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*
- Progress obtained in yields by selecting tolerant lines for the *Empoasca kraemeri*

Rationale

A novel Double Congruity Backcross technique developed at CIAT has permitted the development of fertile interspecific *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 *Acanthoscelides obtectus* and *Empoasca kraemeri*. In 2002 and 2003 we identified several progenies containing both *P. vulgaris* and *P. acutifolius* cytoplasm with very high levels of antibiosis resistance to *A. obtecetus*. In 2004 and 2005, emphasis was placed on the reconfirmation of resistance in previously selected progenies.

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. Each test was evaluated in 5 repetitions. Infestation levels per variety were 2 to 3 mature eggs per seed. The percentage of emergence of adults and the days to emerge of adults were evaluated. In some cases, individual seeds were evaluated, using an infestation level of 2 ripe eggs per seed. Evaluations for resistance to *E. kraemeri* were done in the field under conditions of high levels of natural infestation. A randomized complete blocks design was used for this evaluation with 5 repetitions per genotype. The evaluation for resistance includes a damage score and bean production rating, insect counts, damage counts and in some cases, yield and yield components.

Results and Discussion

Acanthoscelides obtectus. In 2006, emphasis was placed upon the reconfirmation of resistance in previously selected progenies. Seeds of resistant hybrids were multiplied in

2005 in the greenhouse. These materials were then tested in nurseries with 10 repetitions. All the hybrids that had shown intermediate and high levels of resistance became susceptible, as can be seen in Table 2.1.1.

Table 2.1.1. Levels of resistance to *Acanthoscelides obtectus* in selected $F_6 – F_8$ 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_8	{[(G40022 x NI576)x V5] x A3} x VS42-7	95.0	38.5	Susceptible
GNVAV	200H5 F_8	{[(G40022 x NI576)x V5] x A3} x VS42-7	93.8	39.0	Susceptible
GVV	110G F_8	{[(G40022 x NI576)x V5] x A3} x VS42-7	93.8	65.6	Susceptible
GVV	108 N F_8	{[(G40022 x NI576)x V5] x A3} x VS42-7	93.9	40.2	Susceptible
BWG	1F7 F_6	BW-1 FL x GKA-12 F3 FB	93.4	40.2	Susceptible
BWG	1F14 F_6	BW-1 FL x GKA-12 F3 FB	83.5	40.3	Susceptible
BWG	1F18 F_6	BW-1 FL x GKA-12 F3 FB	85.3	40.9	Susceptible
BWG	5N1 F_6	BW-1 FL x GKA-12 F3 FB	89.1	39.6	Susceptible
BWG	5N4 F_6	BW-1 FL x GKA-12 F3 FB	91.0	39.2	Susceptible
BWG	6Y6 F_6	BW-1 FL x GKA-12 F3 FB	95.8	38.4	Susceptible
Checks					
G 40168		Susceptible *Phaseolus acutifolius* accession	88.1	39.4	Susceptible
G 40199		Resistance *Phaseolus acutifolius* accession	7.2	68.1	Resistant
G 25410		Susceptible *Phaseolus lunatus* accession	91.2	42.6	Susceptible
G 25042		Resistance *Phaseolus lunatus* accession	5.8	65.9	Resistant
ICA Pijao		Susceptible *Phaseolus vulgaris* cultivar	96.2	31.9	Susceptible

We evaluated 161 different progenies $F_3 – F_4$ obtained from interspecific crosses *Phaseolus vulgaris* x *P. acutifolius* with *P. vulgaris* cytoplasm.

In Table 2.1.2, 39 different genotypes are shown that have high levels of antibiosis to *A. obtectus*. The multiplication of seeds of the selected genotypes is in progress.

Table 2.1.2. Resistance to *Acanthoscelides obtectus* in pre-selected segregating F_3-F_4 hybrid progenies derived from interspecific *Phaseolus vulgaris* x *P. acutifolius* with *P. vulgaris* cytoplasm.

Code and generation	Cross	Percentage adult emergence	Days to adult emergence	Rating
	Hybrids			
DV-3B F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-6I) x RR-13L26Y]	47.7	53.2	Intermediate
DV-3C F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-6I) x RR-13L26Y]	17.5	55.0	Resistant
DV-3D F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-6I) x RR-13L26Y]	19.0	51.7	Resistant
DV-3G F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-6I) x RR-13L26Y]	45.9	54.3	Intermediate
DV-44D F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D) x STRR-4]	29.2	41.6	Intermediate
DV-45D F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D) x RGRT-2Y]	30.3	47.0	Intermediate
DV-48G F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D) x STRR-4]	39.2	36.5	Intermediate
DV-49 A F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D) x RGRT-2Y]	45.6	34.7	Intermediate
DV-49E F_3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D) x RGRT-2Y]	26.1	44.9	Intermediate
DV-50B F_3	[((((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N) x STRR-7]	47.5	40.4	Intermediate
DV-54C F_3	[((((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N) x STRR-7]	32.6	47.3	Intermediate
DV-81C F_3	[((((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D) x STRR-7]	32.2	43.0	Intermediate
DV-81G F_3	[((((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D) x STRR-7]	37.8	44.0	Intermediate
STZS-1A1	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	5.6	72.0	Resistant
STZS-1B4	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	0.0	N.E[a].	Resistant
STZS-1B5	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	20.0	64.0	Resistant
STZS-1C5	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	17.2	60.3	Resistant
STZS-1C6	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	1.9	46.0	Resistant
STZS-1G3	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	33.8	52.7	Intermediate
STZS-1G4	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	1.4	52.0	Resistant
STZS-1G5	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	16.7	50.8	Resistant
STZS-1G6	[(((((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-7N]	0.0	N.E.	Resistant
STZS-3B3	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant

Table 2.1.2. (Continued)

Code and generation	Cross	Percentage adult emergence	Days to adult emergence	Rating
STZS-3B4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	2.5	58.0	Resistant
STZS-3H1	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	10.7	59.3	Resistant
STZS-3H4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3J2	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	5.0	54.0	Resistant
STZS-3K3	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3K4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3K5	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	7.7	62.5	Resistant
STZS-3K6	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	11.5	56.7	Resistant
STZS-3M4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3M6	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	18.3	54.0	Resistant
STZS-3Q3	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	5.3	76.0	Resistant
STZS-3Q4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	11.0	51.0	Resistant
STZS-3Q5	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	6.4	46.0	Resistant
STZS-3S1	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	7.1	84.0	Resistant
STZS-3S2	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	17.8	44.8	Resistant
STZS-3S3	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3S6	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3U4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3U5	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3V2	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3V6	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3Y4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3Y5	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3Y6	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3Z3	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	11.9	66.5	Resistant
STZS-3Z4	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	15.0	53.3	Resistant

Table 2.1.2. (Continued)

Code and generation	Cross	Percentage adult emergence	Days to adult emergence	Rating
STZS-3Z5	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	0.0	N.E.	Resistant
STZS-3Z6	[(((G12922A x GNV10) x V5) x G25042) x DORSV1) x TZTTZ-24I) x ZX99-15.2.14.A) x STR-5D]	10.5	60.5	Resistant
	Checks			
G 40168[b]		85.2	39.8	Susceptible
G 25410[c]		85.8	43.7	Susceptible
ICA Pijao[d]		96.8	31.0	Susceptible
G 40199[e]		7.8	69.3	Resistant
G 25042[f]		4.4	73.6	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.

We also evaluated 55 different $F_3 – F_4$ obtained from interspecific crosses *Phaseolus vulgaris* x *P. acutifolius* with *P. vulgaris* cytoplasm. As shown in Table 2.1.3, 9 genotypes that have emergence levels similar to G40199, the original resistant parent and genotypes with intermediate resistance levels. Multiplication of selected genotype seeds are in progress.

Table 2.1.3. Resistance to *Acanthoselides obtectus* in pre-selected segregating F_3-F_4 hybrid progenies derived from interspecific *Phaseolus vulgaris* x *P. acutifolius* with *P. acutifolius* cytoplasm.

Code and generation	Cross	% adult emergence	Days to adult emergence	Rating
	Hybrids			
CWBB-18G33 F_4	[(((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BWZ-2F11S) x BWTZBB-2W16]	49.4	45.1	Intermediate
CWBBB-18C 8 F_4	[(((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BWTZBB-2U22) x BB-57U]	37.5	47.7	Intermediate
CWBBC-5D1 F_4	[(((((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BB-58L) x BWTZBB-2W16) x CWB-32J3]	11.2	61.8	Resistant
CWBBG-2A F_3	[(((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BB-58L) x BWTZBB-2W16) x GB-44N]	32.1	48.5	Intermediate
CWBBG-2C F_3	[(((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BB-58L) x BWTZBB-2W16) x GB-44N]	15.3	49.8	Resistant
CWBBG-2D F_3	[(((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BB-58L) x BWTZBB-2W16) x GB-44N]	21.7	50.2	Intermediate
CWBBG-7N F_3	[(((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BWTZBB-2W) x BWTZBB-2W14) x GB-44K]	31.8	56.4	Intermediate
CWBBG-22J 6 F_4	[(((((((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x CTZ-18) x ZXTGX-2D) x TZTE-9B) x BWG-12M) x BWG-20A) x RS-2) x BWTZBB-15Q) x BWTZBB-2W) x BWTZBB-2W14) x GB-44P]	13.3	59.3	Resistant
BBC-5D3 F_4	[((((((((((G40065 x NI576) x V5) x A6) x NGPNMNG-1) x KBNKN-3) x X-2) x T7K-2F281)x GKAX-9B) x UAC-3) x GKVZ-21B) x GKA-12F3) x BWTZBB-5) x CWBB-10]	30.1	54.1	Intermediate
ZX99-15Z-14AF$_3$	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x X-2) x G400199ZS]	28.3	56.1	Intermediate
ZX99-15Z-14B F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x X-2) x G400199ZS]	24.6	53.0	Intermediate
ZX99-15Z-14C F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x X-2) x G400199ZS]	7.2	60.0	Resistant
ZX99-15Z-14D F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x X-2) x G400199ZS]	19.2	58.7	Resistant
Z99ZX-1B-13 F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x G400199ZS) x ZX-11]	5.7	63.5	Resistant
Z99ZX-6A F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x G400199ZS) x ZX-11]	26.7	55.5	Intermediate
Z99ZX-11C F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x G400199ZS) x ZX-11]	14.1	58.6	Resistant
Z99ZX-11D F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x G400199ZS) x ZX-11]	32.7	39.7	Intermediate
ZX99-15-2-14 F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x X-2) x G400199ZS]	2.8	62.0	Resistant
ZX99-15-2-15 F_3	[(((((((G40065 x NI576) x V-5.4) x A-6) x NGPNMNG-1) x KBNKN-3) x X-2) x G400199ZS]	3.7	60.0	Resistant
	Checks			
G40168	Susceptible *Phaseolus acutifolius* accession	78.7	39.1	Susceptible
G40199	Resistance *Phaseolus acutifolius* accession	6.2	65.9	Resistant
G25410	Susceptible *Phaseolus lunatus* accession	91.7	41.8	Susceptible
G25042	Resistance *Phaseolus lunatus* accession	1.8	79.0	Resistant
ICA Pijao	Susceptible *Phaseolus vulgaris* cultivar	95.9	30.7	Susceptible

We evaluated different progenies obtained from interspecific crosses done in *P. lunatus*. These progenies $F_4 – F_5$ were selected in 2005 due to their high resistance levels to *A. obtectus*. With the exception of 2 progenies, all the rest showed high resistance to bruchids (Table 2.1.4).

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

Code and generation	No. of seeds tested	Percentage adult emergence	Days to adult emergence	Rating
		Hybrids		
V5 F_4	300	0.0	N.E.[a]	Resistant
V5 F_4	300	53.0	54.9	Susceptible
V5 F_4	300	2.9	61.7	Resistant
V5 F_4	300	0.2	83.0	Resistant
V5 F_4	300	0.0	N.E.	Resistant
V5 F_4	300	0.9	62.7	Resistant
V5 F_4	150	0.0	N.E.	Resistant
V5 F_4	210	5.0	54.4	Resistant
V5 F_4	210	2.7	64.3	Resistant
V5 F_4	300	5.9	53.5	Resistant
V5 F_4	210	22.0	57.3	Intermediate
$A6F_5$	150	46.9	46.8	Intermediate
$A6F_5$	210	54.7	47.7	Susceptible
		Checks		
G 40168[b]	300	79.4	40.3	Susceptible
G 25410[c]	300	93.3	43.1	Susceptible
ICA Pijao[d]	300	93.2	30.9	Susceptible
G 40199[e]	300	9.4	70.2	Resistant
G 25042[f]	300	5.7	67.8	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.

Zabrotes subfasciatus (Mexican bean weevil)

The improvement method using backcrosses that combine biochemical tests to confirm the presence of arcelin and insect feeding bioassays have had satisfactory results whenever the resistance in bean cultivars should want to be incorporated in the Mexican bean weevil (*Z. subfasciatus*). Preliminary observations (see Annual Report 2005) suggested that the incorporation of arcelin in bean genotypes to develop bruchid resistance affects the reduction of yields. To confirm this hypothesis, in different field tests under field conditions at CIAT's headquarters, we conducted two trials with RAZ lines and one trial with families of Andean red mottled bush (with the presence or not of arcelin), and with their respective recurrent parents.

Table 2.1.5 shows that the incorporation of arcelin into RAZ lines has a negative effect on the yield. Most RAZ lines tested yielded less than their respective recurrent parents. Differences in most cases were not significant, however, not less important. A similar situation is shown in Table 2.1.6, where lines obtained from families derived from the

same recurrent parent (A36) suggest that the incorporation of arcelin does have a depressing effect on yields.

Table 2.1.5. Yields of two trials of RAZ lines and corresponding 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 ∂ respect to recurrent parent		Significance respect to recurrent parent
			(Kg/ha)	Percentage	
RAZ 153	ICA Pijao	2575	414	13.9	ns[a]
RAZ 154	ICA Pijao	2532	457	15.3	ns
RAZ 155	ICA Pijao	2536	453	15.2	ns
RAZ 156	ICA Pijao	2503	486	16.3	ns
RAZ 157	ICA Pijao	2657	332	11.1	ns
RAZ 158	ICA Pijao	2549	440	14.7	ns
RAZ 159	ICA Pijao	2665	324	10.8	ns
RAZ 160	ICA Pijao	2791	198	6.6	ns
RAZ 161	ICA Pijao	2603	386	12.9	ns
RAZ 162	ICA Pijao	2706	283	9.5	ns
RAZ 163	ICA Pijao	2750	239	8.0	ns
RAZ 164	ICA Pijao	2263	726	24.3	*
RAZ 165	ICA Pijao	3020	+31	1.0	ns
RAZ 166	ICA Pijao	2735	254	8.5	ns
Mean backcrosses to Pijao		2635			
ICA Pijao		2989			
RAZ 151	EMP 250	2566	21	0.8	ns
EMP 250		2587			
RAZ 190	TALAMANCA	2682	0	0	ns
TALAMANCA		2682			

[a] ns, not significant; *,significant at the 5% level by Dunnett's test. For comparing all treatment means with the mean of a control. CV = 16.1%.Rep = 8.

Table 2.1.6. Yield of Andean red mottled bush bean line families and corresponding recurrent parents. Families are selected for the presence (resistance) or absence (susceptibility) of arcelin to the Mexican bean weevil.

Identification	Crosses	Rating	Yield (kg/ha)	Difference ∂ respect to recurrent parent		Significance respect to recurrent parent
				(Kg/ha)	Percentage	
3750-51	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	2280	210	8.4	ns[a]
3754-55	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	2406	84	3.4	Ns
3759-60	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	2579	+89	3.6	Ns
3771-72	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	2415	75	3.0	Ns
3775-76	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	3311	+821	33.0	*
3777-78 6MClar	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	2405	85	3.4	Ns
3777-78 6MOsc	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	2582	+92	3.7	Ns
3786-87	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Resistant	2471	19	0.8	Ns
3810-11	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2543	+53	2.1	Ns
3814-15	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2932	+442	17.8	Ns
3833-34	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2196	294	11.8	Ns
3859-60	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	1931	559	22.4	Ns
3896-97	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2203	287	11.5	Ns
3908-	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2300	190	7.6	Ns
3911-12	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2547	+57	2.3	Ns
3920-21	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2550	+60	2.4	Ns
3929-30	A 36 x (A 36 x ((RAZ 44 x ROYAL RED)x(CATRACHITA x WILK 2)))	Susceptible	2107	383	15.4	Ns
	Mean backcrosses to A 36		2456			
A36	Checks (recurrent parents)	Susceptible	2490			
RAZ 44	Check	Resistant	2804	+314	12.6	Ns
	Checks (no recurrent parents)					
ROYAL RED		Susceptible	2260	230	9.2	Ns
CATRACHITA		Susceptible	3019	+529	21.2	Ns
WILK 2		Susceptible	1762	728	29.2	Ns
ICA Pijao	Check	Susceptible	3392	+902	36.2	Ns

[a] ns, not significant; *,significant at the 5% level by Dunnett's test. For comparing all treatment means with the mean of a control. CV =7.3%, LSD = 730.5.

Another case to confirm the hypothesis of arcelin is when it is incorporated to a family without making a backcross to it (A483), as seen in the results on Table 2.1.7, where yields continue decreasing in an important way, although not statistically significant.

Table 2.1.7. Yield of Andean red mottled bush bean line families and corresponding parents. Families are selected for the presence (resistance) or absence (susceptible) of arcelin to Mexican bean weevil.

Identify	Crosses	Rating	Yield (kg/ha)	Difference ∂ respect to recurrent parent		Significance respect to female parent
				(Kg/ha)	Percentage	
3795-96	A 483 x ((MAR 1 x RAZ 50)x (PVA 9576-34-1 x G 17340))	Resistant	2689	716	21.0	ns[a]
3797-98	A 483 x ((MAR 1 x RAZ 50)x (PVA 9576-34-1 x G 17340))	Resistant	2799	606	17.8	Ns
3799-38	A 483 x ((MAR 1 x RAZ 50)x (PVA 9576-34-1 x G 17340))	Resistant	2814	591	17.4	Ns
3802-03	A 483 x ((MAR 1 x RAZ 50)x (PVA 9576-34-1 x G 17340))	Resistant	3542	+137	4.0	Ns
3808-09	A 483 x ((MAR 1 x RAZ 50)x (PVA 9576-34-1 x G 17340))	Resistant	2747	658	19.3	Ns
3793-94	A 483 x ((MAR 1 x RAZ 50)x (PVA 9576-34-1 x G 17340))	Susceptible	3225	180	5.3	Ns
	Mean of crosses derivates of A 483		2969			
A 483	Checks (female parent)	Susceptible	3405			
RAZ 50	Check	Resistant	3722	+317	9.3	Ns
	Checks (male of two simple cross parents)					
MAR 1		Susceptible	3144	261	7.7	Ns
G 17340		Susceptible	2720	685	20.1	Ns
ICA Pijao	Check	Susceptible	3384	21	0.6	Ns

[a] ns, not significant; *,significant at the 5% level by Dunnett's test. For comparing all treatment means with the mean of a control. CV = 6.6%, LSD = 913.9

Empoasca kraemeri (Leafhopper)

Yield tests were conducted using EMP resistant lines and old EMP lines with different seed color. These were compared with commercial varieties to evaluate if there was any change in relation to yield, throughout different recurrent selection cycles; EMP lines developed tolerance, which expresses the ability of the genotype to have better yields than a susceptible one at all infestation levels of the leafhopper.

Table 2.1.8 shows that the means of EMP lines, when are subject to high levels of *Empoasca kraemeri* (non-protected), they are significantly higher than the media of commercial lines, showing a better response and reproductive adaptation, having less loss percentage than the commercial varieties.

Table 2.1.8. Yields of selected EMP lines and commercial varieties tested for tolerance to *Empoasca kraemeri.*

Line or variety	Seed color	Damage score[a]	Reproductive adaptation scores[b]	Yield (kg/ha)		% yield loss	Susceptibility index[c]
				Protected	Non-protected		
EMP 103	Yellow	7.3	4.6	1695	1226	27.7	0.91
EMP 209	Cream	6.0	6.2	1896	1315	30.6	0.92
EMP 226	Red	6.4	6.0	1782	1325	25.6	0.87
EMP 231A	Red	6.5	4.4	1571	1066	32.1	1.04
EMP 253	Red	7.6	2.6	923	462	49.9	1.68
EMP 270	Red	8.0	2.0	748	397	46.9	1.82
EMP 279	Cream	7.6	5.6	1696	1301	23.3	0.83
EMP 316	White	6.5	6.8	1570	1357	13.6	0.70
EMP 406	Carioca	6.1	6.4	2227	1398	37.2	0.98
EMP 409	Carioca	5.8	6.8	2063	1387	32.8	0.93
EMP 413	Carioca	5.9	7.6	2341	1627	30.5	0.85
EMP 436	Carioca	6.4	6.4	1614	1243	23.0	0.85
EMP 443	Carioca	6.6	7.2	2011	1397	30.5	0.90
EMP 449	Carioca	6.2	7.8	1849	1365	26.2	0.85
EMP 486	Red	5.9	6.6	2213	1454	34.3	0.94
EMP 514	Carioca	6.6	6.2	2252	1311	41.8	1.07
EMP 515	Carioca	6.5	7.0	1762	1527	13.3	0.65
EMP 537	Red	7.4	5.6	1783	921	48.3	1.26
EMP 541	Red	6.0	5.8	2011	1215	39.6	1.04
EMP 571	Cream	5.9	6.6	2168	1507	30.5	0.87
EMP 585	Carioca	6.3	6.8	1994	1284	35.6	0.99
EMP 588	Red	6.4	5.8	2311	1662	28.1	0.81
Mean EMP lines		6.5	5.9	1840 bc	1261 a	31.5 c	-
Commercial varieties checks							
Carioca	Carioca	7.2	5.0	2000	1142	42.9	1.12
Exrico 23	White	8.0	5.4	1915	1034	46.0	1.21
BiBri	Red	7.9	4.4	1539	951	38.2	1.15
FEB 115	Cream	7.0	5.4	1984	1015	48.8	1.23
Mean commercial varieties		7.5	5.1	1860 b	1036 b	44.3 b	-
ICA Pijao	Black	6.7	6.4	2191 a	1434 a	34.6 bc	0.93
BAT 41[d]	Red	8.8	2.8	1609 c	641 c	60.2 a	1.52
CV(%)		5.7	9.5	13.8	14.3	-	-
LSD%		0.5	0.7	319	218	-	-

[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. Mean within a column followed by different letters are significantly different, separation by Schaffé's *F* method of significance testing for arbitrary linear contrasts with 139 *df* ($P < 0.05$).

