and microflora. Antimicrobial proteins and peptides have been isolated from seeds of maize (*Zea mays L.*), radish (*Raphanus sativus L.*) and various other plants. They are believed to play a role in plant defense because of their strong antimicrobial activity. This belief is supported by their ability to confer resistance (to pathogens) to transgenic plants containing genes that encode them.

In a previous study, we examined seeds from several tropical forage legumes, for antifungal properties. Of those examined, we isolated, purified, and characterized a protein, designated 'finotin', from seeds of Clitoria ternatea (L.) that exhibited, in vitro, strong antifungal activity on the test fungus Rhizoctonia solani Kühn (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). This protein has antifungal, antibacterial and insecticidal properties.

In this study, we examined the potential use of finotin as a biopesticide for disease control under field and greenhouse conditions.

Materials and Methods

Treatment of P. griseola conidia with the protein finotin: Twenty-σl of a conidial suspension (10^{-4}) was placed on a slide and subsequently covered with a thin layer of potato dextrose agar medium. A 200-σl crude antifungal protein preparation (the same concentration that was used to spray onto bean plants) was applied on the agar. Protein preparation protocols were as described previously (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). Control slides had only water. These were placed in Petri dishes containing wet filter paper and incubated at room temperature. Pictures of conidia were taken under the microscope at 0, 32 and 96 hours to observe the development of individual conidia.

Plant inoculation and extract applications: A highly virulent isolate of the pathogen *Phaeoisariopsis griseola*, causal agent of angular leaf spot, was grown on V8 agar at 24^{o}C for 12 days. Conidia were collected and suspended in sterile distilled water at a concentration of 2 x 10^4 conidia per mL. This inoculum was used on *Phaseolus vulgaris* variety Sprite (a susceptible one) bean plants.

*Greenhouse testing**:*** Seventeen-day old bean plants (15 plants per treatment) were sprayed with either the fungicide benlate (500 εg/ml), crude antifungal protein preparation, or sterile water. Two hours later all the plants were inoculated with *P. griseola* conidia (2 x 10^4 conidia per mL). The inoculated plants were placed in a humidity chamber for 4 days, then transferred to the greenhouse for symptom development. Treatments with crude antifungal protein, benlate or sterile water continued every 2 days. Disease evaluations were conducted 10 days after inoculation.

*Field testing**:*** Thirty days old seedlings of tomato variety Manalucie were transplanted to the field in a randomized design with 3 replications (8 plants per treatment in each replication). Treatments were; 1) control treatment with water alone, 2) spray application (till plants were completely wet) of crude protein preparation once a week, and 3) spray application of crude protein twice a week. Various diseases developed under natural infections.

Results and Discussion

Effect of antifungal protein Finotin on bean angular leaf spot: The crude protein extract from seeds of *C. ternatea* CIAT 20692 showed antifungal activity *in vitro* on the pathogen *P. griseola* (data not shown). Conidia treated with the crude protein failed to germinate 32 or 96 hours after treatment (Figure 2.23.1). Plants treated with the crude antifungal protein preparation consistently developed fewer angular leaf spot disease lesions than the control plants that were treated with sterile distilled water (Figure 2.23.2). Had a purified protein been used to control the disease on bean plants, the level of disease control would perhaps have been even higher.

