Output 2: Grass and legume genotypes with known reaction to pests and diseases and interaction with symbiont organisms are developed

2.1 Pre-selection and propagation of apomictic and sexual *Brachiaria* hybrids

Highlights

- The *Brachiaria* improvement program continued to advance during 2003. A new cycle of the sexual population was sown, propagated, and established in field nurseries in two sites in Colombia. Over 3,000 seedlings are included in these two nurseries.
- A set of 64 "pre-selected" SX x AP clones was propagated for detailed assessment of reaction to three Colombian spittlebug species, reaction to *Rhizoctonia* foliar blight, and aluminum tolerance.
- Open-pollinated (OP) progenies of these pre-selections were established in a field trial to assess reproductive mode.

2.1.1 Pre-select and propagate sexual x apomictic hybrids from field nursery for spittlebug screening

Contributors: J.W. Miles, F. Feijoo, and A. Betancourt (CIAT)

The *Brachiaria* improvement program is based on recurrent, cyclical improvement of a synthetic tetraploid, sexual population. A two-year cycle is currently used. Phenotypic selection among several thousand genotypes is conducted sequentially, first on visual assessment of unreplicated spaced plants in field nurseries at two contrasting sites, and subsequently on more detailed assessment of specific traits such as resistance to spittlebugs, reaction to *Rhizoctonia* foliar blight, and tolerance to aluminum. Each cycle, a set of selected sexual clones is identified for recombination to produce the subsequent cycle of the sexual population, and also to cross with elite apomicts to generate hybrid populations from which superior hybrid apomictic clones can be selected for possible development into commercial cultivars.

In 2002, a set of over 2,000 SX x AP (sexual-by-apomictic) hybrids were generated, propagated, and established in field trials. These were culled to 64 by simple visual assessment by 01 Jan 2003. These 64 pre-selections were propagated for more detailed assessment of reaction to spittlebugs, *Rhizoctonia* foliar blight, and Al tolerance. OP progenies were established for assessment of reproductive mode.

2.1.2 Evaluation of sexual populations and pre-selection of clones for spittlebug screening

Contributors: J.W. Miles, F. Feijoo, and A. Betancourt (CIAT)

Spittlebug resistance remains a prime-breeding objective. Only sexual clones with resistance to several spittlebug species are included as parentals of the sexual population. This year, just over 3,000 sexual (SX) recombinants were established in field nurseries in April and May 2003. The population is being culled down to approx. 1,000 "pre-selections" by periodic visual evaluation. By year's end, we shall have a list of "pre-selections", which will be propagated for screening for resistance to three Colombian species of spittlebug.

As of reporting date (08 September 2003), a large number of plants have been culled, based on undesirable phenotype in the testing sites.

Vegetative propagation from CIAT-Quilichao to CIAT headquarters will begin in October.

Propagation for spittlebug screening will commence in early January 2004.

2.1.3 Propagation of pre-selected sexual x apomictic and sexual hybrids to screen for *Rhizoctonia* reaction

Contributors: J.W. Miles, S. Kelemu, and C. Zuleta (CIAT)

Rhizoctonia foliar blight is a devastating disease of susceptible *Brachiaria* genotypes. The current *Brachiaria* breeding populations have generally high levels of susceptibility. Reliable, massive screening methodology has been lacking. Recent developments in refinement of screening methodology are promising. An initial test population includes 64 "pre-selected" SX x AP genotypes generated in 2002, plus 20 promising hybrid clones involving the highly *Rhizoctonia*-resistant *Brachiaria brizantha* accession CIAT 16320 as pollen parent.

In 2002, a set of over 2,000 SX x AP (sexual-by-apomictic) hybrids were generated, propagated, and established in field trials. These were culled to 64 by simple visual assessment by 01 Jan 2003. These 64 pre-selections were propagated for more detailed assessment of several traits, including reaction to *Rhizoctonia* foliar blight. Also in 2002, a set of 387 hybrids involving the highly *Rhizoctonia*-resistant *B. brizantha* accession CIAT 16320 as pollen parent were established in the field at CIAT-Quilichao. Twenty-nine of these hybrid clones were pre-selected on visual assessment in this trial. The aim is to identify sexual clones among the hybrids with CIAT 16320 that are *Rhizoctonia*-resistant so as to introgress genes for resistance into the synthetic tetraploid, sexual *Brachiaria* breeding population.