Activity 2.2. Screening for sources of resistance to major insect pests

Contributors: J. M. Bueno, J. F. Valor and C. Cardona.

Highlight:

- New accessions and lines with insect resistance were identified

Rationale

Identification of sources of resistance to major insect pests of beans is a continuous activity. Additional work is conducted trying to identify and characterize the mechanisms of resistance to specific major pests.

Materials and Methods

Bruchids nurseries are tested in the laboratory simulating normal storage conditions (20ºC. 80% R.H. and 14% seed humidity). Genotypes are tested using 3-5 replications of 50 seeds per genotype. Evaluation units (replicates) are infested with seven couples of *Z. subfasciatus* per each 50 seeds or two eggs per seed in the case of *A. obtectus*. *T. palmi*, leafhopper and pod weevil nurseries are planted 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 score and bean production ratings, insect counts, damage counts and in some cases, yield components and yields.

Results and Discussion

***Acanthoscelides obtectus* (Storage weevils)**

The search for sources of resistance to *A. obtectus* continued in 2006. No useful sources of resistance to *A. obtectus* were found among 25 wild *P. vulgaris* and 20 *P. acutifolius* accessions tested.

***Empoasca kraemeri* (Leafhopper)**

No useful sources of resistance to the leafhopper were found among about 200 accessions of bean germplasm evaluated in 2006. In trials done under field conditions in CIAT's headquarters, mesoamerican parents with different resistance sources were evaluated with 378 lines in replicate nurseries. From these, 28 were selected to be evaluated again and confirm their resistance in 2007.

Activity 2.3. Screening for virus resistance transmitted by *Bemisia tabaci* biotype B in snap beans

Contributors: P. Sotelo, J. M. Bueno, C. Cardona, M. Castaño, F. Morales and S. Beebe

Highlight:

- Conducted successful screening for sources of resistance to the new virus disease affecting snap beans

Rationale

In Colombia's Cauca Valley region, the area planted with beans and snap beans has been drastically reduced. This is caused by the bean-leaf crumple virus, a Begomovirus transmitted by *Bemisia tabaci* biotype B. It is urgent to develop varieties of snap beans that resist the virus to replace the "Blue Lake" variety that is preferred in the zone. It is also necessary to know the inheritance mechanism of this virus in order to develop an improvement method.

Materials and Methods

With the cooperation of the Bean Improvement Section and the Virology Unit, 148 genotypes were evaluated. These genotypes are backcrosses derived from families G17723 x EAP9510-77 and G17723 x TIO CANELA 75 with snap bean characteristics of resistance to the new virus. The nurseries were planted in the "La Tupia" and "Pradera" zones with a high incidence of virus and high natural levels of *Bemisia tabaci* biotype B. In addition, in this zone we study the inheritance of the resistance to the new Begomovirus in snap beans in the Cauca Valley. For this study, we obtained simple crosses of a susceptible parent (Blue Lake) with a resistant parent (Porrillo Sintético). We also obtained populations (F_1, F_2 and backcrosses with susceptible and resistant parents) to be evaluated in a randomized complete block design in 3 repetitions. Evaluations were done to each plant on the 30^{th} and 50^{th} day after planting. The symptoms of the virus were evaluated using a visual scale from 1 to 5 (1 = no apparent damage; 5 = severe damage), dwarfism (stunts) and pod deformation.

Results and Discussion

At first in generation F_2, we evaluated all the obtained genotypes from families G17723 x EAP951077 and G17723 x TIO CANELA 75. We selected and harvested 8 genotypes from the crosses G17723 x EAP951077 and 32 genotypes from the cross with the TIO CANELA 75 parent. They were planted again in F_3 and 9 individual selections were done in 4 genotypes derived from the cross with the TIO CANELA 75 parent. These materials will continue being evaluated. In the inheritance study, the analysis of variance

and media separation for each evaluated characteristic showed that the scores for resistant and susceptible parents are highly contrasting and that the backcrosses show a tendency to score in a similar way to the parents that had been backcrossed (Table 2.3.1).

Table 2.3.1. Media differences in population derived from the Blue Lake x Porrillo Sintético cross for each evaluated characteristic.

Generation	No. plants	Mosaic[a]	Leaf deformation[b]	Dwarfism[c]	Pod deformation[d]
L.A. (S)	122	4.20 a[e]	4.10 a	3.54 a	4.73 a
L.A.x (L.A.xP.S.)F_1	253	3.13 b	3.04 b	2.89 b	4.51 b
L.A.x P.S. F_1	148	3.09 b	2.86 b	2.57 c	3.60 c
L.A.x P.S. F_2	243	2.52 c	2.14 c	1.84 d	3.48 c
P.S.x (L.A.xP.S.)F_1	323	2.19 d	1.55 d	1.38 e	2.96 d
P.S.(R)	150	2.09 d	1.27 e	1.22 e	2.57 e

The evaluation scores are as follows; [a]1= no presence of mosaic, 9 = mosaic in all the plant; [b]1 = No evident damage, 9 = deformation of all the leaves; [c]1 = normal development of the plant, 9 = severe stunting; [d]1 = Undamaged pods, 9 = severe deformation of most of the pods; [e]means within a column followed by the same letter are not significantly different *df*= 1152, (P<0.05).

A genetic analysis was also done with the media and generational variances method by the means of the statistical program Genes, calculating the phenotypic and genotypic environment variances as well as the inheritance in an ample and narrow meaning, which is very useful information to improvers (Table 2.3.2).

Table 2.3.2. Genetic parameters of F_2 variances derived from the Blue Lake x Porrillo Sintético cross for each evaluated characteristic.

Parameter	Mosaic	Leaf deformation	Dwarfism	Pod deformation
Phenotypic variance	0.589	1.289	1.32	1.102
Environmental variance	0.628	0.698	0.548	0.500
Genotypic variance	-0.039	0.592	0.585	0.602
Additive variance	0.439	1.053	0.832	1.293
Ample inheritability (%)	-6.578	45.89	51.622	54.652
Narrow inheritability (%)	74.569	81.65	73.438	117.33
Amount of genes	2.560	1.90	2.504	1.546

The analysis estimated the amount of genes involved in the expression of resistance to the virus.

In addition to the study, 7 individual selections were done in F_1, 18 in F_2, 3 in backcrosses towards the susceptible parent and 32 in backcrosses towards the resistant

parent. These materials will continue being evaluated and backcrossed again if necessary.

Activity 2.4. Evaluation of *Brachiaria* hybrids for resistance to *Rhizoctonia solani* under field conditions in Caqueta

Contributors: G. Segura, W.Mera, X. Bonilla, J. Miles, S. Kelemu

Rationale

Rhizoctonia foliar blight, caused by *Rhizoctonia solani* Kühn, is an important disease on a wide range of crops around the globe. 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).

R. solani is a basidiomycete fungus that does not produce any asexual spores (called conidia). In nature, the fungus reproduces mainly asexually and exists as vegetative mycelia and/or dense sclerotia. In the absence of a susceptible host, these sclerotia, that are irregular-shaped, brown to black structures, can survive in soil and on plant debris for several years. The fungus can also survive as mycelia by colonizing soil organic matter as a saprophyte. When a susceptible host is available, sclerotia can germinate and produce hyphae that can infect host plants. The fungus is a very common soil-borne pathogen that primarily infects below ground plant parts in a great diversity 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. As symptoms progress, 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. A number of constitutive factors including cell wall calcium content, and cuticle thickness may contribute to resistance. Other factors expressed after infection also play a role in resistance. These components of resistance may also be influenced by factors such as age and maturity of the plant as well as other external factors such as plant nutrition and environmental conditions (e.g. field vs controlled environmental growth conditions). 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. Measurement of resistance is based on quantification of disease symptoms or the growth and expansion of the pathogen on its host. 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: Two-hundred nine *Brachiaria* genotypes (127 with BR05 series and 82 with RZ 05 series) 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^{o}C and an annual rainfall of 3793 mm.

Field layout, artificial inoculations and disease evaluations: Twelve 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 2 m between blocks. The entries were replicated 4 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, 25, 35, 45, 55 and 65 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 in susceptible genotypes 10-15 days after inoculations. There was a high degree of correlation in disease evaluation data among the various evaluation dates.

The resistant control CIAT 16320 was consistently evaluated at scale less than 2. Ten genotypes, RZ05/3635, BR05/0262, RZ05/2721, RZ05/3551, RZ05/3634, RZ05/3738, RZ05/2738, RZ05/2919, RZ05/3394, and RZ05/3575 were evaluated at an average between 2.0 and 2.5. Eighty-one others, R05/0048, BR05/0377, BR05/0555, BR05/0591, BR05/1482, CIAT 36087, BR05/0753, BR05/0760, BR05/0777, BR05/1359, RZ05/2699, RZ05/2816, RZ05/2842, RZ05/3021, RZ05/3362, RZ05/3397, RZ05/3405, BR05/0156, BR05/0537, RZ05/3063, BR05/0114, BR05/0115, BR05/0118, BR05/0379, BR05/0629, BR05/0714, BR05/0744, BR05/0913, BR05/0914, BR05/1455, RZ05/2682, RZ05/2764, RZ05/2942, RZ05/3173, BR05/0071, BR05/0092, BR05/0408, BR05/0549, BR05/0586, BR05/0701, BR05/1352, RZ05/3226, RZ05/3343, RZ05/3472, RZ05/3524, RZ05/3579, BR05/0731, BR05/0746, RZ05/3361, RZ05/3645, BR05/0303, RZ05/0462, RZ05/3158, RZ05/3541, RZ05/3576, RZ05/3434, BR05/0605, BR05/0707, BR05/1467, BR05/1717, RZ05/2838, BR05/0830, BR05/1433, BR05/1434, BR05/1469, BR05/1494, BR05/1865, BR05/1872, RZ05/2786, RZ05/3106, RZ05/3262, RZ05/3335, RZ05/3452, RZ05/3483, RZ05/3539, BR05/0150, BR05/1149, RZ05/2937, RZ05/3630, BR05/0475, BR05/0561 scored with an average rating scale of 2.6-2.9. All remaining 118 materials, BR05/1830, BR05/0637, BR05/0642, BR05/0933, BR05/1440, BR05/1609, BR05/0406, BR05/0733, BR05/1449, BR05/0265, BR05/1401, RZ05/2938, RZ05/2985, BR05/1853, RZ05/3101, RZ05/3332, BR05/1879, RZ05/3371, RZ05/3528, RZ05/3608, RZ05/3333, RZ05/3590, BR05/1426, BR05/1462, BR05/1611, RZ05/2932, BR05/0508, BR05/0545, BR05/0671,

BR05/0708, BR05/1460, RZ05/2801, RZ05/2847, RZ05/3107, RZ05/3355, RZ05/3574, BR05/0120, BR05/0284, BR05/0351, BR05/1574, BR05/1706, RZ05/3128, RZ05/3312, RZ05/3495, BR05/0117, BR05/0334, BR05/0609, BR05/0931, BR05/1464, RZ05/3244, RZ05/3466, BR05/0702, BR05/1302, RZ05/2802, RZ05/3485, RZ05/2992, BR05/0990, BR05/1520, BR05/1857, RZ05/3365, BR05/0354, BR05/0743, BR05/1173, BR05/1308, BR05/1402, BR05/1447, BR05/1623, RZ05/3589, BR05/1249, BR05/1586), BR05/0577, BR05/0627, BR05/0995, BR05/1344, BR05/1435, BR05/1444, BR05/1475, BR05/1738, RZ05/2831, RZ05/3311, BR05/0563, BR05/1361, BR05/1420, BR05/1479, BR05/1835, BR05/0244, BR05/0267, BR05/0891, BR05/1019, BR05/1429, BR05/1826, BR05/1493, BR05/1702, RZ05/3377, BR05/1376, BR05/0020, BR05/0159, BR05/0293, BR05/1040, BR05/1480, RZ05/3359, BR05/0209, BR05/1490, RZ05/2668, RZ05/2873, RZ05/3253, BR05/1331, BR05/1610, BR05/1883, RZ05/3398, CIAT 36061, RZ05/2641, RZ05/3378, RZ05/3391, RZ05/3367, RZ05/3629, BR05/1059, BR05/1647, RZ05/3585, RZ05/3616 scored between 3.0-5.0. Figure 2.3.1 shows a graphical representation of the results using data from representative genotypes from each of these groups.

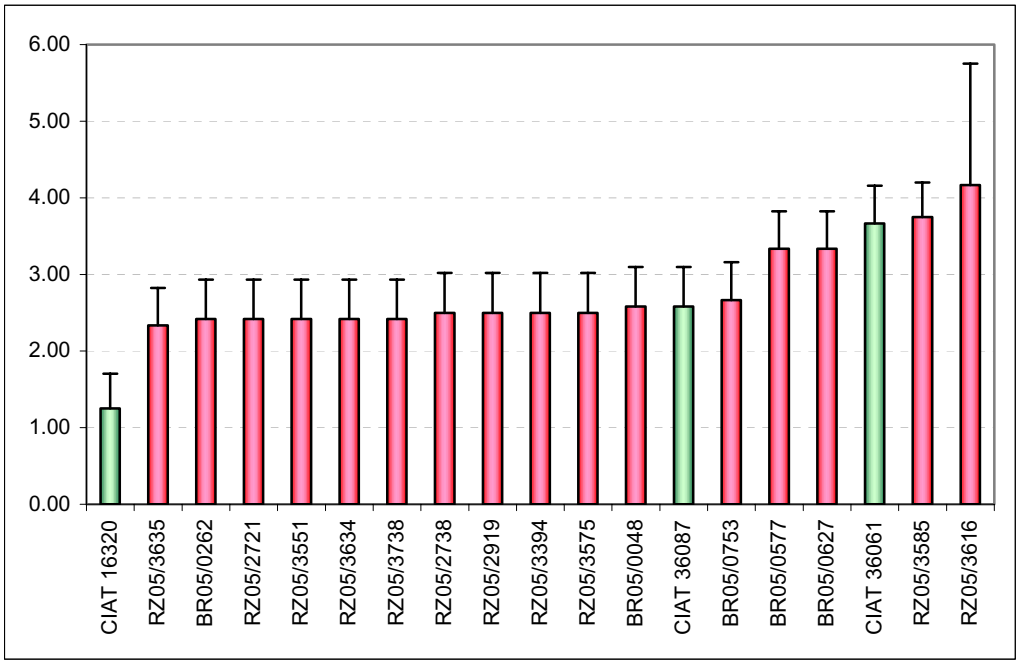

Figure 2.4.1. Ratings of *Brachiaria* genotypes for foliar blight disease reaction on a 1-5 scale 65 days after inoculations with sclerotia of *Rhizoctonia solani* under field conditions, Caquetá, Colombia. Bars indicate standard deviation.

The disease evaluation data taken 65 days after inoculations represented well-developed disease symptoms that correlated well with data taken at various dates.

Activity 2. 5. Bacterial endophytes in *Brachiaria*

Contributors: J. Abello, P. Fory and S. Kelemu

Rationale

Bacterial endophytes are known to reside in plant tissues without doing harm to their host. These bacteria are often isolated either from surface-sterilized tissues or extracted from internal plant parts. They can enter plants mainly through the root zone, although other plant parts such as stems, flowers and cotyledons can also be entry points. In general, many of the entry points for pathogenic bacteria can serve the same purpose for the endophytic ones. Several different endophytic bacteria may reside within a single plant. These endophytes may either remain localized at their entry points or spread in other parts of the plant. Various bacterial endophytes have been reported to live within cells, in the intercellular spaces or in the vascular system of various plants. Although variations in the endophyte populations have been reported in various plants depending on a number of factors, generally bacterial populations are higher in roots and decrease in stems and leaves.

Several endophytic bacteria have been reported to enhance growth and improve plant health in general (Sharma and Novak, 1998. Can. J. Microbiol. 44:528-536; Stoltzfus et al., 1998. Plant Soil 194:25-36). Many plant-growth-promoting bacteria (PGPB) that include a diverse group of soil bacteria are thought to stimulate plant growth by various mechanisms such as plant protection against pathogens, providing plants with fixed nitrogen, plant hormones, or solubilized iron from the soil.

Endophytic bacteria that reside in plant tissues without causing any visible harm to the plant have been isolated from surface-sterilized *Brachiaria* tissues. Three bacterial isolates 01-36062-R2, 02-36062-H4, and 03-36062-V2 were isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively, that tested positive for sequences of the *nif*H gene (the gene that encodes nitrogenase reductase) [IP-5 Annual Reports 2003, 2004]. Because nitrogen fixation is performed by diverse groups of prokaryotic organisms, detection of a marker gene that is unique and is required for nitrogen fixation may be useful to conduct our studies. The *nifH* gene has been used with a number of PCR primers that amplify the gene from microbes and other samples by a number of researchers.

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. This work describes the establishment of a transformation protocol and expression of the green fluorescent protein (GFP) gene in an isolate of a bacterial endophyte associated with species of *Brachiaria*. The purpose of this study is to evaluate the use of GFP in host-parasite interactions.

Materials and Methods

Bacterial isolate and growth conditions: a bacterial isolate designated as CIAT 36062R2 (PE 1 Annual Report 2005), isolated from roots of *Brachiaria* hybrid CIAT 36062, was marked for antibiotic resistance (rifampicin, rif^r). This isolate tested positive for *nif*H gene (the gene that encodes nitrogenase reductase) sequences (IP-5 Annual Report 2005; Kelemu *et al*., 2006, Phytopathology 96:S59) Bacterial cells were collected from a single colony and cultured on Luria agar medium containing rifampicin (LB; tryptone 10 g/l, NaCl 5g/l, yeast extract 5 g/l and agar 15 g/l; rifampicin 50 σg/ml) and incubated at 28 ^{o}C for 24 hours in darkness.

Plasmid: Plasmid pGT-Kan was kindly provided by Dr. Steve Lindow of the University of California, Berkeley. pGT-kan was constructed using plasmido pPROBE-GT (Miller *et al*., 2000, Molecular Plant-Microbe Interactions 13: 1243-1250) as a base and it contains gfp under the promoter *nptII* and confers resistance to Kanamycin as well as gentamycin.

Transformation of the bacterial endophyte CIAT 36062R2: E. coli strain DH5Ŭ was electrotransformed with the plasmid pGT-kan for maintenance of the plasmid. CIAT 36062R2/rif^r was electrotransformed using a protocol described by Dulk-Ras and Hooykaas (1995, Methods Molecular Biology. 55: 63-72) with some modifications. To prepare competent bacterial cells, the cells were grown in LB medium at 28^{o}C with shaking at 250 rpm for 16 hours till a growth density of OD_{600}= 0.5. The cells were collected after centrifugation at 4,000 rpm, 4^{o}C for 15 minutes. The cells were rinsed three times with 20 ml solution of 10% glycerol and 1mM HEPES (pH: 7.0). They were then resuspended in 3 ml of 10% glycerol, 200 σl aliquots were made and stored at -80^{o}C for subsequent use. Electroporation was conducted using a BIO-RAD® gene pulser at 12,5 Kv/cm, 200 T of resistance and 25 σF of capacity. Forty σl of competent cells were mixed with 100ng/σl of plasmid pGT-kan and electric pulse was applied to the mixture. The cells were then transferred to a 1 ml LB medium and incubated for 3 hours at 28^{o}C. One hundred σl of this culture was plated on Luria agar plates containing 50 σg/ml rifampicin and 15 σg/ml of gentamycin for selection of transformants. Putative transformants appeared on the selection plates after 48 hours of incubation.

PCR analysis of bacterial transformants: Genomic DNA was isolated from putative transformants using a protocol described by Cheng *et al.* (2006, Biotechnology Letters. 28: 55-59.). Identification of GFPmut1 gene in transformants was conducted using specific primers T14GFP5′ (5′ATTCCCTAACTAATAA-TGATTAACTTTATAAGGAGGAAAAAC 3′) and T1GFP3′ (5′ GATGCCTGGA-ATTAATTCCTATTTGTATAGTTCATCC 3′) (Miller *et al*., 2000, Molecular Plant-Microbe Interactions. 13: 1243-1250). Amplifications were carried out in a Programmable Thermal Controller (MJ Research, Inc) programmed to 30 cycles comprised of I minute denaturation step at 95^{o}C (3 minutes for the first cycle), followed by 2 min at 50^{o}C, and primer extension for 3 minutes (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.

Plant inoculation: Tillers of about a month old were prepared from a single mother plant of *Brachiaria* hybrid CIAT 36061 (cv. Mulato), their roots washed with sterile distilled water and made ready for inoculations. The roots of these tillers were immersed in a beaker containing 200 ml of bacterial (transformant 36062R2/gfp) suspensions. All plants were kept immersed for 48 hours, after which they were removed and rinsed 3 times with sterile distilled water. They were then each transplanted to pots containing sterile sand and soil in 3:1 proportion and maintained in the greenhouse under natural daylight and at temperatures between 19 and 30°C. At 1, 2 3 and 5 months after inoculations, tissue samples were taken and examined under the microscope.