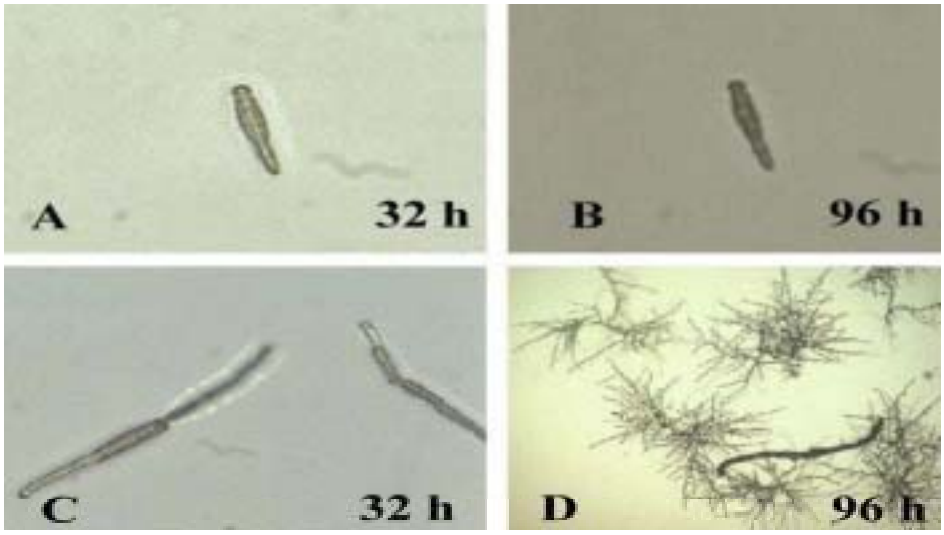

Figure 2.23.1. Treatment of *Phaeoisariopsis griseola* conidia with the antifungal protein finotin. Conidia failed to germinate in the presence of the antifungal protein finotin, 32 and 96 hours (A and B) after treatment, whereas those treated with sterile water germinated (C and D). [Annual Report 2004].

Effect of antifungal protein finotin on tomato diseases: Tomatoes are generally susceptible to a number of diseases under natural conditions. The purpose of these experiments is to develop a simple disease control strategy for small producers using this antifungal protein. Plants sprayed with the crude protein preparation once or twice a week developed better, had fewer disease symptoms, had more plant biomass, and produced more tomatoes than control plants (Figures 2.23.3, 2.23.4).

The protein finotin, is shown to be inhibitory to the growth of a range of important plant pathogenic fungi and at least one important bacterium pathogenic to common bean, as well as two important species of bruchids, *Z. subfasciatus* and *A. obtectus* (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). These findings raise the possibility that finotin may contribute to the high level of disease and insect resistance observed in *C. ternatea* in the field.

Finotin is released from seeds when the seed coat is mechanically damaged creating a zone of fungal growth inhibition *in vitro*. The antifungal activity of finotin is not affected by high temperatures, which made attractive for the direct use of this protein in disease management under field and greenhouse

conditions. The results presented here demonstrate that a disease control strategy can be developed for small producers using this antifungal protein.

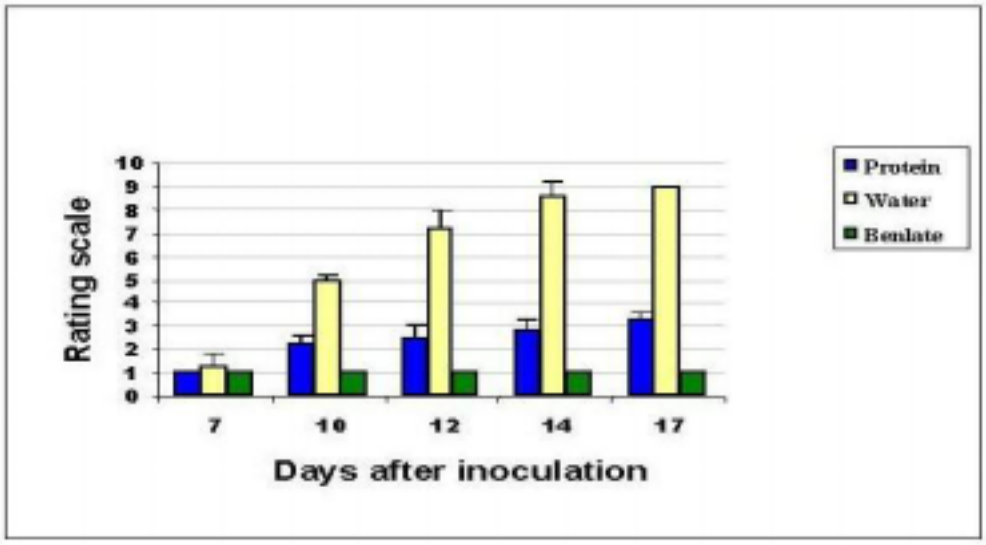

Figure 2.23.2. Angular leaf spot disease development in artificially inoculated bean plants following treatment with crude antifungal protein preparations isolated from *C. ternatea* CIAT 20692, the fungicide benlate, or water control (from AR-2004).

Figure 2.23.3. Tomato plants sprayed with crude antifungal protein preparations twice (3), or once (2) a week, and control (water) [1].

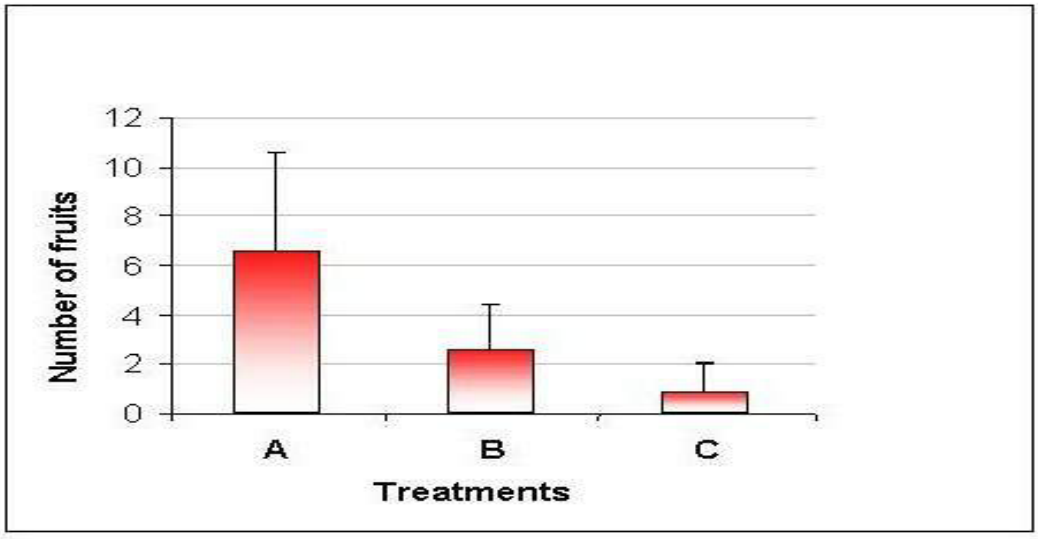

Figure 2.23.4. Average tomato fruit yield per tomato plant in plants treated with crude protein preparations twice a week (A), once a week (B) and water only (C).

Activity 2.24 . Isolating the gene encoding a biocidal protein named Finotin

Contributors: Martin Rodriguez and S. Kelemu

Rationale

Diseases and pests are major biological production constraints in a wide-range of crops. Plants, when attacked by harmful agents, can trigger an array of defense mechanisms. Pathogens and pests, in turn, have an array of matching mechanisms and evolve to overcome and compromise plant defense systems. One type of plant defense mechanism is the synthesis of proteins/peptides or low-molecular weight compounds following mechanical wounding or attack by biological agents. Biocidal or antimicrobial proteins are widely distributed in nature and are synthesized by various organisms. A number of plant-derived proteins that have antimicrobial or insecticidal properties have been isolated and characterized from various plants. One such example is the isolation, purification and characterization of a highly basic small protein, designated 'finotin', from seeds of *Clitoria ternatea* (Kelemu *et al*., 2004, Plant Physiology and Biochemistry 42: 867-873). This protein has broad and potent antifungal, antibacterial and insecticidal properties, indicating that it may contribute to the high level of disease and insect resistance observed in *C. ternatea* in the field. We have subsequently demonstrated that plants sprayed with the crude protein preparation consistently developed fewer lesions of various diseases than the control plants both in greenhouse and field experiments (Kelemu *et al*., 2005, Phytopathology 95:S52).

In light of these findings, it is important to isolate the gene encoding finotin for application of non-host resistance in various crops to combat diseases and pests of economic importance. We report here the progress we have made towards polymerase chain reaction-based cloning of a cDNA corresponding some amino acid sequences of the protein.

Materials and Methods

*Plant material***:** Fully-developed but not dried seeds of *Clitoria ternatea* CIAT accession #20692 were collected directly from the pods and used in this study.