Test for stability of bacterial transformants: Transformant colonies were isolated and plated on Luria agar media without selection antibiotics and subsequently transferred for several cycles on media without selection pressure. These colonies were then examined for expression of GFP.

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. For observations of GFP expressions inside plant tissues, young roots and leaves were sectioned with diameters of approximately 0.5-1.5 mm

Results and Discussion

Transformation of endophytic bacterium CIAT 36062R2/rif^r*:* Putative transformants appeared on selection plates after 48 hours of incubation. Colonies with a diameter of approximately 1-mm were isolated for analysis. Bacterial cells grown to an optical density (OD_{600}) = 1.0 were examined for green fluorescence. All cells examined demonstrated strong fluorescence indicating successful expression of gfp. Control colonies showed no fluorescence. 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.

PCR analysis of putative transformants: The putative bacterial transformants selected on the selection media were further examined using fluorescence microscope, and PCR analysis. DNA isolated from these transformants was examined for gfp sequences using PCR analysis. Transformants that contain gfp gene sequences produced an amplified DNA product of 750 bp-size, confirming successful transformation of endophytic bacterial cells with gfp. Negative controls produced no amplified product. The PCR method allowed us to quickly examine and further confirm putative transformants that have been selected on antibiotic selection media.

Test for stability of transformants: Selected bacterial transformants were cultured sequentially 15 times on media without selection antibiotics. Although stable in expression of gfp, the fluorescence intensity declined after the 9th transfer on media without the selection pressure for some of the transformants. This indicates that the gene of interest was not incorporated with the bacterial genome in some of these colonies that showed a decline in fluorescence intensity when maintained on media without antibiotic selection.

Microscopic examination: Microscopic examinations of selected bacterial transformants demonstrated strong expression of *gfp* as evidenced by the intense fluorescence emission at a range of wavelength (Figure 2.5.1; Figure 2.5.2) he strongest emission was observed at a 355-425 nm range with Leica D filter. The emission intensity was somewhat lower when a filter Leica H3 was used with a 420-490 nm range.

Root and leaf tissues from *Brachiaria* plants inoculated with endophytic bacterial cells transformed with gfp were examined under the microscope at 1, 2, 3 and 5 months after inoculations. Bacterial cells were localized in intercellular spaces. No fluorescent bacteria were observed in young leaves during the period of evaluations. It is possible that the transformed bacteria largely remained localized in the root zone within the period of the evaluations.

Although the transformation protocol functioned well for the endophytic bacteria, the recombination of the introduced gene to that of the bacterial genome was not evident, as the transformed bacteria lost their green fluorescence with time. Preliminary data showed that *Brachiaria* tissues taken from plants inoculated with GFP-transformed bacterial endophytes expressed fluorescence emission (Figure 2.5.1) This will allow us to study the endophyte-*Brachiaria* interaction, endophyte distribution within the plant tissue, and the correlation of endophytic bacterial colonization with *Brachiaria* plant growth and other related benefits.

Although various transformation systems have been developed and reported for many microbes, successful application of the technology is still not routine in many species. Furthermore, developing an efficient transformation system for a previously untransformed microbe can be a technical obstacle. This work describes the transformation and expression of the GFP-encoding gene in an isolate of an endophytic plant growth promoting bacterium associated with species of *Brachiaria*. To the best of our knowledge, this is the first report on transformation of this endophytic bacterium. This is also the first report of a plant growth promoting endophytic bacterium associated with *Brachiaria* that contains *nif*-gene sequences.

Figure 2.5.1. *Brachiaria* tissues from plants inoculated with bacterial endophyte transformed with green fluorescent protein gene *egfp*). a) fluorescence emission under UV light with Leica H3 filter, b) under normal lighting, c) fluorescence emission under UV light with Leica D filter.

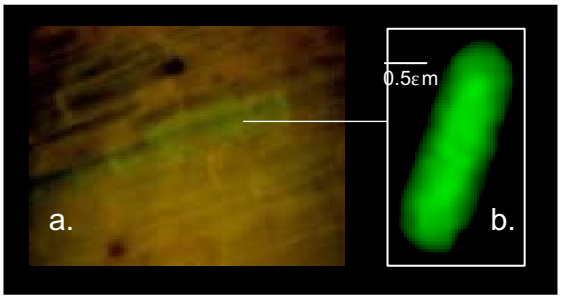

Figure 2.5.2. Endophytic bacterial cells transformed with a gfp gene. a) initial colonization of *Brachairia* roots inoculated with transformed bacterial cells one month after inoculation; b) a single bacterial cell (average length of 2,5 εm). Photographed with Leica D filter.

Activity 2. 6. Endophytic plant growth promoting bacteria associated with *Brachiaria*

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

Rationale

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

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

In 2005 Annual Report, we demonstrated the effect of endophytic bacteria on the growth of *Brachiaria* hybrid CIAT 36061 (cv. Mulato). *Brachiaria* hybrid CIAT 36061 had indigenous endophytic bacteria that are difficult to eliminate. Because of the difficulty to eliminate these indigenous bacteria, we set out to introduce three different strains of bacteria, originally isolated from *Brachiaria* hybrid CIAT 36062, into CIAT 36061, in addition to the indigenous bacteria that this hybrid already has. In general, the introduction of these bacteria had a positive effect on plant growth and development in the recipient plant CIAT. More tiller and root development were observed in artificially inoculated CIAT 36061 plants than plants containing only indigenous endophytic bacteria.

In nitrogen- and other nutrient-deficient conditions, *Brachiaria* plants inoculated with the three bacterial strains had significantly higher average values in all evaluated parameters (with the exception of soluble proteins in leaves) than those control plants containing just indigenous bacteria (PE-1 Annual Report 2005).

Analysis of variance showed that the total biomass production (leaf, stem and root) collected from control *Brachiaria* CIAT 36061 plants was significantly ($P< 0.05$) less than that from inoculated ones (PE -1 Annual Report 2005). The data presented indicate that a close and beneficial interaction existed between the introduced as well as

indigenous endophytic bacteria and *Brachiaria* hybrid CIAT 36061, resulting possibly in nitrogen fixation and enhancement of plant growth.

In this study, we artificially introduced strains of endophytic bacteria into *Brachiaria brizantha* CIAT 6294 cultivar Marandu and examined the effect on plant growth.

Materials and Methods

Plant materials: Twelve *Brachiaria brizantha* CIAT 6294 (cv. Marandu) that are approximately one month old were used for inoculation. These plants were selected after examining with nested PCR, and showed no amplified products for sequences of *nif*H (the gene that encodes nitrogenase reductase) gene, indicating the absence of endophytic bacteria containing these sequences.

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

Plant inoculation: Twelve tillers of *Brachiaria brizantha* CIAT 6294 that are about a month old were prepared, their roots washed with sterile distilled water and made ready for inoculations. The roots of six of these tillers were immersed in a beaker containing a mixture of equal volumes (50-ml each) of the three strains of endophytic bacterial suspension described above. The remaining six plants were immersed in a beaker containing the same volume of sterile distilled water. All plants were kept immersed for 48 hours, after which they were removed and rinsed 3 times with sterile distilled water. They were then each transplanted to pots containing sterile sand (95%) and soil (5%) and maintained in the greenhouse under natural day light and at temperatures between 19 and 30°C. No nutrients were applied.

Plant evaluations: Sixty days after inoculations of *B. brizantha* CIAT 6294, the following measurements were taken in control and treated plants: 1) plant growth and development such as plant height, number of tillers, number of leaves, leaf area model LI-300 (LI-COR, inc., Lincoln, NE), 2) leaf chlorophyll content 3) nitrogen content, and 4) soluble protein content in leaves.

Plant height was measured in centimeters from stem base to the highest part of the plant. Number of leaves per plant and the number of tillers were determined. Leaf area was determined in cm^2/plant and measured using a LI-300 leaf area meter (LI-COR, inc., Lincoln, NE). In addition, dry matter distribution among leaves, stems and roots was determined after drying each tissue separately in an oven at 70ºC for 48 hours. Leaf chlorophyll content was measured with a chlorophyll meter SPAD 502 (Minolta), taken across the third fully developed leaf as an average of 6 measurements. Soluble leaf

protein was measured as described by Rao and Terry (Plant Physiol 90: 814-819). Nitrogen content in leaves and stems was determined using methods described by Salinas and García (1985, CIAT, Working document 83 p).

Bacterial population in the roots: Approximately 1 g of root samples was taken from each individual plant *Brachiaria brizantha* CIAT 6294, surface sterilized (in 1% NaOCl solution for 2 min, in 70% ethanol for one min, then rinsed 3 times in sterile distilled water) and macerated in mortar and pestle in 1 ml of sterile distilled water. One hundred-ε1 of this macerated sample was taken and a dilution series performed. These were plated on nutrient agar medium and incubated for 24 h at 28°C to determine bacterial colony growth, and calculate the number of bacterial cell per gram of root weight.

Experimental design and statistical analysis: The experiment had two treatments (with and without artificial inoculations) each with 6 plants (6 repetitions) and arranged in a completely randomized design. Analysis of variance was determined using Statistics Analysis System (SAS⊇). A t-test was conducted.

Results and Discussion

B. brizantha CIAT 6294 had no indigenous endophytic bacteria that have *nif*H gene sequences. We introduced three strains of bacteria, originally isolated from *Brachiaria* hybrid CIAT 36062, into CIAT 6294. In general, the introduction of these bacteria had a positive effect on plant growth and development in the recipient plant CIAT 6294. There was more tiller and root development in artificially inoculated CIAT 6294 plants than control plants.

Analysis of variance showed that the total biomass production (leaf, stem and root) collected from control *Brachiaria* CIAT 6294 plants was significantly ($P< 0.05$) less than that from inoculated ones (Figure 2.6.1). The data presented indicate that a close and beneficial interaction existed between the introduced bacteria and *B. brizantha* CIAT 6294, resulting possibly in nitrogen fixation and enhancement of plant growth. These results are consistent with the results reported in PE 1 Annual Report 2005 with *Brachiaria* hybrid CIAT 36061 (cv. Mulato)

In nitrogen- and other nutrient-deficient conditions, *Brachiaria* plants inoculated with the three bacterial strains had significantly higher average values in all evaluated parameters, plant height, number of tillers, number of leaves, and leaf area than those control plants (Figure 2.6.2).

Analysis of variance showed that the chlorophyll content (SPAD units) collected from control *Brachiaria* CIAT 6294 plants (43.4 SPAD units) was significantly ($P< 0.05$) less than that from inoculated ones (50.34 SPAD units).

These data strongly suggest that endophytic bacteria have a direct beneficial effect on plant growth and development, and possibly on associated nitrogen fixation in *Brachiaria*. The possibility that this plant growth is through associated nitrogen fixation

is further corroborated by the endophytic bacteria sequence data described in the report in section IV.

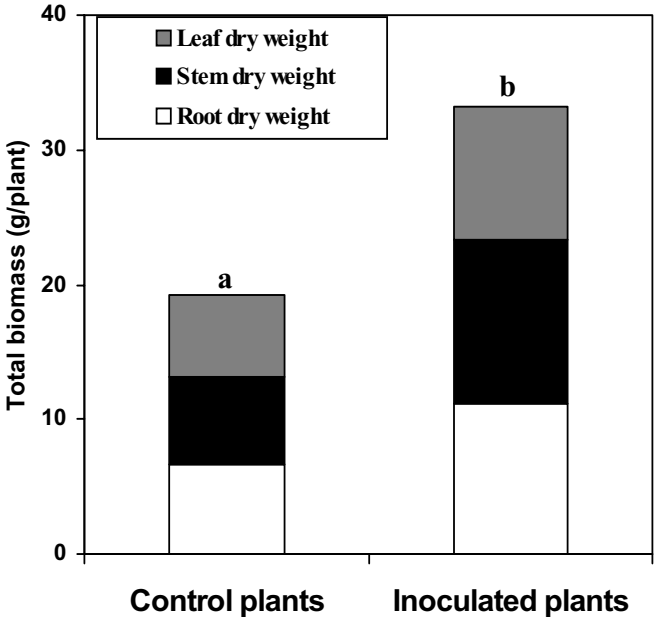

Figure 2.6.1. Total tissue biomass production in *Brachiaria brizantha* CIAT 6294 control plants, and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062), 60 days after inoculations and maintained under greenhouse conditions with no nutrients. Values are average of 6 plants per treatment.

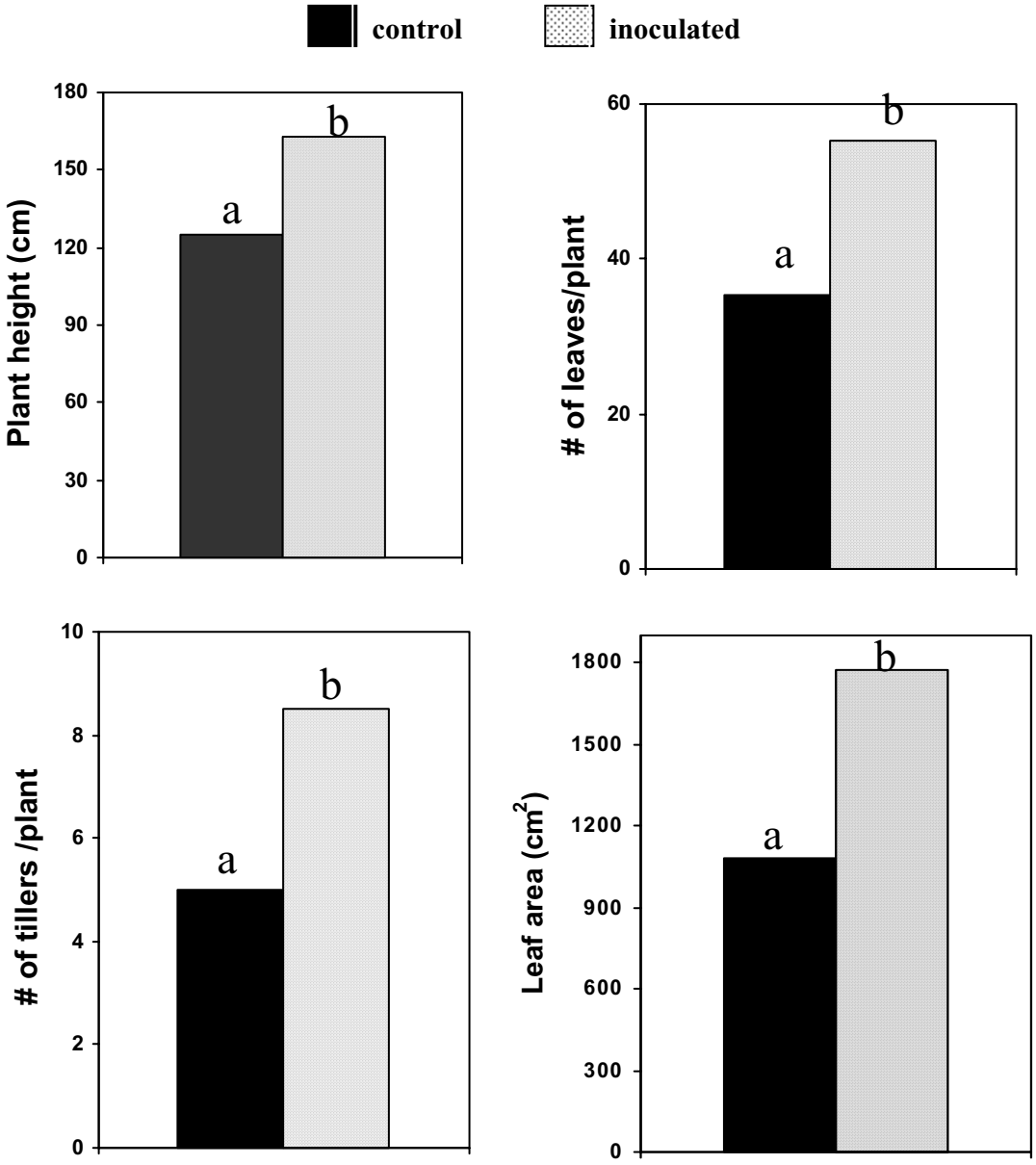

Figure 2.6.2. Effect of bacterial isolates (a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) on the growth of *Brachiaria brizantha* CIAT 6294 60 days after inoculations and maintained under greenhouse conditions with no nutrients. Values are average of 6 plants per treatment.

Activity 2.7. Characterization and comparison of partial sequence of *nif*H gene in four strains of endophytic bacteria associated with *Brachiaria* genotypes.

Contributors: P. Fory and S. Kelemu

Rationale

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

We have reported the isolation of three strains of bacteria from *Brachiaria* hybrid CIAT 36062 (BR97-1371) from roots, leaves and stems that were designated 01-36062-R2, 02-36062-H4, and 03-36062-V2, respectively. Using nested PCR and three primers designed for the amplification of the *nifH* gene sequences, amplified products were generated with template DNA from these bacterial strains. We have also reported previously (IP-5 Annual Report 2004) that fatty acid analysis conducted on these 3 strains resulted in matching them with various bacteria that are known to be nitrogen fixers and/or plant growth promoters (for example with *Flavimonas oryzihabitans*).

We reported (IP-5 Annual Report 2005) the cloning and sequencing of a 371 bp nested PCR amplified product (with *nif*H gene specific primers) isolated from an endophytic bacterium strain 01-36062-R2 associated with *Brachiaria* hybrid CIAT 36062. Using this sequence data, specific primers were designed and synthesized in order to develop a simple diagnostic tool that enables us to do one step PCR analysis and avoiding nested PCR methods, that will eventually allow us to detect, evaluate and select *Brachiaria* genotypes that harbor *nif*H gene containing endophytic bacteria (PE 1 Annual Report 2005). In this study, we continued to clone and analyze the sequences of *nif*H gene in other native strains of endophytic bacteria associated with *Brachiaria* genotypes.

Materials and Methods

Bacterial isolates: For cloning and sequence analysis, endophytic bacterial strains isolated from roots, leaves and stems of *Brachiaria* hybrids CIAT 36062 (designated 01-36062-R2, 02-36062-H4, 03-36062-V2) and a strain isolated from *Brachiaria* hybrid CIAT 3061 were used for this study.

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

added just before use. The method involves cleaning with phenol-chloroform and chloroform/isoamyl alcohol and precipitation with ethanol. The quality of DNA was checked on 1 % agarose gel.

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

Amplification of DNA inserts for sequencing: PCR reactions (25-σl) contained 20 ng/σl plasmid DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, primers T7 (5'-GTAATACGACTCACTATAGGGC-3') and Sp6 (5' –TATTTAGGTGACACTATAG-3') each at 0.1 σM concentration, 0-5U Taq polymerase and amplified in a programmable thermal controller (MJ Research, Inc, MA) programmed with 35 cycles of a 30 sec (2 min for the first cycle) denaturation step at 94°C, annealing for 30 sec at 50°C, and primer extension for 1 min (4 min in the final cycle) at 72°C. Samples of amplified products were separated on a 2% agarose gel by electrophoresis for further confirmation of the expected size insert.

Cloning and digestion of amplified DNA fragments: Amplified products were eluted from agarose gel using Wizard ® PCR Preps DNA Purification System (Promega) according to instructions supplied by the manufacturer. The purified fragments (size 320-322 bp) were ligated to the cloning vector PCR ® 2.1 (Invitrogen, Carlsbad, CA, USA) [see Figure 2.7.1] and used to transform *E. coli* DH5Ŭusing standard procedures (Sambrook et al., 1989. Molecular Cloning: a laboratory manual. 2nd ed. Cold spring harbor laboratory, USA)

Transformed colonies of *E. coli* DH5Ŭwere selected on Luria agar supplemented with n 1-mM of Isopropyl ɓ-D-1-thiogalactopyranoside (IPTG) and 40-µg of X-Gal. Plasmids were extracted from transformed *E. coli* DH5Ŭcells using a Wizard® Plus Mini-preps DNA Purification System (Promega) using the protocol supplied by the manufacturer. To confirm whether the transformants contained the desired size of insert, the plasmid DNA was digested to completion with the restriction enzyme *Eco*RI (Gibco/BRL). The digested products were separated by electrophoresis on a 1% agarose gel (Bio-Rad, NJ), stained with ethidium bromide and photographed under UV light.

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

Phylogenetic anaysis: Phylogenetic analysis of the nucleotide sequences was conducted using *Neighbor-Joining* (NJ) method applying the parameters described in the program MEGA 3.1 (Kumar *et al*., 2004, Brief Bioinform 5:150-163). *Bootstrap resampling test* with 1.000 replications was applied.