RNA Extraction: Various extraction methods described by several authors were evaluated. Of those evaluated, the methods described by Azevedo *et al* (2003, Plant Mol Biol Reporter 21: 333-338), and Chang *et al* (1993, Plant Mol Biol Reporter 11: 113-116) resulted in good quality RNA comparable to that obtained with an RNA isolation kit from Promega. mRNA was isolated from this total RNA using Oligotex™ Direct mRNA kit (QIAGEN) according to the manufacturer's instructions.

*Synthesis of cDNA***:** Although there are various methods for doing so, complementary DNA (cDNA) is often synthesized from mature (i.e. fully spliced) mRNA using reverse transcriptase enzyme, which operates on a single strand of mRNA and generating its complementary DNA based on the pairing of RNA base pairs (A, U, G, C) to their DNA complements (T, A, C, G). In this study, the synthesis was conducted using 200 ng mRNA, 12-σM of BD SMART II**™** A oligonucleotide and 12-σM of *Primer* 5'-RACE CDS in a 10-σl volume. This was incubated at 70°C for 2 min, then placed on ice. Two-σl of 5x buffer, 1-σl of DTT (20 mM), 1-σl of 10 mM dNTP and 1-σl of BD PowerScript Reverse Transcriptase were subsequently added to the mixture and incubated at 42°C for 2 h (the reverse transcriptase scans the mature mRNA and synthesizes a sequence of DNA that complements the mRNA template). Fifty-σl of Buffer Tricina-EDTA was added and further incubated at 70 °C for 7 minutes to deactivate the reaction. For synthesis of cDNA for the 3´ end, 500-ng mRNA and 1-σl (100-ng/σl) of primer oligo-$(dT)_{25}$ were mixed and incubated at 70°C for 8 min followed by cooling the mixture on ice. Subsequently, 10-σl of 5X buffer, 1-σl of 25mM dNTP, 2-σl of 100 mM DTT and 1-σl of SuperScript™ III RT (200 U/σl) were added to the mixture and incubated for 1 h at 50°C. At the end the mixture was deactivated by heat treatment at 70°C for 10 min. Information on oligonucleotides used in this study is given in Table 2.24.1.

Table 2.24.1. Sequences of primers and adaptors used in this study.

Name	**Sequence**
Oligo dT	5'-$(dT)_{20}$-VN*-3'
5' RACE CDS-primer A	5'-$(T)_{25}$-VN*-3'
BD SMART II™ A Oligonucleotide	5'AAGCAGTGGTATCAACG- CAGAGTACGCGGG 3'
primer UPM (Universal Primer A Mix)	5'CTAATACGACTCACTATAGGGCAAGCAGTGGTA TCAACG CAGAGT3'
primers NUP (Nested Universal Primer A)	5'AAGCAGTGGTATCAACGCAGAGT3'
MERF 1	5'-TGYGARCGNGCNTCNCTNACNTGG-3'*
MERF 2	5'-GARMGNGCNWSNYTNACNTGGACN-3'*
MERF3	5'-ACNGGNAAYTGYGGNAAYACNGGNCA-3'*
MERF4	5'-AAYYTNTGYGARMGNGCNWSNYT-3' *
MERF5	5'-ACNTGGACNGGNAAYTGY-3' *
FINOR 5	5'-CARTCRAARTARCARAARCAYTT-3'*
FINOR 6	5'-RTTNCCNCKYTTRTGRCANGCNCC-3'*
** N=A, C, G o T; V=A, C o G H=A, C o T D=A, G o T R=A o G Y=C o T M=A o C*	

*Polymerase chain reaction of cDNA***:** We contracted Cornell University's biotechnology unit to sequence finotin. However, the protein (finotin) sequence data obtained from Cornell Biotech was not satisfactory,

and as a result we used sequences of an antifungal protein (from Clitoria ternatea) reported by Osborn *et al* (1995, FEBS Lett. 368: 257-262) to generate degenerate primers. A 25-σl PCR mixture contained 1.5-mM of $MgCl_2$, 200-ε Mof dNTPs, 0.5-ε M of each olgonucleotide (UPM and FINOR5), 200-ng cDNA, 1 unit of Taq DNA polymerase, and 1x PCR buffer. Amplifications were programmed with 35 cycles of a 30 second (3 min for the first cycle) denaturation step at 94 °C, annealing for 45 seconds at 50 °C, and prime extension for 45 seconds at 72 °C.

Cloning and sequencing: The amplified product was excised and eluted from the agarose gel using a BIO-RAD DNA purification kit. This was cloned in pGEM-T Easy vector (Promega, USA), and sequenced using ABI Prism 377-96 DNA Sequencer. The sequence data were aligned using Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI).

```
1          acgcggggatagtagataagagtaaataaggtagctagcttagtacctggtttaaagtta
61         aggagagtATGGCAAAGTGTAACACAATGGTAATAGCATTAGCAGCAGTAGTAGTAGTGT
1                    M  A  K  C  N  T  M  V  I  A  L  A  A  V  V  V  V
121        TGCTGATTGATGGTGGAGAAAGTTTTGCAATATGTAACGTAGATTCAAGTCAGTTAAGCT
18         L  L  I  D  G  G  E  S  F  A  I  C  N  V  D  S  S  Q  L  S
181        TGTGTCGTGCAGCAGTTAGTGGTGGTAATCCGCCACCACCAGATGAAAAGTGTTGTGCTG
38         L  C  R  A  A  V  S  G  G  N  P  P  P  P  D  E  K  C  C  A
241        TCATTCGTCAGGTCAATCTGCCCTGCCTCTGCCAATACAGGGGATTCCTACTTCGGTTTG
58         V  I  R  Q  V  N  L  P  C  L  C  Q  Y  R  G  F  L  L  R  F
301        GAATCAATCCCAAAAATGCTTTTGCTACTTCGACTG
78         G  I  N  P  K  N  A  F  A  T  S  T
```

Figure 2.24.1. Sequence analysis of a cDNA clone synthesized from mRNA of seeds of *Clitoria ternatea.* The figure shows nucleotide sequences and the corresponding deduced amino acid sequences The sequence highlighted in green represents a possible TATA box sequence where as the yellow highlighted region represents a sequence commonly present in various LTP genes. The cysteine amino acid sequences are circled. The sequence of primer FINOR5 (anti-sense) is underlined.

Results and Discussion

Amplified DNA fragments (using cDNA as a template) ranging from 120 to 650 bp were isolated and cloned. Ten combinations of primers, 7 sense and anti-sense orientations and a universal primer, were used on 4 different preparations of cDNAs. A total of 37 clones were generated and of these, 17 have been sequenced so far. The sequence of one such clone is shown in Figure 2.24.1. The sequence data demonstrated homology to genes encoding nonspecific lipid transfer proteins (nsLTPs) from plants. These findings may be significant because nsLTPs have been reported to play a role in plant defense systems. A number of peptides (small proteins with sizes ranging 2-10 kDa) including nsLTPs have been reported to be involved in plant defense mechanisms. It is generally believed that seed proteins with antimicrobial activity may play a role in the protection of seeds against harmful microbes. Nonspecific lipid transfer proteins are basic, 9-kDa proteins with conserved cysteines and present in high amounts in plants. One promising clone with homology to nsLTPs is tentatively designated CtLTP. The clone is not complete, but based on sequences from nsLTP of other plants, only a small portion from the 3' end is missing (Figure 2.24.2).