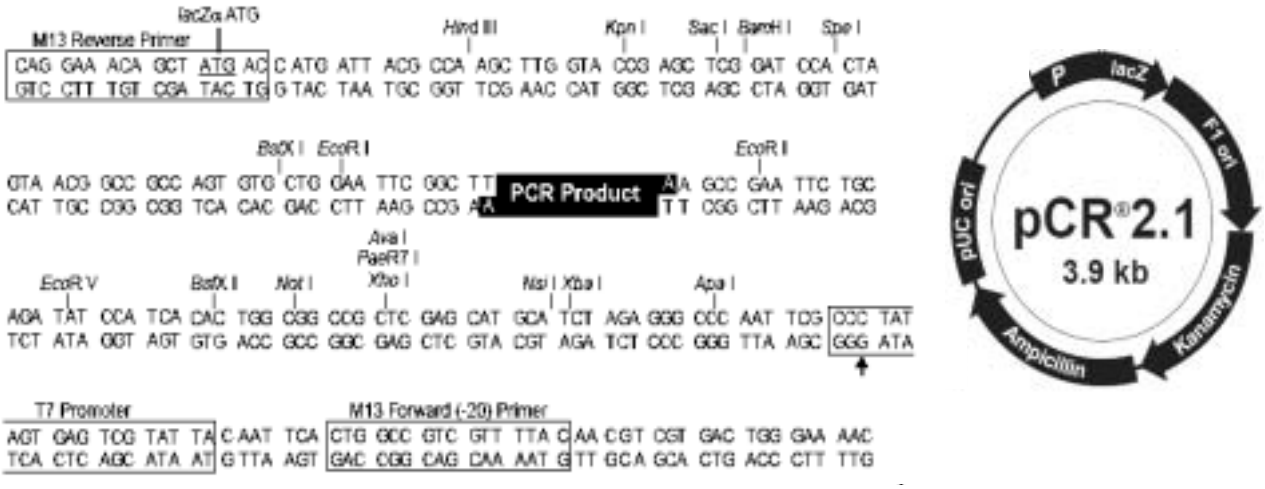

Figure 2.7.1. Cloning vector PCR ® 2.1. The vector has genes for resistance to the antibiotics ampicillin (Amp^r), kanamycin (Km^r), and *lac*Z.

Results and Discussion

Nucleotide similarity comparison: The sequences corresponding to *nif*H gene sequence were edited, cleaned and assembled using the program Sequencher v 3.0 (Sequencher 3.0 User Manual, 1995). The fragments that showed homology were aligned using the program Clustal version W (1.8). The sequences that correspond to strains designated as 01-36062-R2; 02-36062-H4 and 03-36062-V2, isolated from roots, leaves and stems, respectively, were identical to each other.

The sequence analysis demonstrated the presence of *nif*H gene sequences in these sequenced clones, with a similarity of 89% in 283 bp with the *nif*H gene sequence of *Klebsiella pneumoniae* with a GenBank as AF303353.1. Further more the sequences had an 88% similarity with *Klebsiella* sp. Y83 (DQ821727.1) and *Enterobacter* spp. (Y79DQ821726.1) The clone from the endophytic bacterium isolated from *Brachiaria* hybrid CIAT 36061 had a 97% sequence similarity in 290 bp with three accessions registered in the GenBank, designated as DQ982313.1, DQ982300, and DQ982299.1. These sequences correspond to clones isolated from uncultured diazotrophes (nitrogen fixing organisms) isolated from roots and stems of maize plants. *Nif* genes that encode the nitrogenase complex and other enzymes involved in nitrogen fixation have consensus sequences identical in various nitrogen-fixing bacteria

Sequence comparison: The strains 01-36062-R2; 02-36062-H4, 03-36062-V2, and 36061 were compared with 16 nucleotide sequences that were selected with a maximum

identity, Score and E-value, registered in the *GenBank* (Table 2.7.1). Furthermore, 18 nucleotide sequences of nitrogen-fixing organisms used in the studies by Franke *et al*., (1998, Lett. Appl. Microbiol. 26:12-16). Bacteria with other characteristics were also included in these comparisons. Figure 2.7.2 clearly shows the sequences analyzed are phylogenetically grouped in three groups, A, B, and C, with high bootstrap values of 80, 82, 100 %, respectively. Group A contained 20 accessions, 8 of these belonging to protobacteria, one actinobacteria, one unidentified bacterium, 6 clones from uncultured organisms, and the four endophytic bacterial strains used in this study.

The three sequences from the endophytic bacteria isolated from *Brachiaria* CIAT 36062, designated 01-36062-R2; 02-36062-H4, and 03-36062-V2, are closely related to *Klebsiella, Enterobacter* and *Micrococcus*. Microorganisms in these three genera are known to be nitrogen-fixers. These results are in agreement with biochemical analysis (fatty acid analysis) of the isolate 01-36062-R2 conducted in earlier studies that showed a match with *Leclercia adecarboxylata*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, at 0.879, 0.841, and 0.820 similarity index, respectively (IP-5 Annual Reports 2003/4). The sequence of the endophytic bacterial strain isolated from *Brachiaria* hybrid CIAT 36061 grouped 100% with three clones coded as DQ982313.1, DQ982300, and DQ982299.1, and 87 % with the rest in Group A (Figure 2.7.2).

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

Group B consists of 15 accessions subdivided into three subgroups B-1, B-2, B-3. The sub-group B-1 contains 2 species of *Frankia* (a soil-inhabiting nitrogen-fixing bacterium) and one betaprotobacteria. The subgroup B-2 consists of three accessions that belong to the genus *Azotobacter*. Sub-group B-3 consists of 9 accessions. The group C consists of 2 species of the genus *Clostridium*

For a better understanding of the endophytic microbial diversity and identity associated with species of *Brachiaria*, it is important to extend the work to include more enophytic bacterial isolates from a number of *Brachiaria* hybrids. This work further complements the results reported in IP-5 Annual Report 2005 on the role of these bacterial endophytes in *Brachiaria* plant growth possibly through nitrogen-fixation.

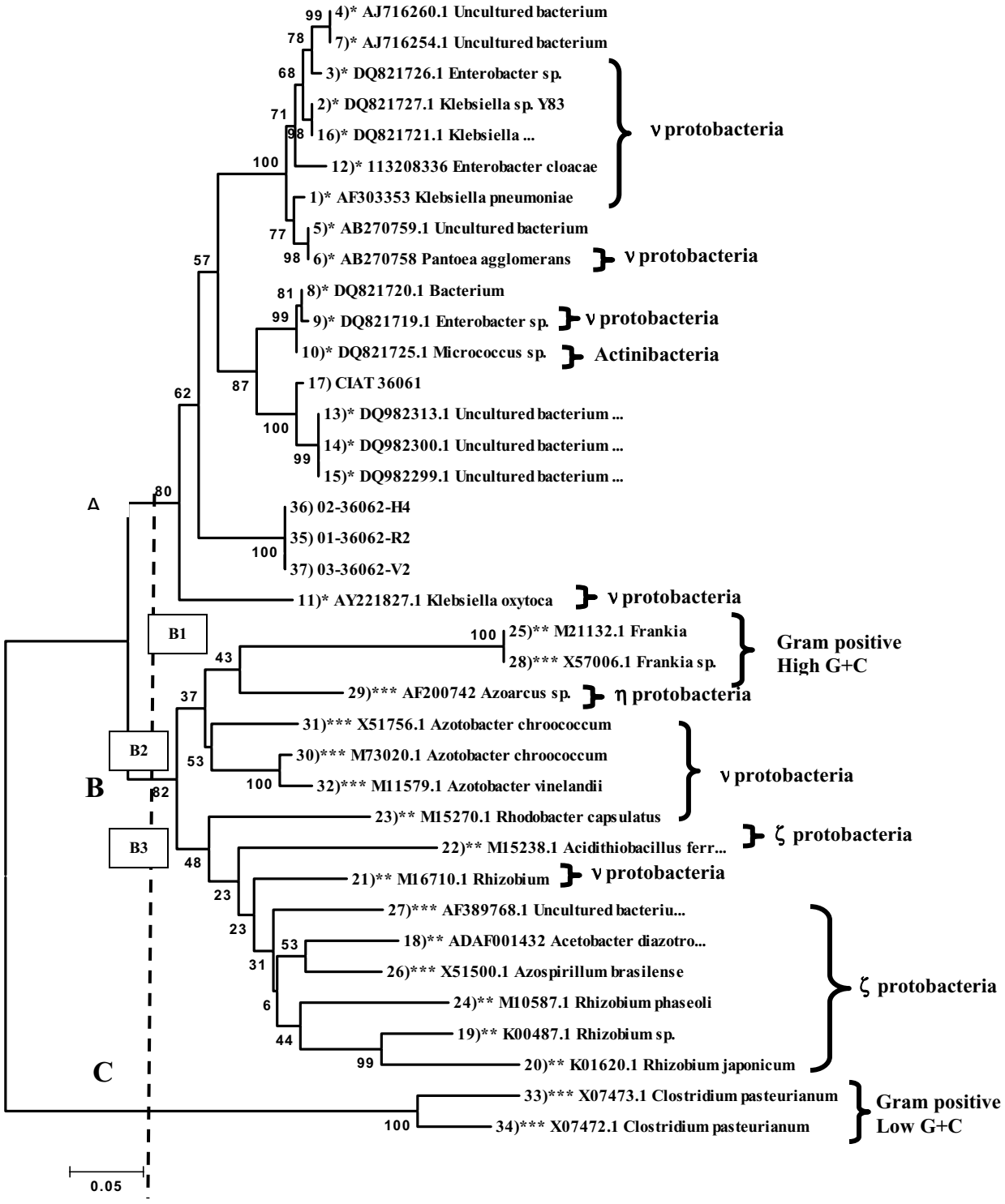

Figure 2.7.2. Phylogenetic tree generated for nucleotide sequences evaluated using Neighbor Joining analysis. The values represent 1,000 replications in bootstrap method.

Table 2.7.1. Nucleotide sequences and values generated using *GenBank* data for four endophytic bacterial isolates associated with *Brachiaria* hybrids in relation to other microbes.

No	Accession	Description	bp	*Organism*	Score	*E value*	Max Identity (%)
1	DQ982300.1*	uncultured bacterium	322	Bacteria; environmental samples	531	$5e^{-148}$	97
2	DQ982313*	uncultured bacterium	518	Bacteria; environmental samples	531	$5e^{-148}$	97
3	DQ982299.1*	uncultured bacterium	322	Bacteria; environmental samples	531	$5e^{-148}$	97
4	DQ821721*	*Klebsiella pneumoniae*	322	Bacteria; Proteobacteria; Gammaproteobacteria Enterobacteriales; Enterobacteriaceae; *Klebsiella*	385	$7e^{-104}$	90
5	AF303353	*Klebsiella pneumoniae*	518	Bacteria; Proteobacteria; Gammaproteobacteria Enterobacteriales; Enterobacteriaceae; *Klebsiella*	365	$7e^{-98}$	89
6	DQ821727	*Klebsiella* sp. Y83	322	Bacteria; Proteobacteria; Gammaproteobacteria Enterobacteriales; Enterobacteriaceae; *Klebsiella*	377	$1e^{-89}$	88
7	DQ821726	*Enterobacter* sp. Y79	322	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Enterobacter	377	$1e^{-89}$	88
8	AJ716260	uncultured bacterium	360	Bacteria; environmental samples	325	$6e^{-86}$	87
9	AB270759.1	uncultured bacterium	360	Bacteria; environmental samples	321	$9e^{-85}$	88
10	AB270758	*Pantoea agglomerans*	360	Bacteria; Proteobacteria; Gammaproteobacteria Enterobacteriales; Enterobacteriaceae; Patoea	321	$9e^{-85}$	88
11	AJ716254	uncultured bacterium	360	Bacteria; environmental samples	317	$1e^{-83}$	87
12	DQ821720	Bacterium Y41	322	Bacterium Y41	313	$2e^{-82}$	87
13	DQ821719.1	*Enterobacter* sp. Y11	322	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae; Enterobacter	305	$5e^{-80}$	87
14	DQ821725.1	*Micrococcus* sp. Y70	322	Bacteria; Actinobacteria;Actinobacteridae;Actinomycetales Micrococcineae; Micrococcaceae; Micrococcus.	297	$1e^{-77}$	87
15	AY221827.1	*Klebsiella oxytoca*	327	Bacteria; Proteobacteria; Gammaproteobacteria Enterobacteriales; Enterobacteriaceae; *Klebsiella*	293	$2e^{-76}$	86
16	AB270754	*Enterobacter cloacae*	359	Bacteria; Proteobacteria; Gammaproteobacteria;	281	$8e^{-73}$	86

Note: The first 4 accessions noted with * correspond to the endophytic bacterial strain isolated from *Brachiaria* hybrid CIAT 36061

Activity 2.8. Validation of thermotherapy of stem cuttings, plant extract and *Trichoderma* to manage cassava diseases in the Eastern Plains region and in Cauca (Colombia).

Contributors: E. Álvarez, G. Llano, J. Loke, J.F. Mejía, V. Montaña, J. Jaramillo (Petrotesting Colombia S.A.), and B. Muñoz (CORFOCIAL, Cauca)

Highlight:

- Cassava root rots were successfully reduced under field conditions by using *Trichoderma viride* and *Trichoderma harzianum* as biocontrol agents.

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 the use of biocontrol agents, have been evaluated recently for managing root rots in cassava. In this report, we discuss the progress made towards the objective: with farmer participation, to adjust and validate strategies of integrated management of the constraining diseases found in each region.

Materials and Methods

With farmer participation, to adjust and validate strategies of integrated management of the constraining diseases found in each region: 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

- 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

Eastern Plains: Two semicommercial plots were established on the farms "La Vega" (Yopal, Casanare) and "Cantaclaro" (Puerto López, 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.*

Cantaclaro (Pto. López, Meta: 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.

CIAT-14PDA-4 is a strain of the fungus *T. viride*, and an antagonist and plant growth stimulator. It 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*) was added 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*, Biotropical).

Results and Discussion

Germination was similar in both stake treatments. Evaluations of 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-bioethanol production from cassava.

Variety 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.

At harvest, significant differences were observed between yields of varieties, with Vergara and CM 4574-7 being the best. However, the latter variety was the most affected by rots at 20% when the AgroGuard strain of *T. harzianum* was used.

Except for variety Vergara, no significant differences were observed among yields after strains of the antagonistic *Trichoderma* fungus were applied. For Vergara, yields were highest after treatment with the strains CIAT-14PDA-4 and AgroGuard.

In terms of dry matter content, CM 4574-7 and La Reina had the highest values (30.8% and 29.2%, respectively). After treatment with the strains, dry matter increased with the Bioderma strain, enabling the highest value (30.9%).

Figure 2.8.1 shows the effect of the three *Trichoderma* strains evaluated for yield and percentage of root rots. Yield with the CIAT strain was more than 10 t/ha higher than the other two in varieties Vergara and La Reina, whereas in variety CM 4574-7, the Bioderma strain surpassed the AgroGuard strain by more than 5 t/ha, which itself surpassed the CIAT strain by almost 9 t/ha.

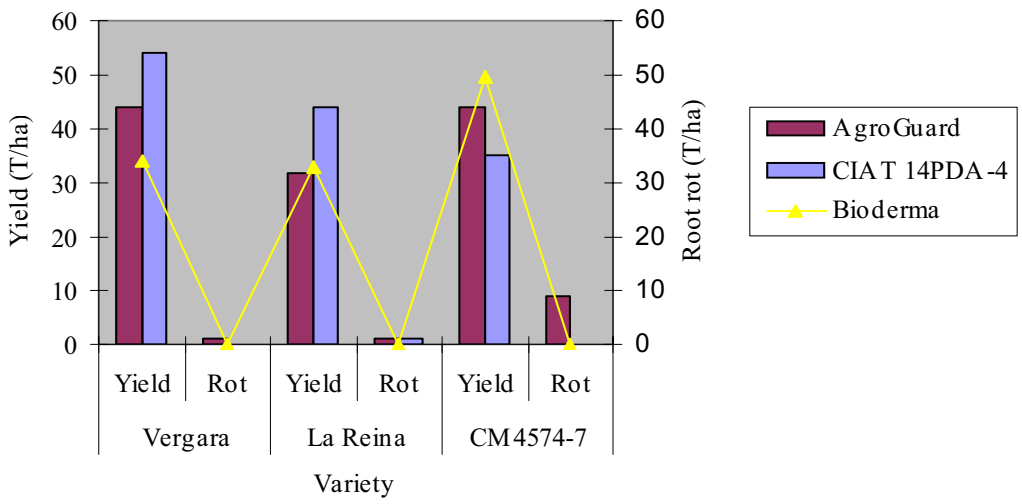

Figure 2.8.1. Effect of three *Trichoderma* strains on the yield of three cassava varieties, Cantaclaro Farm, Puerto López, Meta.

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

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 fungal 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.

Results and Discussion

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.

We observed that the overall average yield of the two varieties was very similar, with

31.6 t/ha for Catumare and 31.8 t/ha for La Reina, whereas root rots were 2.46% and 3.73%, respectively. Yield for Catumare stakes treated with Lonlife was 31.7 t/ha and with chemicals, 34.6 t/ha, whereas the percentage of root rots was 4.09% and 1.66%, respectively.

On applying the AgroGuard strain of *T. harzianum* to variety Catumare, we obtained 40.5 t/ha of cassava, with 2.92% of roots rotting. In contrast, with the CIAT strain (*T. viride*), yield was 31.1 t/ha, but the percentage of rot was much lower (1.85%). For its part, the check with no applications of *Trichoderma* spp. yielded only 25.9 t/ha. That is, 14.6 t/ha less than the treatment with AgroGuard and 5.2 t/ha less than the treatment with the CIAT strain. Rot in the check reached 2.58%.

For variety La Reina, yield of plants whose stakes were treated with Lonlife was 33.5 t/ha and with chemical treatment was 31.6 t/ha. Root rot was 1.58% and 4.57% for the Lonlife and chemical treatments, respectively (Figure2.8.2).

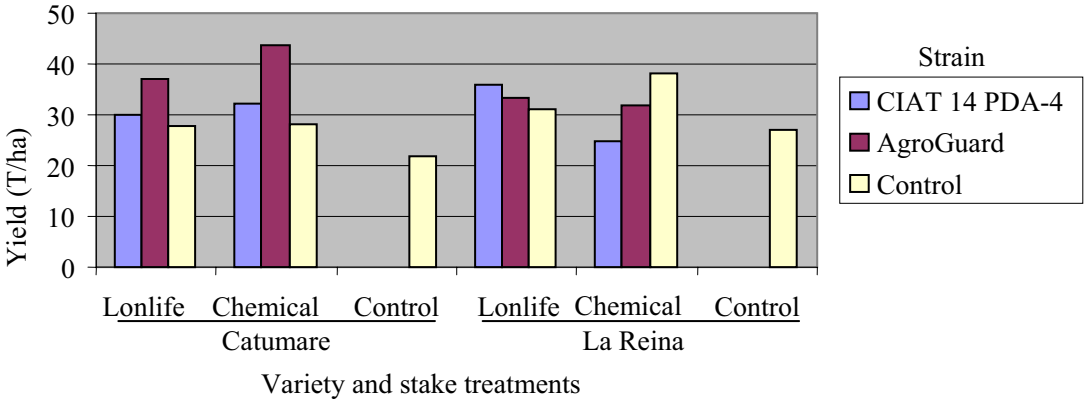

Figure 2.8.2. Effect of treating stakes with Lonlife® or a chemical, together with strains of Trichoderma harzianum (AgroGuard®) and T. viride (CIAT) on the yield of two cassava varieties, Yopal, Casanare.

In terms of yield obtained with the applications of *Trichoderma* spp., the highest yield was achieved with AgroGuard (32.7 t/ha), followed by the check with no application (32.0 t/ha) and the CIAT strain (30.5 t/ha). However, with the latter strain, root rots were lower (0.84%) than for AgroGuard (3.47%) or the check with no applications (5.84%).

Figure 2.8.2 shows that, for variety Catumare, treatment with AgroGuard was found to range between 35 and 44 t/ha, depending on the product used to treat the stakes, with the chemical treatment being the higher. With the CIAT strain, yield was about 30 t/ha, whereas the check with no *Trichoderma* spp. reached 28 t/ha. The check for which stakes were not treated nor received applications of *Trichoderma* spp. only barely surpassed 20 t/ha.

For variety La Reina, yields were very similar, with or without applications of *Trichoderma* spp. When stakes were treated with Lonlife, yield was only slightly higher than the treatment with the CIAT strain (36 t/ha). When stakes were treated chemically, no reaction was observed to applications of *Trichoderma* spp. The effect of the *Trichoderma* applications apparently depended on variety.

Figure 2.8.3 shows that cassava rots declined considerably with the application of *Trichoderma* spp., the effect being most marked when the CIAT strain was used. According to sampling data from the Yopal plots, AgroGuard was not very effective in reducing rots, probably because the roots conserved a greater quantity of water when the AgroGuard strain was used (i.e., dry matter was 32.9% with the CIAT strain and 31.7% with AgroGuard for La Reina and 34.8% with the CIAT strain and 32.7% with AgroGuard for Catumare). It should be pointed out that rots occur in foci, which would explain why the disease was less in some checks.

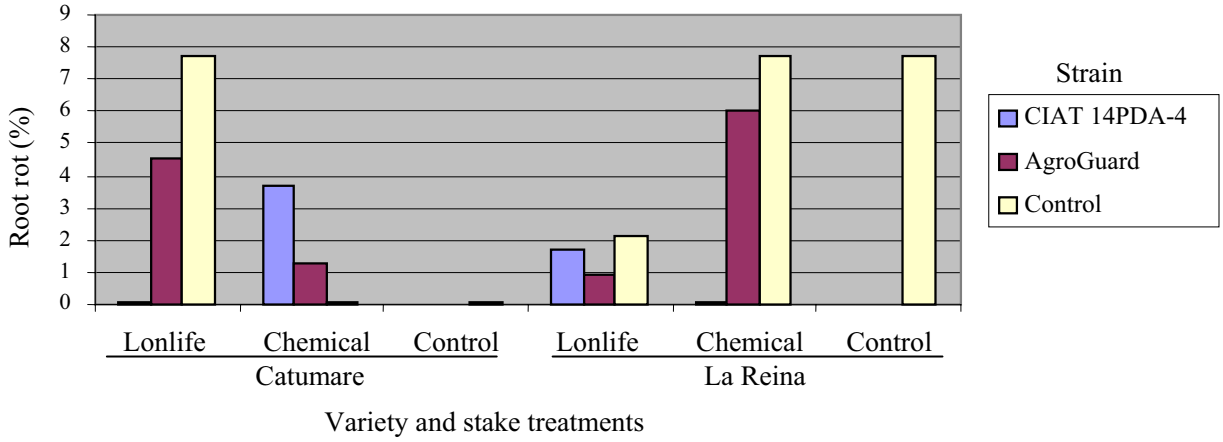

Figure 2.8.3. Effect of treating stakes with Lonlife® or a chemical, together with strains of *Trichoderma harzianum* (AgroGuard®) and *T. viride* (CIAT) on root rots in two cassava varieties, Yopal, Casanare.