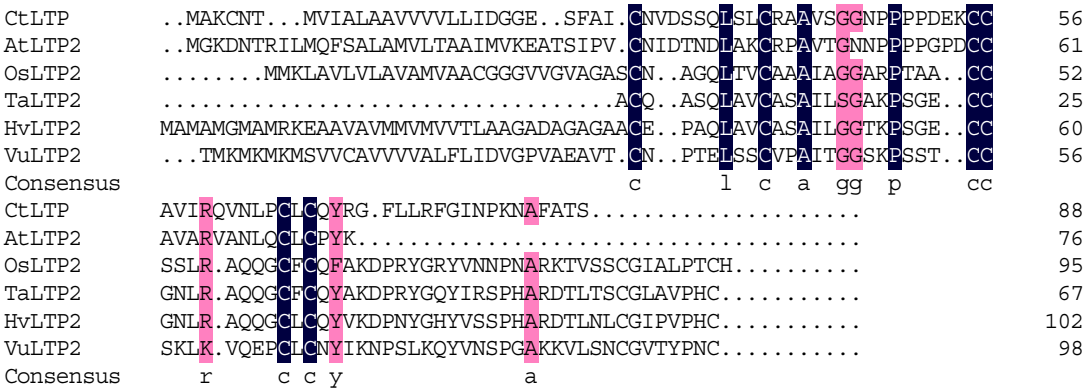

Figure 2.24.2. Amino acid sequence comparisons among various plant nsLTPs that are associated with plant defense systems. CtLTP: sequences deduced from this study; AtLTP: nsLTP of *Arabidopsis thaliana*; OsLTP: nsLTP of *Oryza sativa*; TaLTP: nsLTP of *Triticum aestivum*; HvLTP2: nsLTP of *Hordeum vulgare*; VuLTP2: nsLTP of *Vigna unguiculata*.

The biological function of nsTLPs is still well understood. However, a number of studies have demostrated growth inhibition of a range of pathogens by nsTLPs. For example Cammue *et al* (1995, Plant Physiol. 109:445-455) isolated a protein from seeds of onions (*Allium cepa* L.) that had sequence homology to nsLTPs and that was a potent groth inhibitory effect against 14 different pathogens.

Our preliminary data presented in this report indicate that the cDNA clone that we generated may be classified as a member of the nsLTP protein family based on the deduced amino acid sequence. Work is in progress to generate a full length cDNA clone, to complete the sequences of the remaining 20 clones, and to determine the antimicrobial activity of a successful complete clone.

Activity 2.25. Inducing symptoms of bacterial wilt of plantain with a strain of *Ralstonia solanacearum* isolated from tomato

Contributors: Elizabeth Álvarez, Germán Llano, Juan Pablo Castillo, and John Loke

Highlight:

- First occurrence of an emerging tomato strain of *R. solanacearum* race 1, a new pathotype that genetically clusters with plantain strains (race 2). Greenhouse inoculation showed that the tomato strain was pathogenic to plantain, hence, bringing into question the use of plantain–tomato rotations.

Rationale

The *R. solanacearum* species complex, which includes the banana blood disease bacterium (BDB) and *Pseudomonas syzygii*, is a highly diverse group of organisms. Traditional methods of characterizing this organism, including biovar typing and race assessment, have been very useful in defining diversity within *R. solanacearum*. However, recent genetic evidence has indicated that these phenotype-based schemes are not sufficient to cover the entire diversity of strains represented by the species *R. solanacearum*. In our study, the pathogenicity of *R.* solanacearum obtained from tomato was determined in plantain seedlings, confirming Fegan and Prior's hypothesis (Fegan and Prior, 2002, 3rd International Bacterial Wilt Symposium, South Africa).

Materials and Methods

Samples of leaves and stem bases were taken from an infected plant growing in a tomato crop with symptoms of wilt. The crop was located on a farm in the Municipality of Montenegro (Quindío) and was being cultivated beside a plantain crop that had foci of bacterial wilt. The samples were processed in the laboratory. They were first washed for 20 min under running water, disinfected with 1% sodium hypochlorite for 1 min and then in 50% alcohol for another minute. They were then rinsed twice with sterilized distilled water, and finally macerated in sterilized distilled water. The mash was cultured onto SMSA medium and incubated at 28 ∀C for 4 days.

The mucoid and reddish colonies grew in the medium, and were purified by droplet transfer to SMSA. After 3 days, they were transferred to nutrient agar (NA) medium, where the bacterium grew for 36 h. A bacterial suspension was then prepared with the bacterium grown on NA by scraping the dishes in sterilized distilled water and passing through a spectrophotometer at an absorbance of 0.1 at 600 nm wavelength (1×10^8 cfu/mL). This suspension was injected at 0.5 mL/plant into 4 seedlings of plantain variety Africa. The control used was strain CIAT 1008, isolated from plantain. The inoculated plants were incubated for 5 days with continuous wetting at 22 ∀C at night and 30 ∀C during the day, and 95% relative humidity, after which they were moistened daily for 1 h.

Once the symptoms were reproduced in the plantain, the bacterium was re-isolated. The pathogenic strains were again inoculated into plantain and into 15-day-old tomato seedlings by injection of a bacterial suspension.

In another trial, the soil was inoculated with a bacterial suspension of strain CIAT 1008 at 0.1 absorbance at 600 nm wavelength in a 25-mL volume that was added to 1-kg flasks carrying 30-day-old tomato plants. In some flasks, perpendicular cuts were made into the soil, 5 cm from the stem thus causing root wounds. This treatment was compared with unwounded plants. Plants were also inoculated by injection of 0.5 mL of the bacterial suspension. The plants were incubated as described for plantain.

Results and Discussion

Symptoms began appearing in plantain plants in the second week after inoculation. The plants showed wilting of leaves and later death in a manner very similar to that of the control (Figure 2.25.1). Before the plants totally wilted, the bacterium was re-isolated and cultured onto SMSA, where the typical morphology of *R. solanacearum* was observed. The inoculated tomato plants showed wilting within 6 days and died 3 to 5 days later.

Figure 2.25.1. A) Bacterial wilt in tomato cultivated in the plantain-growing area of Montenegro (Quindío, Colombia). B) Symptoms of bacterial wilt are reproduced in plantain seedlings inoculated with a strain of *Ralstonia solanacearum* isolated from the tomato plants observed in A.

Plants that received the soil inoculation treatment causing root wounds showed typical symptoms of bacterial wilt 6 days after inoculation. The plants that were not wounded showed symptoms 4 days later (Figure 2.25.2).

Figure 2.25.2. Bacterial wilt in tomato caused by isolate CIAT 1008 of *Ralstonia solanacearum* obtained from plantain, 10 days after inoculation. A) Plant with wounds to the roots. B) Plant without wounds to the roots

The results contradicted reports that suggested that race 2 is specific to the Musaceae and does not attack tomato. On the contrary, they confirmed Fegan and Prior's findings where they had identified several phylotypes of *R. solanacearum* attacking both plants. The strains we isolated from both plantain and tomato classified as phylotype II.

Activity 2.26. Evaluating ecological practices of soil management in foci affected by bacterial wilt (*Ralstonia solanacearum*) in two plantain crops in the Department of Quindío

Contributors: E. Álvarez, G. Llano, M. Vargas, O. Zuluaga, L. A. Meza Becerra, V. H. Treviño Henao, J. L., and J. F. Mejía

Rationale

Farmers consider current recommendations for managing moko as inefficient and demand that formalin, used to disinfect soil, be replaced with a nontoxic alternative. In earlier work, our greenhouse experiments have shown that French marigold (Tagetes patula) reduces bacterial populations in the soil by 85%. To evaluate the effect of incorporating into the soil several different nontoxic alternatives to inhibit *Ralstonia solanacearum*, field experiments were conducted in the Quindío region of Colombia.

Materials and Methods

Field: Three foci affected by bacterial wilt were chosen in two plantain crops on "La Guaira" and "Cataluña" Farms (Montenegro, Quindío). To evaluate their effects on the bacterium *R. solanacearum*, causal agent of bacterial wilt, the following treatments were applied:

- Phosphoric rock (29% P_2O_5), lixiviate, and entire *T. patula* plants were incorporated at rates of 25 kg, 24 L, and 4 kg, respectively, at each affected site

- Check with 50% formalin

- Check with no treatment

The treatments were distributed in a randomized complete block design, with 3 replications in "La Guaira" and 6 in "Cataluña". The experimental unit corresponded to an affected site (i.e., one plant with daughter and granddaughter).

Before applying treatments, samples were collected from soil at 20–25 cm deep and from affected plants in 25-cm-long pieces that encompassed both above and below ground parts, as well as tissues. The samples were used to detect the bacterium.

Once sick plants in the foci were identified, these plants were uprooted and chopped into pieces of about 30 to 40 cm long, stacking them on the affected site. Above the pieces the phosphoric rock was applied first, then the lixiviate, and finally the *T. patula* plants, cut into pieces on application to favor release of the plants' thiophenes, which are probably responsible for inhibiting the bacterium (Arenas *et al*., 2005, Fitopatol Colomb 28:76–80.). The mixture was covered with soil and with black polyethylene to prevent weeds growing.