Department of Cauca: We conducted two trials with the collaboration of the Local Agricultural Research Committee (CIAL, its Spanish acronym) "La María", in the Municipality of Piendamó, and a farmers' group in Cabuyal, Municipality of Caldono. 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.

La María (Piendamó): Varieties, Three elite clones were evaluated: 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 fungal 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.

Results and Discussion

Stakes treated with Lonlife had higher germination rates and greater vigor.

When the AgroGuard strain of *T. harzianum* was used, yield of clone SM 1495-5 reached 31.6 t/ha, surpassing that of SM 1498-4 and SM 707-17. With strain CIAT 14PDA-4 of *T. viride*, a yield of 37.5 t/ha was obtained with variety SM 1498-4, whereas SM 1495-5 yielded only 9.9 t/ha, indicating a strain × variety interaction. Treatment with *Trichoderma* spp. permitted an increase in yield of more than 20% than that of the control. For both strains, yields increased slightly when the stakes were also treated with an extract of citric seeds (i.e., Lonlife) at planting. The differences, however, were not significant (Figure 2.8.4). No root rots appeared in this trial.

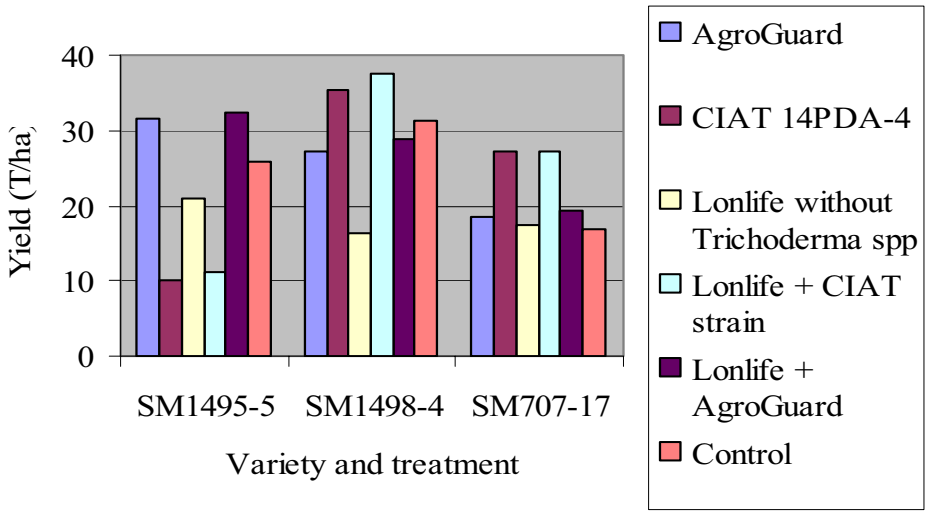

Figure 2.8.4. Effect of treating stakes with Lonlife® and strains of *Trichoderma harzianum* (AgroGuard®) and *T. viride* (CIAT) on The yield of three elite cassava clones, Piendamó, Cauca.

Cabuyal (Caldono): With the same objective as for the trial at La María, another trial was established in the village district of Cabuyal, Municipality of Caldono, Cauca.

Varieties: Three elite clones were evaluated: SM 1707-41, SM 1834-20, and SM 1992-1.

Results and Discussion

Stakes treated with Lonlife had higher germination rates.

The highest cassava yields were obtained when *T. harzianum* (AgroGuard) was used, with or without treating stakes with Lonlife, reaching 52 t/ha with clone SM 1834-20. This finding corroborates the results obtained in Piendamó. In contrast, the effect of strain CIAT 14PDA-4 of *T. viride* was not effective. The treatment of stakes with Lonlife and no applications of *Trichoderma* spp. gave yields of more than 50 t/ha, probably because of the site, which had been chosen at random. Yields of the controls fluctuated between 7.2 and 23 t/ha (Figure 2.8.5).

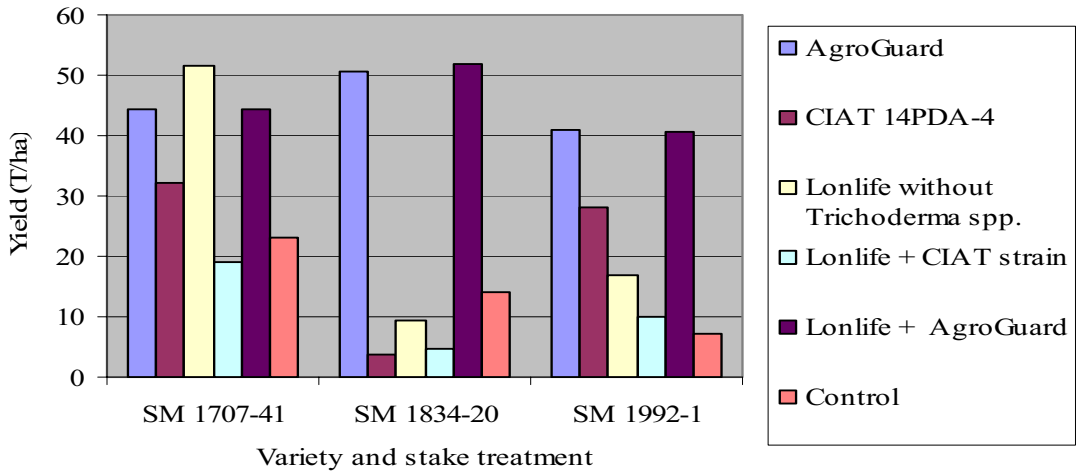

Figure 2.8.5. Effect of treating stakes with Lonlife® and strains of *Trichoderma harzianum* (AgroGuard®) and *T. viride* (CIAT) on yields of three elite cassava clones, Caldono, Cauca.

Figure 2.8.6 indicates that clone SM 1707-41 was least affected by root rots, showing higher resistance than the other two clones evaluated. For this clone, all treatments surpassed the control, which had 11.6% of roots with rots. With Lonlife + AgroGuard, only 2.4% of roots had rot; with Lonlife only, 1.1%; and with the CIAT 14PDA-4 strain of *T. viride*, 1.2%. The other treatments were not affected by rots.

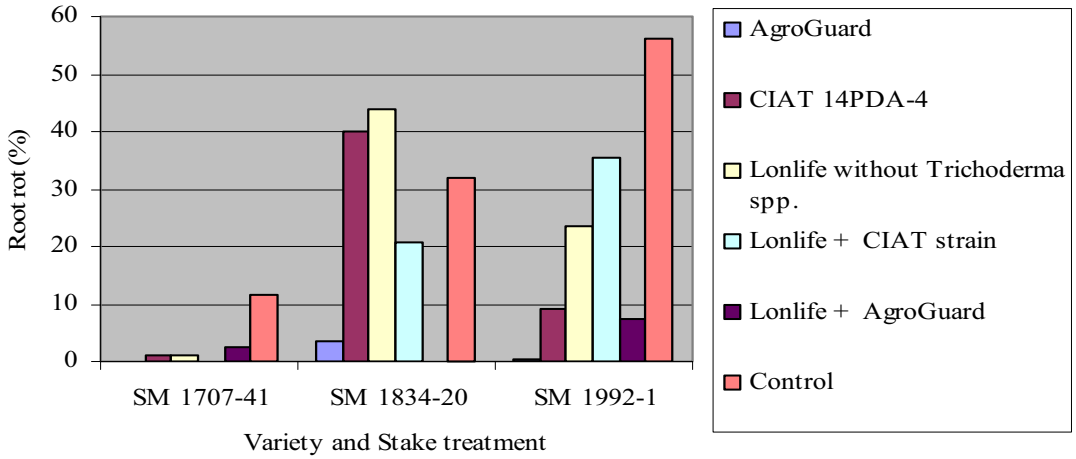

Figure 2.8.6. Effect of treating stakes with Lonlife® and strains of *Trichoderma harzianum* (AgroGuard®) and *T. viride* (CIAT) on the control of root rots in three elite cassava clones, Caldono, Cauca.

Apparently, the soil and environmental conditions affect the *Trichoderma* strains evaluated. In Yopal (Casanare), strain CIAT 14PDA-4 was more effective in reducing rots but, in Caldono (Cauca), the AgroGuard strain of *T. harzianum* was more effective. Lonlife, although it encouraged better germination of the stakes, did not much influence cassva yields.

In a culinary and tasting test carried out by a group of seven farmers, clone SM 1834-20 was the most accepted. However, clones SM 1707-41 and SM 1992-1 surpassed it in terms of dry matter content, a characteristic preferred by farmers in Cauca, who produce most of their crop for the starch industry.

Activity 2.9. Improving Nutritional Management for the Preventive Control of Downy Mildew of Roses (*Peronospora sparsa*)

Contributors: E. Álvarez, E. Gómez, G. Llano, and F. Castillo

Rationale

Downy mildew is the principal phytosanitary problem of roses in Colombia. It affects plant productivity, product export quality, and production costs. The extra costs caused by this disease lie in the increase in the number of applications of fungicides, use of specific products that are more expensive than those used to control other phytopathogens, and cultural management of this disease.

The search for new alternatives of pest-and-disease management in commercial crops contributes to friendly agricultural production by improving product quality and reducing toxins, and thus protecting the environment. Plant nutrition has a significant impact on the predisposition of plants to be affected by pests and diseases. In this sense, plant nutrition can contribute as much to the increase or reduction of resistance and/or tolerance of downy mildew, affecting growth patterns, anatomy and morphology, and particularly chemical composition.

Strengthening the host through adequate nutrition is a common preventive practice to manage disease and so eventually make the plants less susceptible to the pathogen. However, the relationships between specific associated nutrients and resistance of rose plants to *Peronospora sparsa* are currently unknown. Some nutritional elements such as nitrogen, potassium, calcium, boron, and silicon, and the N-to-K ratio are believed to be important for inducing resistance to obligate parasites. Hence, one objective of this study is to improve the nutritional balance of the rose crop to prevent downy mildew. This study evaluates, under hydroponic conditions, the effect the elements N, K, Ca, Bo, Si, and Mn would have on roses and on the incidence and severity of downy mildew.

Materials and Methods

We first established rose plants of the varieties Charlotte and Classy, which are susceptible to downy mildew, and Malibu, which is considered to have an intermediate reaction to the disease. For the optimal development of these activities, we identified the best environmental conditions for the expression of symptoms of the disease, improved the efficiency in producing inoculum, and adapted a scale for evaluating disease severity.

Sites. The activities and trials were conducted in laboratories and greenhouses, under controlled conditions of temperature and relative humidity.

Establishing different environmental conditions for disease development: Spray inoculation. Inoculation was carried out by spraying sporangia, using a DeVilbiss spray connected to a compressor. Both the upper and lower surfaces of all the leaves of the plants were sprayed from a distance of 40 cm. Thus, we could ensure that the leaflets were covered by a film of free water. Inoculum was obtained from rose leaves with typical symptoms of the disease and from sporangia of the pseudofungus, obtained from rose plants grown under the conditions of the Bogotá Sabana on flower farms where no previous treatments with fungicides had been applied for 1 week. Young leaflets with signs of sporulation on their back surfaces were collected from the upper third of the plants. They were then placed and agitated for 20 min in containers carrying a solution of 0.1% Tween 80 prepared with sterilized deionized water. They were later examined under an optical light microscope at 10X. The concentration of sporangia was calculated in a hemacytometer to later adjust the suspension to a concentration of 3×10^4 sporangia/mL. Plants were inoculated by spraying with an aqueous suspension of

sporangia on the upper and lower leaf surfaces, using an atomizer at a distance of about 40 cm from the leaves to ensure the presence of a film of free water on each foliar.

*Encouraging the development of downy mildew under different environmental conditions***:** The inoculated plants were incubated under different conditions of humidity and temperature to identify the best conditions for producing inoculum and developing the disease. The environments evaluated were:

1. Petri dishes in which the pseudofungus was inoculated onto healthy leaflets and incubated under the conditions of a humid chamber.

2. Growth room with permanent humidification to maintain relative humidity between 27% and 95% and temperatures between 14°C and 25°C.

3. Humid chamber, which was 1 m high, 1.50 m wide, and 1 m deep. Temperatures fluctuated from 35°C during the day to 19°C at night. Relative humidity ranged between 31% and 98%, using an electric humidifier for 6 hours continuously between 9 a.m. and 3 p.m. for 3 days and then four times a day for 30 min each time.

4. Once inoculated, the plants were placed inside a humid chamber, which had a stable relative humidity of more than 95%, obtained by using an electric humidifier.

5. Greenhouse with a relative humidity ranging between 64% and 97%, obtained by using microsprays that functioned for 1 min at 0, 4, 8, 11, 13, 16, and 19 h. Temperatures were between 34.4°C during the day and 22.2°C at night.

Designing the evaluation scale: The disease was evaluated according to a scale of severity developed by Gómez (Gómez, 2004, Determinación de componentes de la biología de *Peronospora sparsa* Berkeley, y caracterización de la respuesta de tres variedades de rosa a la infección del patógeno bajo condiciones de laboratorio e invernadero. Universidad Nacional de Colombia, Bogotá, 72 pp.) with some modifications. The scale had four levels, where 0 corresponded to leaflets with mild crinkling; 1 to leaflets with pronounced crinkling, sporulation, and presence of chlorotic mottling; 2 to leaflets that presented green islands, chlorosis, and initial necrosis; and 3 to leaflets that showed advanced necrosis and the presence of purple or brown spots.

Establishing the nutrition trial: Rose plants of the varieties Charlotte, Classy, and Malibu were transplanted, without removing the peat, to flowerpots with 5-inch diameters and placed in a screen house (Figure 2.9.1). The substrate used was 1100 g of washed quartz sand that was sterilized and hydrated with distilled water. The peat was kept so not to damage the plant's root system on transplanting. To evaluate the effect of changes in nutrition with six elements (N, K, B, Ca, Mn, and Si), nutritive solutions were adjusted, following the recommendations of Raúl Cabrera, Associate Professor, Department of

Horticultural Sciences, Texas A&M University (Appendix 1). The pH of the solutions was maintained between 5.5 and 6.5.

Figure 2.9.1. Establishing rose plants for the nutrition trial in the screen house. The roses are planted in sterilized sand. We first observed and calculated evapotranspiration rates and measured surface tension with a tensiometer to measure water retention. From these data, we determined when to initiate watering the plants with the respective nutritive solution for each treatment. Plants were watered with 50 mL of nutritive solution in the morning and afternoon.

To maintain uniformity in the number of leaves for evaluating incidence and severity, we pruned the plants on initiating applications of nutritive solutions.

The experimental unit was eight inoculated plants for each of the 14 treatments, four uninoculated check plants, and four uninoculated check plants with fungicides applied. The 14 treatments comprised modifications of a standard solution that complied with nutritional requirements for roses (Table 2.9.1), and were as follows: K as 50% and 150% of the standard solution, Ca as 50% and 150%, B as 50% and 150%, N as 50% and 150%, Mn as 50% and 150%, the standard solution itself, and Si as 50% and 150% of a concentration of 100 ppm, which was established according to previous studies on the integrated management of pests in gerberas and roses (Parrella, 2006, Forum on innovation in Colombian flower culture and demonstration of technologies for efficiency, Bogotá, Colombia). This concentration was also used for a treatment.

Table 2.9.1. Concentration of macroelements and microelements for the standard nutrient solution for roses.

Cations (meq/L)				**Anions (meq/L)**			**Microelements (ppm)**					
Mg	K	Ca	NH_4	NO_3	H_2PO_4	SO_4	Fe	B	Mn	Zn	Cu	Mo
24	137	80	14	112	15.5	32	1	0.25	0.25	0.025	0.01	0.005

Source: Raúl Cabrera, Associate Professor, Department of Horticultural Sciences, Texas A&M University.

Results and Discussion

Encouraging the development of downy mildew under different environmental conditions: We succeeded in producing inoculum under laboratory conditions by inoculating individual leaves from the upper third of the plant and incubating them under humid chamber conditions in petri dishes. However, the best production of inoculum was from complete inoculated plants in a humid chamber in the greenhouse with alternative periods of humidity, and on whose leaves was maintained a film of water that favored the germination of sporangia, infection, and later sporulation of the pathogen. For the first 2 days, the plants were under conditions of continuous humidity for 6 h and then exposed to four daily applications of 30 min each. The plants in the growth room, where temperatures were lower, developed typical symptoms of the disease, with its characteristic purple to brown, or black spots, irregular in shape. Under these conditions, the plants also presented symptoms of green islands on the leaflets that were as large as 1 cm in diameter, followed by yellowing and premature defoliation. The plants presented white mycelial masses on the stems, which then suffered necrosis and fissuring. These symptoms were so drastic that they affected the evaluation of disease severity and incidence in the plants. We could not determine differences among plants treated with Agrifos® and its effect on disease development. The plants incubated under greenhouse conditions with microspraying showed effective levels of disease, with symptoms appearing quickly. This allowed us to evaluate more clearly the severity found for each treatment.

Designing an evaluation scale: A severity scale was designed on the basis of expression of foliar symptoms, taken as the percentage of foliar area of young leaves affected in the upper stratum of the plant (Figure 2.9.2) and of mature leaves in the middle stratum (Figure 2.9.3).

Figure 2.9.2. Scale of severity of downy mildew in leaves of rose variety Charlotte taken from the plant's upper stratum. Severity was expressed as a percentage of foliar area affected by the disease.

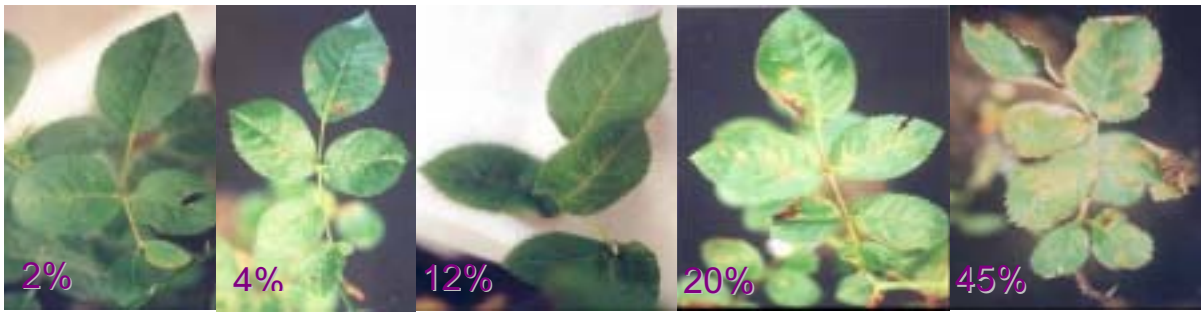

Figure 2.9.3. Scale of severity of downy mildew in leaves of rose variety Charlotte taken from the plant's middle stratum. Severity was expressed as a percentage of foliar area affected by the disease.

Appendix 1: Preparing nutritive solutions
(according to recommendations made by Raúl Cabrera, Associate Professor, Department of Horticultural Sciences, Texas A&M University)

1. Use as sources: ammonium nitrate, phosphate of ammonia, nitric acid, phosphoric acid, potassium nitrate, caustic potash, calcium nitrate, magnesium sulfate, copper sulfate, manganese sulfate, ferrous sulfate, zinc sulfate, boric acid, and ammonium molybdate.

2. Prepare a 20-L solution for each source of nutrient.

3. When preparing a solution, use a 1000-mL beaker in which 500 mL of deionized water has been added and shake constantly with a magneto.

4. Then add the quantity of ammonium nitrate, phosphate of ammonia, phosphoric acid, and caustic potash to make 20 L.

5. For 20 L, separately prepare a stock of copper sulfate, zinc sulfate, and ammonium molybdate.

6. For the minor elements Mn, Fe, and B, use manganese sulfate, ferrous sulfate, and boric acid, measuring the respective quantities, which are then added to the beaker.

7. Then heat the potassium nitrate to improve solubility and add to the beaker.

8. Finally, add the calcium nitrate to the solution in the beaker and leave, agitating, for 5 min. Then add the magnesium sulfate without forming precipitates.

9. To prepare the solution with Si, carry out the same procedure, adding the potassium silicate immediately before the potassium nitrate and magnesium sulfate, thus preventing the formation of precipitates.

Activity 2.10. Resistance induction in roses to reduce severity of downy mildew by applying potassium phosphate.

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

Rationale

Previous studies seeking new alternatives for controlling powdery mildews have demonstrated the effectiveness of salts of phosphorous acid (mono- and di-potassium phosphites) on reducing the incidence of these diseases of flower crops (Álvarez et al. 2001, Rev Asocolflores 62:31–40) and mango (Reuveni et al. 1998, Eur J Plant Pathol 104:853–860). The salts also control downy mildew of grapevines (Reuveni 1997, J Small Fruit & Viticult 5:27–38). The phosphites, derived from phosphoric acid, improve crop nutrition and stimulate the plant's natural defense mechanisms into producing phytoalexins, as has been observed for cucumber and fruit plants (Reuveni et al. 2000, Crop Prot 19:355–361). Moreover, they act as fungistats; the fungi cannot metabolize the phosphites. Hence, mycelial growth and the formation of reproductive structures are inhibited. Our study aimed to determine the effectiveness of potassium phosphites for controlling downy mildew of roses.