To apply formalin to the affected site, the soil was perforated at five points with a ¼-inch-diameter rod to as deep as 60 cm. Two liters of 50% formalin were applied, filling up every orifice. The treated site was then covered with black polyethylene.

The check consisted of plant material that was chopped up and covered with black polyethylene, receiving no treatment.

Once the treatments were applied, a ditch was dug around each site to prevent contamination among sites.

Every 30 days, samples were taken of the soil at 20 and 50 cm deep in the same site where the affected plant was found. Three months after the trial was established, the treatments were re-applied.

Isolation: From each soil sample, 3.3 g were taken and 30 mL of TE buffer (10 mM blend of TRIZMA® base and TRIZMA® HCl, and 1 mM of EDTA) at pH 7.6 were added and homogenized by vortexing. This suspension became the base solution.

From this base solution, dilutions at 10^{-1} to 10^{-3} strengths of TE buffer were prepared. Of each dilution, 100 εL were placed in petri dishes containing SMSA (1 liter of medium contained 10 g Bacto™ Peptone, 5 mL glycerol, 1 g casamino acids, 18 g Bacto™ Agar, 26 mg bacitracin, 100 mg polymyxin-ϸ sulfate, 5 mg chloramphenicol, 0.5 mg penicillin, 5 mg crystal violet, and 50 mg 2,3,5 chlorotriphenyltetrazole). The samples cultured onto this medium were incubated at 28 ∀C for 7 days.

After 7 days, colonies that looked like *R. solanacearum* (reddish color, mucoid, and amorphous) were transferred to SMSA and incubated for 4 days. Colonies confirmed as possibly positive were transferred to nutrient agar (20 g/L), incubated at 28 ∀C for 24 h, and inoculated onto plants of plantain variety Africa that had derived from meristem culture.

Inoculation: For inoculation, a bacterial suspension of 0.1 absorbance at 600 nm was prepared and 0.5 mL injected into each plant. The inoculated plants were incubated for 10 days in a humid chamber at 29

∀C during the day and 24 ∀C at night and relative humidity between 80% and 91%. From day 10 to day 30, records were made of those plants that produced typical symptoms of bacterial wilt. Detection by PCR was also carried out with specific primers, as described in Activity 2.

Indicator plants: Three methodologies were carried out for evaluations with indicator plants:

1. In one focus in "La Guaira" Farm, with two replications, 55 plantain plants were planted in an area of 4 × 4 m to act as indicators of the presence of *R. solanacearum* in the soil.

2. Because the bacterium was difficult to isolate from the soil, apparently because of its small population, 4 plantain plants were planted in each experimental unit 4 months after the trials were established.

3. Fegan and Prior (2005, *In*: Allen *et al*. (eds), Bacterial wilt disease and the *R. solanacearum* species complex, APS press, pp 449–461) reported phylotype II of *R. solanacearum* as being infectious in tomato and plantain. Although no other report is known in Colombia, a tomato growing in Montenegro (Quindío) was found to have symptoms of bacterial wilt, infected by the same phylotype of *R. solanacearum* pathogenic in plantain. Based on this report, tomato plants were also planted as indicators, on each site.

Results and Discussion

In direct form, the soil samples were detected as having very low levels of the bacterium, even in the check with no treatment. Hence, 1 mL of each of the base solution and 10^{-1} to 10^{-3} dilutions were mixed with 1 mL of liquid broth medium to enrich the bacterial population before culturing onto SMSA medium. Of the samples collected before applying the treatments, the bacterium was detected in two sites in each of "La Guaira" and "Cataluña" Farms.

At 1 and 5 months of applied treatments, the bacterium was detected in a check established on the "Cataluña" Farm. The presence of the bacterium in these samples was verified by inoculating plantain and amplifying through PCR.

At 4 months after establishing the trial in "La Guaira", one of the indicator plants (planted in 4 m square) manifested symptoms of bacterial wilt. This plant was located on the margin of a ditch that separated a check treatment with no applications.

After 8 months of applying the treatments in "Cataluña", four indicator plantain plants, planted on the site, were recorded as infected. These plants corresponded to two treatments with formalin and two controls, indicating a possible re-inoculation of the bacterium. In "La Guaira", no new infected plants were detected.