Matherials and Methods

Activities and trials were conducted in the laboratories and greenhouses of the Cassava Pathology Program at the Centro Internacional de Agricultura Tropical (CIAT), in Palmira, Valle del Cauca. We used plants of the rose varieties Charlotte and Malibu, which are susceptible to the disease but are of interest to flower growers. The plants were kept in greenhouses in sacks containing a mixture of sand, clayey loam, and sterilized rice husks at a rate of 6:4:1. Relative humidity ranged from 64% to 98% during watering and temperatures were between 22°C at night and 34.4°C during the day.

To evaluate the effectiveness of potassium phosphites for controlling downy mildew of roses, we established a trial, using a randomized complete block design with five treatments for the rose varieties Charlotte and Malibu. Three treatments involved inoculations with the causal agent of downy mildew. Two foliar applications per week were conducted with Agrifos® (mono- and di-potassium phosphites, equivalent to 400 g of phosphorous acid per liter) in doses of 0.5% mixed with the coadjuvant 0.1% INEX-A in deionized water.

The study also included a rotation of commercial foliar fungicides mixed with the coadjuvant 0.1% INEX-A in deionized water (Table 2.10.1) and a rotation of fungicides and phosphites in the concentrations already mentioned in two applications per week. Checks were uninoculated plants that were (1) treated with 0.5% phosphites and (2) not treated. These plants were isolated from the other treatments by plastic separators.

Table 2.10.1. Rotation of the fungicides used to evaluate the effect of phosphites on the incidence and severity of downy mildew of roses.

Day	Fungicide	Active ingredient	Concentration in water (g/L)
	Before inoculation		
1	Aliette®	Fosetyl-al	1.5
4	Invento®	Propineb	13.0
8	Forum®	Dimethomorph	0.6
11	Previcur®	Propamocarb	1.25
15	*Inoculation*		
18	Mildex®	Fenamidone	1.0
22	Sandofan-M®	Oxadixyl	1.6
25	Invento®	Propineb	13.0
29	Previcur®	Propamocarb	1.25
32	Mildex®	Fenamidone	1.0

Applications of Agrifos® began 2 weeks before the plants were inoculated and continued two times a week for the 34 days of the experiment. Inoculation involved applying suspensions of the pathogen at a concentration of 3×10^4 sporangia/mL under greenhouse conditions, as described previously. Disease incidence was evaluated throughout the experiment, using a scale of severity that was based on the expression of foliar symptoms. That is, the percentage of infected foliar area was determined in young leaves from the upper stratum of the plant and in mature leaves from the middle stratum.

The experimental unit for each variety was six plants for each of the treatments with phosphites (4 replications) and without phosphites (3 reps), and four for each of the treatments (a) rotation of fungicides (2 reps), (b) rotation of Agrifos® and fungicides (4 reps), (c) uninoculated check with Agrifos® (2 reps), and (d) absolute check with absence of pathogen and no fungicide (2 reps).

Results and Discussion

Applying potassium phosphites two times a week until the end of the experiment effectively controlled the disease. By day 34, when the evaluations were finalized for variety Charlotte, incidence had increased from the day of inoculation by 8.9% under the phosphite treatments and 20.1% under the fungicides. Plants that had not been treated with phosphites showed an increase of 51.0%. The lowest incidence, at 11.5%, was observed for the check with Agrifos®. The absolute check showed an incidence of 34.0% (Table 2.10.2). In inoculated plants, severity increased by 0.8% (phosphites), 1.4% (fungicides), and 45.6% (no phosphites or fungicides) (Table 2.10.3).

Table 2.10.2. Effectiveness of two foliar applications of phosphites per week on the incidence (%) of downy mildew of roses in variety Charlotte.

Treatment	Day						
	1	4	10	16	20	25	34
Phosphites	0.0	0.0	1.7	4.0	17.9	12.3	8.9
No phosphates	0.0	2.8	0.0	40.4	41.8	48.4	51.0
Fungicides[a]	0.0	1.3	0.0	12.3	12.1	17.3	20.1
Phosphites + fungicides[a]	0.0	7.1	12.6	18.5	12.8	26.0	20.8
Check (uninoculated plants) + phosphites	0.0	4.9	10.8	12.9	7.7	17.5	11.5
Check (uninoculated plants), no applications	0.0	2.1	14.2	19.3	34.0	29.8	34.0

a. Mildex®, Aliette®, Invento®, Sandofan-M®, Forum®, and Previcur®.

Table 2.10.3. Effectiveness of two foliar applications of phosphites per week on the severity (% of foliar area infected) of downy mildew of roses in variety Charlotte.

Treatment	Day						
	1	4	10	16	20	25	34
Phosphites	0.0	0.0	0.9	1.9	5.0	1.3	0.8
No phosphites	0.0	3.4	5.7	14.7	16.6	17.6	19.4
Fungicides[a]	0.0	1.9	1.9	3.9	1.8	2.8	3.3
Phosphites + fungicides[a]	0.0	2.1	6.6	8.1	5.2	7.7	7.0
Check (uninoculated plants) + phosphites	0.0	2.2	4.5	4.5	2.6	4.9	2.6
Check (uninoculated plants), no applications	0.0	0.9	2.8	2.8	9.8	7.5	9.7

a. Mildex®, Aliette®, Invento®, Sandofan-M®, Forum®, and Previcur®.

In variety Malibu, the effect of potassium phosphites on incidence (38.9%) was not as marked as for variety Charlotte. Incidence with fungicide treatment was 38.0%, and for plants without phosphites, the increase was 38.5%. In check plants with absence of the pathogen, the lowest incidence observed was for the treatment with phosphites (Table 2.10.4). The least increase in severity was observed for plants of the Malibu variety inoculated with phosphites (4.1%) and the highest values were for plants with fungicides (6.8%) and no phosphites (9.3%) (Table 2.10.5).

Table 2.10.4. Effectiveness of foliar application of phosphites on the percentage of incidence of downy mildew of roses in variety Malibu.

Treatment	Day						
	1	4	10	16	20	25	34
Phosphites	0.0	2.4	17.2	26.7	24.6	41.7	38.9
No phosphites	0.0	2.9	4.1	30.2	29.8	61.5	38.5
Fungicides[a]	0.0	6.8	3.3	33.2	58.5	34.3	38.0
Phosphites + fungicides[a]	0.0	2.9	3.0	8.6	12.1	19.3	44.4
Check + phosphites	0.0	0.0	11.6	15.3	14.6	40.8	41.2
Check	0.0	0.0	2.2	13.7	24.7	52.5	52.5

a. Mildex®, Aliette®, Invento®, Sandofan-M®, Forum®, and Previcur®.

Table 2.10.5. Effectiveness of foliar application of phosphites on the severity of downy mildew of roses in variety Malibu.

Treatment	Day						
	1	4	10	16	20	25	34
Phosphites	0.0	1.6	4.1	4.7	6.3	6.6	4.1
No phosphites	0.0	0.3	2.5	12.2	11.3	14.9	9.3
Fungicides[a]	0.0	2.7	1.2	10.1	14.2	7.0	6.8
Phosphites + fungicides[a]	0.0	1.2	5.5	3.1	3.1	4.8	1.4
Check + phosphites	0.0	0.0	0.0	7.8	3.9	7.1	6.3
Check	0.0	0.0	0.6	5.0	8.8	4.7	3.9

a. Mildex®, Aliette®, Invento®, Sandofan-M®, Forum®, and Previcur®.

Potassium phosphites (mono- and di-potassium phosphites) reduced the incidence of downy mildew of roses by 42.1% and severity by 18.6% in variety Charlotte, compared with the treatment without phosphites. In variety Malibu, severity was reduced by 5.2%, compared with the treatment without phosphites. Severity of disease observed in varieties Charlotte and Malibu gradually increased over time. However, the treatment with phosphites on inoculated plants was observed to reduce the progress of the disease, compared with the treatments of fungicides alone and fungicides with phosphites, demonstrating that phosphites have an inhibitory effect on downy mildew of roses.

Foliar treatment with phosphites of rose plants of the variety Charlotte before exposure to the pathogen reduced incidence and severity of downy mildew to a greater extent than did rotations with fungicides and rotations of phosphites with fungicides. Likewise, potassium phosphites had previously been shown to control downy mildew of grapevines (*Plasmopara viticola*), preventing sporulation of the fungus and colonization of leaves (Reuveni 1997, J Small Fruit & Viticult 5(2):27–38).

Activity 2.11. Microbiological and physicochemical evaluation of lixiviates from decomposing plantain rachises and pseudostems and their effectiveness in managing bacterial wilt

Contributors: E. Álvarez, L. A. Mesa, V. H. Triviño, G.Llano, and J. Loke

Rationale

The plantain crop is affected by the vascular disease *moko* or bacterial wilt, caused by *Ralstonia solanacearum*. Currently, this disease is causing significant losses in Colombia, but it has not been successfully controlled because of a lack of effective management technologies and the nonexistence of resistant plantain varieties. Hence, research is needed to discover efficient alternatives that can be applied at low cost within an integrated management program, while generating a favorable impact on the environment. Preventive management of bacterial wilt of plantain is an excellent approach towards controlling the pathogen. This approach involves the use of natural substances extracted from organic residues such as lixiviate of compost of plantain rachises, pseudostems, and fruit.

Activity 2.11.1. Identifying microorganisms present in lixiviate from decomposing plantain rachises and pseudostems

Highlight:

- Lixiviates from decomposing plantain rachises and pseudostems contain bacteria that are useful for releasing nutrients and for acting as possible antagonists of pathogens.

Materials and Methods

Samples, from which the bacterial strains under study were obtained, came from the plantain variety Dominico Hartón, grown on seven farms located in the Department of Quindío, Colombia.

Isolating the bacteria: To isolate the microorganisms present in samples of lixiviates, we used a nutrient agar culture medium. The organisms were incubated for 24 h at 28ºC, after which different colonies were selected according to their morphology. The potassium hydroxide test was conducted on the various isolates to differentiate between Gram-negative and Gram-positive microorganisms.

Morphology of the bacteria assessed: To identify the type of microorganisms found in the lixiviate samples, we planted them in different culture media that were specific to different types of microorganisms (Table 2.11.1.1).

Table 2.11.1.1. Culture media used to characterize microorganisms in lixiviate from compost of plantain rachises and pseudostems and in a mixture of lixiviate from compost of plantain rachis, phosphoric rock, and french marigold.

Culture medium	Specific to:
Yeast extract, dextrose, and calcium carbonate (YDC)	*Xanthomonas, Erwinia*
Medium B of King et al. (KB)	*Pseudomonas fluorescens*
Casein agar and glucose (CAG)	*Bacillus*
Nystatin, polymyxin, penicillin, cycloheximide (NPPC)	*Streptomyces*
Salmonella–Shigella agar (SS)	*Salmonella, Shigella*
MacConkey	Enterobacteriaceae

Pure strains grown on nutrient agar with 24 h of incubation were planted on different culture media to observe their growth and later conduct biochemical tests to identify each microorganism.

Results and Discussion

We obtained 22 bacterial isolates, of which 8 were Gram-negative and 14 Gram-positive, according to the KOH test. Table 2.11.1.2 presents the results of the microbiological analyses conducted on samples of lixiviate from the decomposition of various plantain parts. The largest number of bacteria were isolated from lixiviate of rachis.

Table 2.11.1.2. Presumed identification of bacteria present in four sources of lixiviates of plantain compost.

Rachis	Pseudostem	Mixture[a]	Fruit
Bacillus	*Bacillus*	*Bacillus*	*Listeria*
Klebsiella oxytoca	*Streptococcus*		*Staphylococcus*
Actinobacillus	*Acinetobacter*		
Eikenella			
Pseudomonas			
Proteus vulgaris			

a. Lixiviate of rachis, phosphoric rock, and french marigold.

We did not identify the bacteria *Escherichia coli* or *Salmonella* spp., corroborating the results obtained by Larco (2004. Desarrollo y evaluación de lixiviados de compost y lombricompost para el manejo de sigatoka negra (*Mycosphaerella fijiensis* Morelet), en plátano. Master of Science Thesis. Program of Education for Development and Conservation, Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Turrialba, Costa Rica), who reported the absence of *Salmonella* in lixiviate from banana and plantain compost. Through biochemical tests, we identified *Pseudomonas* bacteria,

which are ecologically important microorganisms found in the soil and probably responsible for the degradation of many soluble compounds that derive from the monomeric rupture of plant materials in oxygenated habitats. These organisms are typically aerobic and contribute to the decomposition and discharge of nutrients, attacking the organic substrate, including humic acids and synthetic pesticides (Bess VH. 1998. BBC Laboratories, Inc.). *Proteus* bacteria were found because they habituate soils, residual waters, and manure.

Conclusions

The lixiviates, being products generated by organic decomposition, presented different types of microorganisms according to their origin. Notable among them were beneficial bacteria, responsible for the initial and final stages of decomposition. To ascertain its innocuousness, a more specific microbiological characterization of lixiviate of plantain compost must be made.

Through this study, we identified the bacteria present in samples of plantain lixiviates and demonstrated that the variety of microorganisms changed according to the source of lixiviate.

Activity 2.12. Physicochemical characterization of lixiviates from Decomposing rachises, pseudostems, and fruit of plantain

Contributors: E. Álvarez, L. A. Mesa, V. H.Triviño, G. Llano, and J.Loke

Highlight:

- Lixiviates from decomposing plantain rachises, pseudostems, and fruit are ideal ecological resources for use in managing the disease *moko* or bacterial wilt. These lixiviates contain various nutrients and minerals, in particular, high levels of potassium and manganese, which help reduce the disease.

Materials and Methods

To determine the characteristics of lixiviates and thereby recommend suitable use in both their management and application, we conducted various analyses. We used molecular spectrophotometry to identify nitrogen, phosphorus, nitric nitrogen, ammoniac nitrogen, sulfur, boron, and carbon. To identify potassium, we used atomic absorption spectrophotometry (AAS), and the atomic absorption technique for calcium, magnesium, copper, zinc, manganese, and iron (García MN. 2005. Manual de métodos de análisis del laboratorio de servicios analíticos (LSD) CIAT, Cali, Colombia)

To physically characterize the 10 sources of lixiviates from plantain compost collected from seven farms, we visually determined the color of each sample.

Results and Discussion

Lixiviate from fruit was black, whereas lixiviates from other plant parts were either light or dark brown. Lixiviates from rachises also differed among themselves in color (Table 2.12.1). These results on color characteristics agree with those reported by Paúl and Clark (Paúl EA; Clark FE. 1996. Soil microbiology and biochemistry, 2nd ed. Academy Press) when they described a commercially acceptable compost.

Table 2.12.1. Colors of 10 lixiviates from decomposing plantain rachises, pseudostems, and fruit collected from seven farms, Colombia.

Origin	Source of lixiviate	Color
La Yalta Farm, Armenia	Rachis	Dark coffee brown
La Guaira Farm, Montenegro	Rachis	Light coffee brown
Las Américas Farm, Quimbaya	Rachis	Dark coffee brown
Las Américas Farm, Quimbaya	Fruit	Black
Guadualito Farm, Montenegro	Rachis	Dark coffee brown
Santa Elena Farm, Armenia	Rachis	Dark coffee brown
La Diana Farm, Armenia	Rachis	Dark coffee brown
La Diana Farm, Armenia	Pseudostem	Light coffee brown
La Diana Farm, Armenia	Mixture[a]	Light coffee brown
La Manigua Farm, Armenia	Rachis	Light coffee brown

a. Mixture of lixiviate from decomposing rachis and phosphoric rock.

Table 2.12.2 shows the results of chemical analyses of lixiviate from decomposing rachises and pseudostems of plantain and a mixture (phosphoric rock, french marigold, and lixiviate of rachis) obtained from different farms in the Department of Quindío. Differences clearly existed among macroelements, according to the source and origin of lixiviates from compost analyzed in this study.

The largest percentage of phosphorus in lixiviate was found at Guadualito Farm with 404.93 mL/L. The smallest quantity (11.91 mL/L) of phosphorus was found at La Manigua Farm. Except for copper, the overall contents of elements in lixiviate of pseudostem was 10 times less than for lixiviate of rachis. The values for most elements were higher for fruit than for rachises and pseudostems. The average pH value ranged from 8 to 9, except for a pH 3.9 for lixiviate prepared from fruit at Las Américas Farm.

The chemical analyses of lixiviate indicated high concentrations of potassium in most samples. This element tends to be associated with inducing resistance to some diseases (Grajales CX; Villegas J. 2002. Control de *Sphaerotheca pannosa* var. *rosae* en rosas mediante la utilización de lixiviado de compost de rachis de plátano. Faculty of Agroindustrial Engineering, Universidad de San Buenaventura, Santiago de Cali, Colombia). For iron, values were higher than those reported. The lowest value (4.35 mg/L) for ammoniac nitrogen was found at La Manigua Farm and the highest (212.91 mg/L) was for lixiviate from fruit.

The correlation between disease progress of bacterial wilt (expressed as the area under the wilt progress curve over 7 weeks or AUWPC) in trials conducted in the greenhouse and the chemical composition of five lixiviates showed values of -0.77 for potassium content and -0.75 for manganese content. These values indicated that these two elements helped reduce the disease's advance.

The chemical analyses of different sources of lixiviate showed that most presented high values for phosphorus, possibly because of the high quantity of residues (rachises, leaves, pseudostems, and corms) generated in each harvest of banana and plantain. These results agree with those reported by Muñoz (2003. Efecto de los lixiviados producto del proceso de descomposición del rachis del plátano sobre la actividad y biomasa microbiana en épocas de floración y cosecha del tomate *Lycopersicum sculentum* Miller. Faculty of Agricultural Sciences, Universidad Nacional de Colombia, Palmira, Colombia.).

Lixiviates are an ideal resource for use in managing bacterial wilt, as the quantity of nutrients and minerals released is high, especially of potassium and manganese. Another important aspect is the quantity of water present in these residues, as it facilitates rapid decomposition and transformation into organic matter (Mojica E. 1994. Atlas agropecuario de Costa Rica: Suelos de Costa Rica, 1st ed. Edited by G. Cortés. Universidad Estatal a Distancia, San José, Cost Rica. p 29–30). Moreover, lixiviates can be applied in the field or greenhouse to manage diseases or as fertilizer.

Conclusions

Lixiviates produced from decomposing plantain rachises and fruit contain high concentrations of potassium. The chemical composition of lixiviates shows that a relationship exists between the quantities of potassium and manganese in lixiviates and reduced severity of the disease.

The chemical analysis of sources of lixiviate showed that most lixiviates were high in phosphorus.

Table 2.12.2. Chemical composition of samples of lixiviate from decomposing rachises, fruit, and pseudostems of plantain from seven farms in the Department of Quindío and one farm in Ariari (Meta), Colombia.

S[a]	Source (farm and tissue)	pH (null)	C (mg/L)	N (mg/L)	P (mg/L)	K (mg/L)	Ca (mg/L)	Mg (mg/L)	S (mg/L)	B (mg/L)	Na (mg/L)	Fe (mg/L)	Mn (mg/L)	Cu (mg/L)	Zn (mg/L)	N (NH_4) (mg/L)	N (NO_3) (mg/L)
1	Las Américas, rachis	8.67	2780.00	420.09	157.95	18,185.42	82.92	38.66	111.52	0.83	12.55	2.67	1.25	0.16	0.29	6.98	0.00
2	Las Américas, fruit	3.91	11,680.00	1781.61	375.68	11,343.41	5137.17	824.74	197.37	6.61	22.34	2949.81	23.32	0.11	10.33	212.91	4.52
3	La Diana, rachis	8.38	1640.00	278.55	317.16	16,332.48	74.75	51.68	149.93	0.86	7.51	2.94	1.39	0.00	0.31	19.20	0.01
4	Santa Elena, rachis	9.28	1565.00	249.30	212.00	20,937.91	41.37	34.77	183.82	0.67	28.54	1.75	1.01	0.16	0.53	6.27	0.00
5	Ariari (Meta), rachis	9.36	2690.00	466.58	311.05	26,680.96	51.77	51.62	398.46	2.38	6.77	3.82	4.12	0.00	23.36	7.44	0.00
6	Guadualito, rachis	9.34	2996.07	893.38	404.93	28,838.90	69.11	31.30	206.50	0.56	6.05	3.05	1.91	0.04	0.60	144.64	0.00
7	La Guaira, rachis	8.58	2160.06	205.10	187.88	15,588.58	64.58	43.37	55.72	0.12	6.13	0.50	0.51	0.00	0.06	47.21	0.00
8	La Manigua, rachis	8.54	408.95	45.76	11.91	324.83	5.54	2.04	0.00	0.00	4.91	1.62	0.41	0.00	0.27	4.35	2.88
9	Mixture[b]	8.89	2.67	283.63	367.40	18,391.39	379.76	59.82	86.75	2.19	5.40	40.14	0.00	0.00	0.82	32.65	0.00
10	La Diana, pseudostem	8.43	1.29	70.03	66.89	4,458.62	44.91	22.65	21.46	0.98	1.46	0.88	0.00	2.23	0.01	24.97	0.00
11	La Yalta, rachis	8.97	2.84	217.82	161.59	12,749.92	46.89	24.45	87.60	1.16	5.62	0.69	1.11	0.11	0.28	73.24	0.00

a. S = sample.
b. Mixture of phosphoric rock, french marigold, and lixiviate of rachis.

Activity 2.13. Detecting *Ralstonia solanacearum* in lixiviates from decomposing rachises and pseudostems of plantain.

Contributors: E. Álvarez, L. A. Mesa, V. Triviño, J. Loke, and G. Llano

Rationale

Moko, maduraviche, or *ereke* is a bacterial wilt of plantain and banana caused by *Ralstonia solanacearum.* It is the most important bacterial disease of these crops in Colombia, affecting 125,000 families who depend directly on them for their livelihoods. The use of lixiviate from decomposing plantain rachis has been effective as a practice for managing the disease. The presence of *R. solanacearum* in samples of lixiviate from decomposing plantain residues is a phytosanitary risk if it is applied directly to the crop without first verifying if the pathogen is absent. In this study, we determined whether *R. solanacearum* is present in lixiviates.

Materials and Methods

The pseudostems of 6-week-old plantain seedlings, variety Dominico Hartón, were inoculated with 0.5 mL of lixiviate obtained from plantain harvest residues from the Department of Quindío, specifically from the farms of La Guaira, La Manigua, Santa Elena, Guadualito (rachises only), Las Américas (fruit and rachises), La Diana (rachises; pseudostems; and mixture of lixiviate of rachis, phosphoric rock, and french marigold or *Tagetes patula*), and La Yalta (rachises). We used a randomized complete block design, with five replications and an experimental unit of two plants. We evaluated the effect of different sources of lixiviate, using the *R. solanacearum* strain CIAT No. 78 as a positive check and sterilized deionized water as the negative check. The inoculated plants were kept for 3 days under constant wetting and later microsprayed every 24 h for 26 days.

Evaluations of severity were conducted daily, taking into account the development of symptoms of wilt between Days 5 and 30 and recording the appearance of symptoms such as flaccidity in leaves and wilt. To measure disease development, a scale of 0 to 6 was generated, where:

0 = absence of symptoms

1 = leaves presenting flaccidity

2 = leaves showing a slight but noticeable wilt, not only in their shape but also in the loss of their intense green color

3 = leaves showing a highly noticeable flaccidity and, in some cases, yellowing

4 = leaves showing yellowing with necrosis in some sites and highly advanced flaccidity, losing their shape

5 = advanced necrosis and leaves have totally lost their turgidity

6 = plants are entirely dead

In addition to the experiment described above, the SMSA medium was added to two petri dishes and, in each dish, a 0.1-mL sample of each lixiviate was suspended. Incubation was carried out at 28∀C. The dishes were examined every day for the pathogen's presence. Possible colonies were purified and incubated for 2 weeks. The samples were evaluated three times during the experimental period.

Results and Discussion

Healthy plants that had been inoculated with 10 different sources of lixiviate showed no symptoms of the disease. The positive check inoculated with *R. solanacearum* showed typical symptoms of the disease. Some plantain seedlings injected with lixiviate of fruit presented leaves showing some small burns. When these tissues showing burns were cultured on SMSA medium, no *R. solanacearum* isolates were obtained. This symptom was probably caused by a phototoxic substance in the lixiviate or low pH.

Lixiviates planted in SMSA medium did not show colony growths typical of *R. solanacearum*, thus confirming the results obtained in the greenhouse trial.

The absence of *R. solanacearum* in the lixiviate samples collected from the seven farms therefore ascertains the innocuousness of lixiviates for plantain crops for either managing bacterial wilt and sigatoka or using as biofertilizer (García E; Apezteguia H. 2001. Estudio del lixiviado de compost y su efecto sobre el control de sigatoka negra (*Mycosphaerella fijiensis* Morelet) y el crecimiento del cultivo de banano (Musa AAA). Thesis in Agronomy. EARTH, Guácimo, Costa Rica; Larco E. 2004. Desarrollo y evaluación de lixiviados de compost y lombricompost para el manejo de sigatoka negra (*Mycosphaerella fijiensis* morelet), en plátano. MSc thesis. Program of Education for Development and Conservation, Centro Agronómico Tropical de Investigación y Enseñanza (CATIE), Turrialba, Costa Rica).

Conclusions

The different sources of lixiviate evaluated were considered not to contain *R. solanacearum* because the plantain plants inoculated with the lixiviates did not develop symptoms of the disease after 30 days of evaluations under greenhouse conditions. The absence of *R. solanacearum* from the lixiviate samples evaluated demonstrated that applications of lixiviate of plantain compost do not cause either residual or pathogenic effects on plantain plants, thus indicating its suitability for use in this type of crop.

Activity 2.14. Identifying live and dead cells of *Ralstonia solanacearum* exposed to lixiviates from plantain residues, phosphoric rock, and french marigold

Contributors: E. Álvarez, L. A. Mesa, V. Triviño, and J. Loke

Rationale

Laboratory trials on culture medium and in the greenhouse showed that lixiviate of plantain rachis and pseudostem inhibits the *R. solanacearum* bacterium. One limitation of these tests is that nobody knows the effect lixiviates have on the viability of the pathogen's inhibited cells. The bacterial cells could live without multiplying and thus continue to be a phytosanitary risk.

Materials and Methods

Bacterial viability is determined by using the fluorescence kit LIVE/DEAD® L-13152 (Molecular Probes, Leiden, Netherlands), which contains two nucleic acid markers. The fluorochrome Syto9 is a small molecule that can penetrate bacteria that possess an intact plasmatic membrane, giving off a green fluorescence when observed under a Zeiss Axiolab epifluorescent microscope. The fluorochrome propidium iodide (PI) penetrates damaged membranes, which are therefore not viable, giving off a red fluorescence (Defives et al. 1999). The bacterial strain used was CIAT No. 78, isolated from plantain rachis from Montenegro (Quindío, Colombia). Treatments were:

1. Cumbre® (gentamicin sulfate at 10.7% and oxytetracycline hydrochloride at 32.3%)
2. Lixiviate from decomposing rachis of plantain variety Dominico Hartón from La Guaira Farm
3. Lixiviate of rachis from Las Américas Farm
4. Lixiviate from decomposing pseudostem from La Diana Farm with phosphoric rock (29% P_2O_5)
5. French marigold (*Tagetes patula*)
6. Mixture of lixiviate of rachis compost from La Diana Farm with phosphoric rock and french marigold

As checks, we used:

1. Components A (Syto9) and B (propidium iodide) from the kit LIVE/DEAD® without *R. solanacearum*
2. Components A and B with the bacterium
3. Sterilized deionized water
4. Sterilized deionized water with the bacterium
5. Ethanol at 70% (Molecular Probes, 2004)
6. Ethanol at 70% with the bacterium

The treatments were filtered twice with a vacuum pump using Whatman No. 1 filter paper and once through a 0.2-µm Millipore filter (Whatman). They were then stored in sterilized, BD Falcon™, centrifuge tubes of 50 mL. They were kept sealed with Parafilm® and conserved at -80°C. From each solution, 2 mL were placed per microcentrifuge Eppendorf tube, sterilized twice at 121°C and under 20-lb pressure for 20 to 30 min.

Preparing inoculum: A bacterial suspensión was prepared in sterilized deionized water. Under aseptic conditions, the concentration of the suspensión was determined in a Turner spectrophotometer, model 390, with a wavelength of 600 nm, using Fisher cells (ref. Spectronic 20). The concentration was adjusted by diluting with sterilized deionized water until a 0.3 absorbance was obtained, corresponding to about 1×10^6 colony-forming units per milliliter (He et al. 1983).

Observing R. solanacearum cells tinted with LIVE/DEAD® L-13152 with the epifluorescent microscop: We took 100 µL of *R. solanacearum* and added them to the Eppendorf tubes that contained the treatments and vortexed. In another 0.5-mL tube, we mixed the Syto9 coloring (component A) with 0.75 mL of propidium iodide dye (component B). The tube with the mixture of colorings was wrapped in aluminum foil to keep out light and conserved at -20ºC. We took 0.25 µL from each Eppendorf tube containing a treatment with the bacteria and placed them on one microscope slide per treatment. Immediately, 0.25 µL of the mixture of colorings were also added. The mixture was gently moved about with the point of a micropipette and a cover slip placed over it. The slides were kept in darkness for 15 min, after which time, the sample was examined under an epifluorescent microscope with a wavelength of 490 nm to determine the percentage of live (green) and dead bacterial cells (red) in the sample.

Results and Discussion

The total count of the bacterial population was obtained by counting the percentages of green (viable) and red (dead) cells that were observed in the field of the epifluorescent microscope. Observation over time allowed us to determine the number of days needed for each product analyzed to act on the *R. solanacearum* cells. On Day 2, we observed that the mixture of phosphoric rock, french marigold, and lixiviate of plantain rachis from the La Diana Farm destroyed 80% of the pathogen's cells (Table 2.14.1).

Table 2.14.1. Microscopic observations conducted with the kit LIVE/DEAD® backlight L-13152 to determine the effect of different types of lixiviate on *Ralstonia solanacearum* cells.

Treatment	Inocul. with *R. solanac.*	Red cells (dead) of *R. solanacearum* (%) at days after experiment begins						
		1	2	5	7	14	21	24
Checks								
1 Components A (Syto9) + B (PI) from the kit	No	0	0	0	0	0	0	0
2 Components A (Syto9) + B (PI) from the kit	Si	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
3 Sterilized water	No	0	0	0	0	0	0	0
4 Ethanol at 70%	No	0	0	0	0	0	0	0
5 Sterilized water	Yes	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
6 Ethanol at 70%	Yes	100	100	100	100	100	99.9	100
Products								
1 Gentamicin and tetracycline[b]	Yes	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
2 Lixiviate[c] of plantain rachis (La Guaira Farm, Montenegro)	Yes	0[a]	0[a]	25	25	50	90	99.9
3 Pure lixiviate[c] of plantain rachis (Las Américas Farm, Quimbaya)	Yes	0[a]	0[a]	10	0[a]	0[a]	99	99.9
4 Lixiviate[c] of plantain pseudostem (La Diana Farm, Armenia)	Yes	0[a]	0[a]	0[a]	20	20	20	50
5 Phosphoric rock[d]	Yes	0[a]	0[a]	40	20	10	20	40
6 French marigold[e]	Yes	0[a]	0[a]	2	60	90	90	99.9
7 Lixiviate[c] of plantain rachis, with phosphoric rock[d] and french marigold[e] (La Diana Farm, Armenia)	Yes	0[a]	80	90	90	90	100	100

a. Green.
b. Cumbre® (gentamicin sulfate at 10.7% and oxytetracycline hydrochloride at 32.3%).
c. At a pure concentration, with no water added.
d. Concentration at 30 g/50 mL.
e. Concentration at 10 g/50 mL.

Conclusions

Through epifluorescent microscopy, exposure to the mixture of lixiviate from decomposing plantain rachis, phosphoric rock, and french marigold was shown to kill all the cells of the pathogenic bacterium *R. solanacearum.* Lixiviates without disinfection by phosphoric rock and french marigold killed most of the cells, but with a small quantity still viable.

This is the first study that demonstrated through microscopy the effect of different types of lixiviates on the viability of the *R. solanacearum* bacterium.

Activity 2. 15. Determining the Control of Bacterial Wilt in Plantain Seedlings by Different Types of Lixiviate

Contributors*:* E. Álvarez, L. A. Mesa, V. Triviño, J. Loke, and G.Llano

Rationale

Different greenhouse trials were conducted to discover the effect on plantain seedlings of applying lixiviates of rachis and pseudostem to the soil. The idea was to evaluate the potential of lixiviates as an ecological practice for controlling the disease.

Trial 1: Inoculating plantain plants with *Ralstonia solanacearum* at different concentrations

Materials and Methods

We aimed to determine (1) the time between inoculation with *R. solanacearum* and expression of symptoms of bacterial wilt, and (2) the minimum concentration of inoculum needed to produce symptoms. We used the *R. solanacearum* strain No. 78 from the collection held at CIAT, first growing it for 24 h in nutritive agar. We then prepared three suspensions of the strain in sterilized deionized water at different concentrations. Under aseptic conditions, the concentration of each suspension was determined by reading the absorbance in a Turner spectrophotometer, model 390, with a wavelength of 600 nm, using Fisher cells (ref. Spectronic 20). The concentrations were as follows:

Absorbance 0.5 = 1×10^8 colony-forming units (cfu)/mL
Absorbance 0.3 = 1×10^6 cfu/mL
Absorbance 0.1 = 1×10^3 cfu/mL (He et al. 1983)

The inoculation method used was injection with sterilized 1-mL syringes and needle size 27G × 1/2". For each suspension, 0.5 mL was inoculated into the pseudostem of each of eight plantain plants at a height of 2 cm from the soil surface. Another eight plants were used as the negative control and were injected with sterilized deionized water. The 32 inoculated plants were then placed in three randomized complete blocks and left to incubate in a humid chamber for 3 days before being microsprayed for 30 days. The plants were evaluated daily between Days 5 and 30 after inoculation.

Results and Discussion

After inoculating plantain seedlings with *R. solanacearum,* the disease's progress was observed under controlled conditions to determine the speed at which the pathogen infected the plants. Of the three concentrations tested, those seedlings receiving 1×10^8 cfu/mL had symptoms by Day 7, and by Day 18 they were dead. The disease was also observed to advance by the vascular system, as described by Gómez (Gómez E. 2005. Aislamiento, identificación y caracterización del agente causal del moko del plátano, *Ralstonia solanacearum* raza 2, proveniente de plantaciones afectadas en Colombia. Faculty of Sciences, Pontificia Universidad Javeriana, Bogotá, DC, Colombia), beginning at the site of inoculation and advancing along the stems to the leaves. The trial showed that the more concentrated the inoculum, the more quickly the disease was expressed and the more severe the symptoms (Table 2.15.1).

Table 2.15.1. Development of bacterial wilt in plantain seedlings inoculated with different concentrations of *Ralstonia solanacearum* and evaluated for 3 weeks under greenhouse conditions.

Treatment (concentration of *R. solanacearum* at cfu/mL)		Disease progress at day of evaluation[a]		
		7	14	21
1	Water	0	0	0
2	1×10^3	0.8	1.5	4.0
3	1×10^6	2.2	3.0	5.0
4	1×10^8	2.5	3.5	6.0

a. Scale of 0 to 6, where 0 is absence of disease and 6 is plant death.

Conclusions

Through this trial we showed that the three concentrations of inoculum of *R. solanacearum* caused plant death within 3 weeks. By using different concentrations of inoculum of the pathogen, we could better assess the lixiviates' capacity to reduce the damage caused by bacterial wilt.

Trial 2: Inoculating sterilized soil with *Ralstonia solanacearum*

Materials and Methods

The goal was to identify the minimum bacterial concentration detectable in inoculated sterilized soil. We used dilution methodology to determine concentrations and indicator plants to establish disease progress. We first grew the *R. solanacearum* strain CIAT No. 78 for 24 h in nutritive agar and then prepared suspensions of it in sterilized deionized water. Under aseptic conditions, each suspension's concentration was determined by reading its absorbance from the spectrophotometer. The concentration of each bacterial suspension was adjusted with dilutions in sterilized deionized water starting with absorbance 0.3. We added 1 mL in a test tube, completing to 100 mL with sterilized deionized water. From this solution, 1 mL was taken and completed to 100 mL with sterilized deionized water in a test tube. For the last tube, the same procedure was carried out to obtain the fourth concentration. The concentrations of the bacterial suspension were as follows:

Treatment no. 1 = 1×10^8 colony-forming units per milliliter (cfu/mL)
Treatment no. 2 = 1×10^6 (cfu/mL)
Treatment no. 3 = 1×10^4 (cfu/mL)
Treatment no. 4 = 1×10^2 (cfu/mL)
Negative check = Inoculation with sterilized deionized water

For each concentration, 30 mL of bacterial solution was placed in flowerpots containing soil previously sterilized at 121°C under 20 lb of pressure for 30 min. Later, 9-week-old plantain plants were planted into each pot. After inoculating the soil, the plants were incubated in a humid chamber (90% RH) for 3 days. The plants were then transferred to a

greenhouse and microsprayed at intervals of 1 min throughout the day. On Day 7 after inoculation, evaluations were begun, continuing for 1 month.

We inoculated 50 plants, distributed across 5 treatments, with 5 replications and a negative check. The experimental unit was 2 plants in a randomized complete block distribution. Evaluations were carried out every day for 30 days, checking all leaves on each plant to determine wilt. If a leaf presented symptoms of the disease, a tissue sample was taken from the infected plant and cultured onto the semi-selective SMSA medium and again inoculated onto other healthy plantain plants.

Results and Discussion

The healthy plants planted into soil inoculated with *R. solanacearum* presented typical symptoms of the disease, starting from Day 10 (Table 2.15.2).

Table 2.15.2. Reaction of healthy plants to applications of different concentrations of *Ralstonia solanacearum* to the soil.

Treatment (concentration of *R. solanacearum* at cfu/mL)		**Disease progress[a] at day after inoculation**			
		7	**14**	**21**	**28**
1	Water	0	0	0	0
2	1×10^2	0	0.2	0.5	1.2
3	1×10^4	0	0.3	0.7	1.6
4	1×10^6	0	0.8	1.2	2.0
5	1×10^8	0	0.8	2.2	4.3

a. Scale of 0 to 6, where 0 is absence of disease and 6 is plant death.

Table 2.15.2 shows that, at higher bacterial concentrations, symptoms of the disease were expressed in the plantain plants in less time and with greater severity. The table also shows that when the bacterium is inoculated into the soil, symptoms take longer to manifest in the plants (10 days) than when it is directly inoculated into plants (7 days). The disease appeared even at concentrations of 1×10^2 cfu/mL, which is the equivalent to 3000 colonies per flowerpot.

Trial 3: Applying lixiviate before and after inoculating seedlings with *Ralstonia solanacearum*

Materials and Methods

To evaluate the effectiveness of lixiviate of plantain in managing bacterial wilt, 30 mL of pure lixiviate was applied to the soil before or after inoculating with the pathogen. Lixiviate was obtained from plantain compost from the following farms: Las Américas, La Guaira, La Manigua, Santa Elena (all rachises), and La Diana (pseudostem and a mixture of lixiviate of rachis, phosphoric rock, and french marigold). All the farms were located in the Department of Quindío, Colombia.

In the greenhouse, 6-week-old plantain plants were planted in polypropylene sacks with a 1-kg capacity and containing a mixture of sterilized sand and soil at a rate of 3:2. One set of 168 plants were inoculated on Day 15 after planting, using sterilized 1-mL syringes. Their pseudostems were inoculated at a height of 2 cm from the soil surface with a concentration of absorbance 0.1, determined as previously described for preparing inoculum for plantain plants. Each plant received 0.2 mL of the suspension. After inoculation, 30 mL of lixiviate from different sources (rachises, pseudostems, and fruit) was applied at 100% to the soil of each of the 168 plants. Another set of 168 plants received lixiviate before they were inoculated with *R. solanacearum*.

We established 46 treatments, the experimental unit being 3 plants. The experimental design was split-plot in different blocks separated by treatment, with 4 replications. The main plot was the time of applying lixiviate (before or after inoculation) and the subplot was the source of lixiviate. Lixiviate was applied at Days 3, 7, and 15 before inoculation and at Days 3, 5, and 7 after inoculation.

To establish the positive check, 72 plants were inoculated with the *R. solanacearum* strain CIAT No. 78. The negative check comprised another 72 plants that were inoculated with sterilized deionized water and also received the antibiotic Cumbre® (gentamicin sulfate at 10.7% and oxytetracycline hydrochloride at 32.3%) at 8 g/L, injecting 0.5 mL into the stem and 1 mL to the soil for each plant.

The inoculated plants were kept for 3 days under constant humidification and then given 7 lots of microspraying at 1 min per day of 24 h for 30 days. Evaluations were made daily between Days 5 and 30, examining leaf by leaf in each plant for the appearance of symptoms of wilt such as flaccidity and yellowing. The area under the disease progress curve (AUDPC) was calculated for the variable of severity, according to a graded scale of severity of disease and, through the statistical program Statistix 8.0, an analysis of variance was conducted for the AUDPC.

To determine differences between treatments in terms of their effectiveness in controlling bacterial wilt on Days 7, 14, 21, 28, 35, 42, and 49, an analysis of variance was conducted, together with tests on the separation of means (Tukey's; Ŭ= 5%).

Results and Discussion

On average, 10 days after inoculation, leaves showed the first symptoms, presenting flaccidity on touch (grade 1). After about 18 days, the leaves showed some wilting and began losing their intense green color (grade 2). After 4 weeks, the leaves were noticeably flaccid and, in some cases, yellow (grade 3). For grade 4, 40 days after inoculation, the leaves were yellow, necrotic, and very flaccid, having lost their shape.

These findings contrast with those of Gómez (Gómez E. 2005. Aislamiento, identificación y caracterización del agente causal del moko del plátano, *Ralstonia solanacearum* raza 2, proveniente de plantaciones afectadas en Colombia. Faculty of Sciences, Pontificia Universidad Javeriana, Bogotá, DC, Colombia), who reported that,

by Day 5, symptoms of the disease such as flaccidity and/or wilt in the leaves can appear. Possibly, the size and origin of the seedlings influence the disease's progress.

Only those plants inoculated with bacteria and treated with water presented grade 5 on the disease scale, showing the most severe symptoms of advanced necrosis and loss of shape in the leaves. The other treatments did not present this grade of disease until the seventh evaluation (i.e., 49 days after inoculation). The symptoms observed and the re-isolation demonstrated that the symptoms are caused by *R. solanacearum*. Uninoculated plants and treated only with water did not show wilt during the experiment. At 3 weeks after inoculation, no significant differences were observed between treatments, using Tukey's test at 5%.

By the fourth week, significant differences were observed between products and the positive check. During the fourth and fifth weeks, the lixiviate from Las Américas Farm in Quimbaya (Quindío) significantly reduced the disease's progress. At the end of the trial, all the products had a similar effect on the wilt's progress with an average grade of 4.2, as expressed according to the severity scale, where the leaves presented yellowing with necrosis and advanced flaccidity, losing their shape (Table 2.15.3).

The inoculated plants treated only with water suffered grade 6.0, that is, they died (Figure 2.15.1). With respect to the four replications, two had a greater concentration of inoculated pathogen than the other two. Highly significant differences (Tukey, 5%) occurred between replications 1 and 2 and replications 3 and 4, with symptoms appearing much more quickly in replications 1 and 2 than for replications 3 and 4.

Table 2.15.3. Analysis (Tukey, 5%) to compare the effect of time between the application of products and day of inoculation with Ralstonia solanacearum on disease progress in plantain seedlings established in the greenhouse. Values in the table refer to scores on a disease scale of 0 to 6, where 0 is absence of disease and 6 is plant death.

Time between product application and inoculation of seedlings	**Evaluation on day after inoculation**						
	7	14	21	28	35	42	49
Days before inoculation							
15	0.49 bc	1.02 c	1.59 c	2.20 b	3.01 b	3.27 b	3.45 b
7	1.44 a	2.38 a	3.01 ab	3.35 ab	3.84 ab	4.31 ab	4.60 ab
3	0.97 ab	2.08 ab	3.11 ab	3.62 a	4.05 ab	4.42 ab	4.66 a
Days after inoculation							
3	0.26 c	1.43 abc	2.54 abc	3.04 ab	3.53 ab	4.06 ab	4.40 ab
5	0.29 c	1.41 bc	2.31 bc	3.11 ab	3.44 ab	3.88 ab	4.33 ab
7	0.28 c	2.18 ab	3.49 a	4.11 a	4.39 a	4.81 a	5.17 a

Conclusions

The mixture of the two antibiotics gentamicin and tetracycline was effective in inhibiting the bacterium *R. solanacearum* from infecting plantain seedlings. This finding is similar to that reported, where Cumbre® was shown to be effective in controlling this pathogen in tomato. Taking into account that the lixiviates used in this study are as equally effective as the antibiotics, we do not recommend the use of Cumbre®. On observing the effect of the lixiviates on disease progress, we recommend that plantain farms apply this ecological alternative every 2 weeks to reduce disease incidence and expression of wilt symptoms and as a preventive to protect the crop from infection. Of the three lixiviates obtained from plantain rachises, that collected from Las Américas Farm was the most effective in reducing the wilt's progress over time.

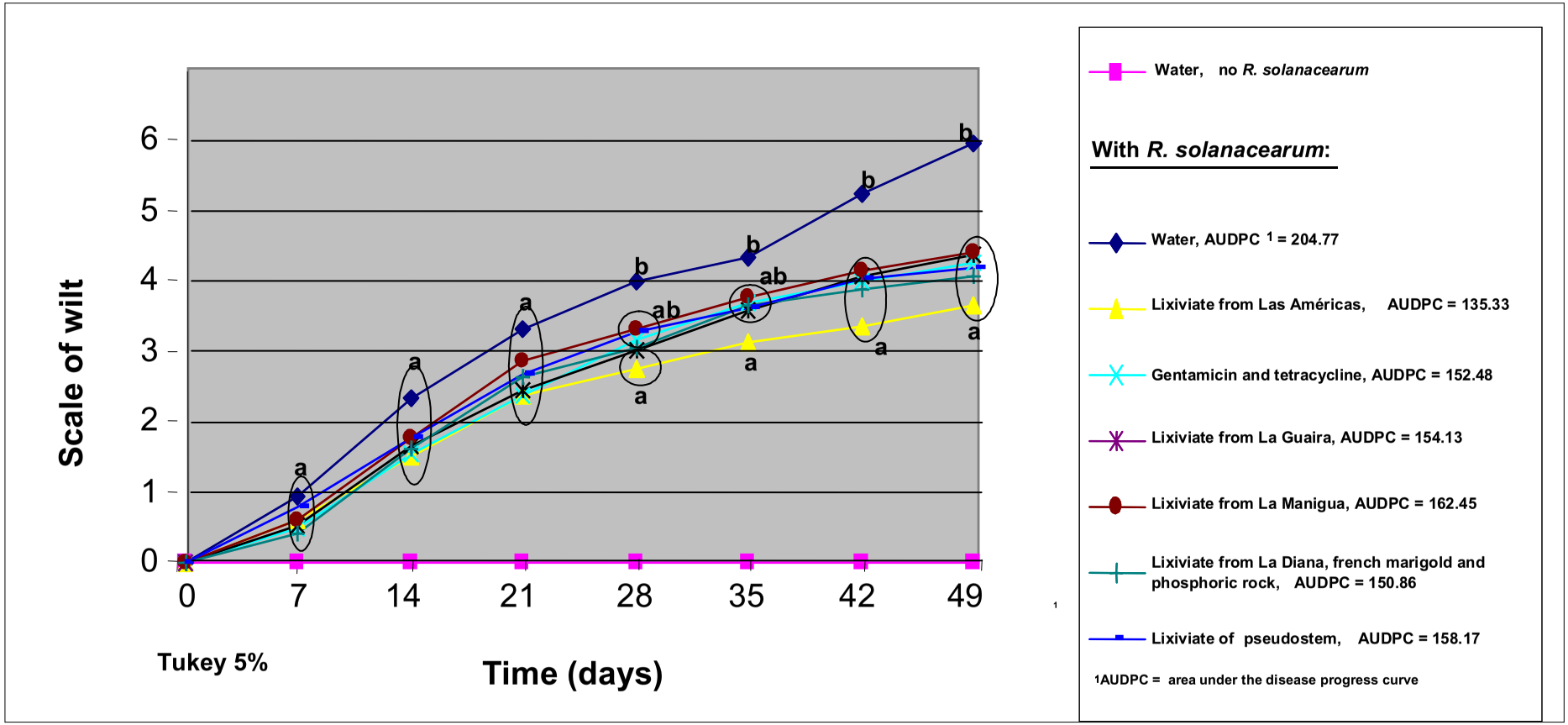

Figure 2.15.1. Effect of lixiviates from decomposing residues of plantain on the progress of bacterial wilt in plantain seedlings growing in the greenhouse over 7 weeks.

Activity 2.16. Effect of lixiviates on controlling bacterial wilt in soil under field conditions at the Santa Elena farm, municipality of Armenia, Quindío

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Rationale

Bacterial wilt of plantain is disseminated not only by work tools but also through survival in the soil and dissemination through water. Plantain producers do not have access to practices for disinfecting or inhibiting the multiplication of the pathogen in the soil. This study aims to evaluate the effect of three sources of lixiviates of plantain rachis, phosphoric rock, and french marigold for managing, in the soil, foci of bacterial wilt of plantain at the Santa Elena Farm, Municipality of Armenia, Quindío.

Materials and Methods

We identified 14 foci on the Santa Elena Farm where plants presented typical symptoms of bacterial wilt. The farm is located in the village district of La Pradera, *corregimiento* El Caimo, Municipality of Armenia in Quindío, Colombia. The foci are managed by surrounding the area with polypropylene fiber and *guadua* or building bamboo to prevent workers from disseminating the disease. Then, for each focus, infected plants and surrounding healthy plants are pulled up and chopped on the site. All tools that had been used in the foci are disinfected with sodium hypochlorite at 2.25% (hypochlorite for pools at 15%, using 600 mL per gallon of water, or Patojito® at half a gallon to half a gallon of water).

Selecting treatments for managing wilt in the field: To evaluate the effectiveness of lixiviate of rachis compost in the ecological management of wilt, we selected at random the treatments described in Table 2.16.1.

The lixiviates were applied only to the site where the diseased plants had been eradicated in each focus. In May, french marigold was chopped up and mixed with the lixiviate 2 days before applying, partly to allow a release of metabolites that inhibit bacteria and partly to facilitate the infiltration of these metabolites, together with the lixiviate, in the area where the roots of the eradicated infected plants are found.

Table 2.16.1. Distribution of soil treatments, number of applications, and plantain plants infected by bacterial wilt on the Santa Elena Farm, Municipality of Armenia, Quindío. The field trial was established in November 2005a and evaluated over 13 months.

Soil treatment	**Unit**					
Lixiviate of plantain rachis from Las Américas Farm applied to plot no.:	1	7	9	12	15	18
Number of plants in that plot infected by bacterial wilt at start of trial, Feb. 2006	5	2	6	3	3	10
Months in 2006 when lixiviate applied	March, April, May	March, April, May	March	March, April, May	March	March
Lixiviate of plantain rachis from La Guaira Farm applied to plot no.:	3	5	6	8	11	14
Number of plants in that plot infected by bacterial wilt at start of trial	3	1	1	3	1	10
Months in 2006 when lixiviate applied	March, April, May	March	March, April, May	March, April, May	March	March
Lixiviate of plantain rachis from Santa Elena Farm applied to plot no.:	13	17				
Number of plants in that plot infected by bacterial wilt at start of trial	2	2				
Months in 2006 when lixiviate applied	March	March, April, May				

a. Plots 1, 7, 9, 12, 15, and 18 received applications of phosphoric rock, extract of french marigold, and lixiviate of plantain rachis.

Treatments established in foci of bacterial wilt: Inside the focus, we applied, over the material of diseased plants that had been eradicated and chopped as described previously, the following products:

- Leaves, stems, and flowers of french marigold (fresh weight) at 1 kg/m^2
- Phosphoric rock at 25 kg (Bolivariana de Minerales Ltda., Bogotá, Colombia; active ingredients = total phosphorus 29%)
- Efficient Microbes (EM), which comprised the principal groups of beneficial microorganisms (phototrophic bacteria, lactic acid bacteria, yeasts, and fungi) at 0.5 L for 20 L of lixiviate
- Lixiviate of plantain rachis at 20 L per site

Each focus was numbered and labeled with the treatment applied. It was kept free of weeds by applying glyphosate and manually eradicating any appearance of plantain shoots. The entrance into each focus was defined by a stake, where a tray containing sodium hypochlorite at 2.25% was set down to disinfect shoes on entering and leaving the focus. The precaution was also taken to shake the earth off the shoes before wetting them with the disinfectant.

Detecting Ralstonia solanacearum in soil and tissue samples taken from foci infected by bacterial wilt: The soil and plant tissue samples were obtained to determine the presence of bacteria in the plots, designated as foci, where wilt-infected plants were eradicated. Samples were taken every 30 days and processed at the Cassava Pathology Laboratory, CIAT.

Processing soil samples: Soil samples were collected from wilt-infected foci at a depth of 20 to 35 cm, placed in 1-lb polypropylene bags that were duly marked, and conserved in a styroform ice-box for transport from the farm to CIAT. In the laboratory, 3.3 g of soil was weighed from each sample and mixed with 30 mL of sterilized deionized water by vortexing. Serial dilutions were conducted in TE buffer at pH 7.6, taking 1 mL of the mother solution for a 10^{-1} dilution and, from this, 100 µL for a 10^{-2} dilution. The diluted solutions were then planted in petri dishes containing semi-selective SMSA medium and incubated at 28ºC for 7 days. As a comparative reference for typical colony growth, we used strain CIAT No. 78 of *R. solanacearum* race 2 from the collection held at the Cassava Pathology Laboratory, CIAT.

After incubation, we selected those colonies whose growth in the SMSA medium presented morphological characteristics that were similar to those of the reference strain. The selected colonies were re-chopped and planted in drop form on nutritive agar (NA) to obtain individual colonies. After 24 h of culturing in NA, a suspension was prepared in sterilized deionized water. Under aseptic conditions, the concentration of the suspension was determined by reading the absorbance from a spectrophotometer (Turner, model 390) with a wavelength of 600 nm, using Fisher cells (ref. Spectronic 20). The concentration was adjusted by diluting with sterilized deionized water until an absorbance of 0.3 was obtained, corresponding to about 1×10^{-6} colony-forming units per milliliter (He et al. 1983).

The colonies were inoculated onto 45-day-old plantain plants of the variety Dominico Hartón, produced by Silverio González in thermic chambers located in the Municipality of La Tebaida, Quindío. The plants, with naked roots, were transported in carton boxes to the CIAT greenhouses, where they were planted in plastic 1-kg bags containing a sterilized mixture of sand and soil at a rate of 3:2. The plants were not watered for 24 h before inoculation (EPPO 1990).

The inoculation method used was injection with sterilized 1-mL syringes and needle size 27G × 1/2". Of the bacterial suspension, 0.5 mL were inoculated into the pseudostem of each plant at a height of 2 cm from the soil surface. After inoculation, the plants were incubated for 3 days in a humid chamber to guarantee optimal development of the pathogen. They were then placed in the greenhouse under controlled conditions with temperatures between 24º and 29ºC (minimum night and maximum day, respectively), relative humidity between 91% and 80% (maximum night and minimum day, respectively), and light at about 13 h.

Processing plant tissue samples: Samples of different types of infected plant tissues (corms, pseudostems, and leaves) were selected from plantain plants that presented

typical symptoms of the disease such as reddish streaks on the pseudostem, and wilt, flaccidity, and yellowing of leaves. These had been ascertained from evaluations conducted during visits to the farm to determine the presence of the pathogen.

Fragments of infected tissue were washed with deionized water for 30 min, disinfected in sodium hypochlorite at 1% for 30 s, then submerged in ethanol at 50% for 1 min, and finally rinsed twice with sterilized deionized water for 10 s to remove residues of the disinfectants. This procedure was conducted in a laminar flow chamber under aseptic conditions, using materials sterilized at 121ºC under 20 lb of pressure for 20 to 30 min. To isolate the bacteria present in the tissue, the disinfected fragments were macerated in a mortar sterilized at 121ºC under 20 lb of pressure with a TE buffer solution (10 mM Tris-HCl and 1 mM EDTA, with a pH 7.6).

The resulting suspension was planted, using a sterilized microspade, in petri dishes containing semi-selective SMSA medium (10 g/L peptone, 5 mL/L glycerol, 1 g/L casaminoacids, and 18 g /L agar). Antibiotics were added under aseptic conditions when the temperature of the SMSA medium was 50ºC. This stock was added as follows: 100 mg/L (600,000 U) polymyxin ƃ-sulfate; 25 mg/L bacitracin (source: 36 mg/L Baneocin®); 0.5 mg/L (82.5 U) penicillin; 5 mg/L chloramphenicol; 50 mg/L 2,3,5 chlorotriphenyltetrazole; and 5 mg/L crystal violet. (Martins, 2000. Polymerase chain reaction in the diagnosis of bacterial wilt, caused by Ralstonia solanacearum (smith) Yabuchi et al. Thesis (Doctor of Agricultural Sciences). Georg-August University, Faculty of Agricultural Science, Gottingen, DE. 127 p). The solution of antibiotics was sterilized by filtering, using Millipore filters with pore size of 0.22 µm, and adjusted for use in syringes.

The dishes that were planted with the suspension were incubated for 5 to 7 days at 28ºC. Colony growth was compared with that of strain CIAT No. 78 of *R. solanacearum* race 2, itself isolated from samples of plantain rachis from Montenegro, Quindío.

The procedure for samples from colonies isolated in SMSA medium was the same as for the soil samples.

Decomposition of materials from infected plants: To accelerate the decomposition of materials chopped up from infected plants, we prepared 12.5 g of *Trichoderma harzianum* or *T. viride* (1×10^{10} conidia/g) in 20 L. Four applications were made every 15 days. The two strains were alternated every 15 days. To compare the plots where the product was applied, we had to take into account the appearances of new cases of plantain plants infected by wilt where the material was chopped. In all the treated foci and 10 others, plants infected with wilt and surrounding healthy plants were chopped up, including corms. Each infected plant received an application of 4 L of a mixture of 200 L of water, 10 kg of molasses, 20 L organic matter decomposer facilitated by Sanoplant (Palmira, Valle del Cauca), 200 g of a mixture of *T. harzianum, T. viride*, and *T. koningii* (concentration at 1×10^{12} conidia/g), and 200 g of *Paecilomyces lilacinus* (concentration at 1×10^{12} spores/g). The application was repeated 30 days later.

Results and Discussion

From the soil samples taken from foci infected with wilt, we found that, of 172 samples analyzed, only 3 were positive. This finding ratified the difficulty in isolating and identifying viable cells of *R. solanacearum* in the soil, where the percentage of detection of this pathogen in this study was low (1.7%), confirming findings by Gómez (Gómez E. 2005. Aislamiento, identificación y caracterización del agente causal del moko del plátano, *Ralstonia solanacearum* raza 2, proveniente de plantaciones afectadas en Colombia. Faculty of Sciences, Pontificia Universidad Javeriana, Bogotá, DC, Colombia), who detected only 8% in soil samples.

The mechanisms of *R. solanacearum* for surviving in the soil are complicated and little studied. One reason that the pathogen was not readily detected in the soil samples is perhaps that populations decay progressively over time to an undetectable level. This finding contrasts with that reported by Martins (Martins, 2000. Polymerase chain reaction in the diagnosis of bacterial wilt, caused by Ralstonia solanacearum (smith) Yabuchi et al. Thesis (Doctor of Agricultural Sciences). Georg-August University, Faculty of Agricultural Science, Gottingen, DE. 127 p), who suggested that the bacterium can persist in many soils under different crops, with diverse conditions of management. The bacterium's survival is linked directly with the presence of water. The soils of most of the farms in Quindío had good drainage, which made it difficult for the pathogen to stay in the soil.

Other tools exist for detecting the bacterium, such as those based on molecular markers, which are more sensitive and specific to the cells of the bacterium in the soil. For example, Álvarez et al. (Alvarez et al., 2006. Diseño y estandarización de una sonda TaqMan para la detección específica de *Ralstonia solanacearum* Raza 2, en plátano mediante PCR en tiempo real. XLVI Annual Meeting Phytopathological Society (APS) Caribbean Division – XXVII Congreso ASCOLFI, Cartagena, Colombia. Sept. 12-16, 2006) used a TaqMan® probe that is specific to *R. solanacearum* race 2 in plantain, using real-time PCR. They detected only pathogenic strains of the bacterium isolated from plantain.

In this study, the bacterium was not detected between November 2005 and March 2006 in any of the foci. The pathogen was isolated only in April and October 2006 (Table 2.16.2). In August and September 2006, no samples were taken because seedling indicator plants had been planted to improve the detection of *R. solanacearum* in the different experimental plots. Indicator plants were planted at 3 per plantain plant infected with bacterial wilt.

Throughout the experiment, we also processed 24 samples of plant tissues from new cases of plants infected with bacterial wilt around the treated foci. Results were positive, thus confirming the presence of the bacterium around the foci being treated.

Table 2.16.2. Detection of *Ralstonia solanacearum* in soil and plant tissue samples, using SMSA culture medium to determine the effect of a mixture of lixiviate from decomposing plantain rachis, phosphoric rock, and french marigold applied to the soil of foci infected by bacterial wilt at the Santa Elena Farm, Quindío, Colombia. Eight samplings were conducted over 13 months.

		Soil samples		Plant tissue samples	
Month and year in which samples were taken		(no.)	Positive for *R. solanacearum* (no.)	(no.)	Positive for *R. solanacearum* (no.)
1	Nov, 2005	21	0	15	1
2	March, 2006	21	0	7	1
3	April, 2006	23	2	3	0
4	May, 2006	23	0	0[a]	0
5	June, 2006	23	0	0[a]	0
6	July, 2006	23	0	0[a]	0
7	Oct, 2006	18	1	0[a]	0
8	Nov, 2006	20	0	0[a]	0
	Total of samples	172	3	25	2
	Efficiency of detection of *R. solanacearum* (%)		1.7		8.0

a. The sampling of plant tissue could not continue because of the chopping up of infected plants. The gradual decomposition of plant tissue made sampling impossible.

Visits to the farm allowed us to monitor the area around the treated plots and so identify the pathogen's dissemination by the appearance of new cases. We saw an increase in August, noting that new cases of infected plants consistently appeared very close to the old foci, probably because of deficient eradication of healthy plants around the infected plants at the beginning of the experiment. To create soil conditions more conducive to pathogen development, we planted indicator plants in plastic bags and placed them inside the experimental plots. The bags were placed below soil level, thereby permitting the accumulation of moisture in each bag for lack of drainage. In each of the 18 plots, 3 indicator plants were planted. However, none of these plants became diseased with this method of detection.

Of 145 plantain indicator plants planted in July 2006 in 15 experimental plots, none were diseased 4 months later. Nor, in October 2006, was *R. solanacearum* detected in 16 plants processed in the laboratory, using SMSA culture medium.

The two products applied to the pseudostems, leaves, and corms chopped up from destroyed plants were compared with untreated residues for speed of decomposition. The two products decomposed more quickly than the untreated residues. Because this research theme was not an objective of this study, we recommend that tests be conducted in different farms, evaluating residues in cloth bags, with and without treatment, and monitoring weight loss over time.

Conclusions

A very low (1.7%) efficiency of detection of *R. solanacearum* was observed for soil samples from the experimental plots, using SMSA medium. For future studies, we recommend using molecular markers and real-time PCR to detect the pathogen in the soil and plant tissues. During the frequent visits made to the farm, we went through fields recording the appearance of plants newly infected with bacterial wilt. In the site occupied by each infected plant in each focus, we planted three plantain plants after the last application of lixiviate to discover the efficacy of the treatments established. On Day 60, a sampling of soil and plant tissues was conducted to detect the presence of *R. solanacearum* in the soil and living materials of plantain plants.

Over 9 months, we watched 54 new plants become diseased with bacterial wilt. We concluded that the disease spread in this farm largely because inadequate management practices were used such as the use of tools (e.g., machete and hacking knife) without disinfecting them, lack of early detection and eradication of foci, and not marking infected areas. We did not find evidence dissemination of the disease through the soil. After 2 months, we saw that the 48 plants planted in plastic bags did not become diseased, even with the rainy conditions of the zone. We conclude that the pathogen was not found in plot soil. Because *R. solanacearum* was absent from the soil, we could not determine the effect control practices had over bacterial wilt. We recommend that plans be established to prevent losses in crops and, hence, drastic applications of chemical products. Likewise, we suggest the use of integrated management packages to control this disease.