Refinements are still being made in screening methodology. A detached-leaf technique is being used. Preliminary results suggest that this method will be adequate at least to identify the most resistant genotypes with reasonable reliability. The method is quick (results in 5 days) and cheap. Only a very small amount of plant material is needed. Of the 93 genotypes being tested, a selected sub-set of 36 has been identified. Reactions of the genotypes in this group are being reconfirmed, while refinements in the screening technique itself are being made.

2.1.4 Propagation of pre-selected SX x AP clones for screening of Al tolerance in solution culture

Contributors: J.W. Miles and I.M. Rao (CIAT)

Candidates for commercial cultivars need to be assessed for reaction to aluminum toxic soils. Sixty-four clones were pre-selected from an initial set of over 2,000 hybrids produced in 2002.

"Pre-selected" hybrid clones were vegetatively propagated and delivered to the plant nutrition group for screening.

2.1.5 General adaptation and agronomic traits of sexual seedlings and pre-selection for Al tolerance

Contributors: J.W. Miles and I.M. Rao (CIAT)

Tolerance to high soil aluminum levels is a desirable attribute in *Brachiaria* cultivars. It is one of the traits we are attempting to incorporate in bred cultivars.

Owing to inherent difficulties in assessment of Al tolerance, rapid screening of large (~1,000 genotypes) populations has not been possible. We are attempting this year to make "pre-selections"

earlier (October), so that vegetative propagation from the field at Quilichao can be initiated opportunely to provide timely availability of adequate volumes of plant material for aluminum screening.

We are on schedule to begin propagation of "pre-selections" in October.

2.1.6 Establishment of replicate sets of open pollinated sexual plants in field nurseries in Puerto López (Matazul) and CIAT-Quilichao

Contributors: J.W. Miles, F. Feijoo and A. Betancourt (CIAT)

The *Brachiaria* breeding strategy is based on recurrent, cyclic improvement in a synthetic tetraploid, sexual breeding population.

A new breeding cycle was initiated in 2003, with the generation of a large (>3,000) recombinant population from 22 parental clones selected from the previous cycle. Recombinant seedlings were established in the greenhouse and vegetatively propagated to establish two, un-replicated, space-planted field nurseries. These nurseries were established in April and May, 2003. Visual assessment of these two nurseries allows culling of plants with undesirable phenotypes. By year's end, no more than 1,000 "pre-selected" individuals will remain. These will be propagated for more detailed assessment of reaction to spittlebugs, *Rhizoctonia*, and aluminum.

Field nurseries were successfully established. Several ratings of plant merit have been conducted. Beginning in October, "pre-selected" plants will be propagated to CIAT-Palmira for further evaluations.

2.2 Selection of *Brachiaria* hybrids for resistance to spittlebug

Highlights

- A mass rearing of *Aeneolamia reducta* was successfully established which will facilitate simultaneous evaluation of *Brachiaria* genotypes for resistance to different species of spittlebug.
- Four hybrids showing high levels of antibiosis resistance to *Aeneolamia varia*, *A. reducta*, and *Zulia carbonaria* were identified.
- Several hybrids showing field resistance to *Zulia pubescens* and *Mahanarva trifissa* were identified

2.2.1 Continuous mass rearing of three spittlebug species in Palmira and Macagual

Contributors: C. Cardona and G. Sotelo (CIAT)

This is a continuous activity. A permanent supply of insects is essential in the process of evaluating genotypes for resistance to spittlebug. Progress made in the logistics of mass rearing of nymphs and in obtaining eggs from adults collected in the field has allowed us to screen *Brachiaria* genotypes for simultaneous resistance to six major spittlebug species: *Aeneolamia varia*, *A. reducta*, *Zulia carbonaria*, *Z. pubescens*, *Mahanarva trifiss*a, and *Prosapia simulans*. Insect material produced in our mass rearing facilities is used for greenhouse evaluations in Palmira and field evaluations in Caquetá.

2.2.2 Glasshouse screening of *Brachiaria* genotypes for spittlebug resistance

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

2.2.2.1 Greenhouse screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species (*Aeneolamia varia, A. reducta, Zulia carbonaria, Z. pubescens*)

Rationale

The correct identification of resistant hybrids is an essential step in the process of breeding superior *Brachiaria* cultivars at CIAT. This is why assessment of resistance to spittlebug received special attention in 2003. Based on results obtained in 2001 and 2002, simultaneous but independent screening for resistance to three key spittlebug species was fully implemented.

Materials and Methods

A set of 64 "pre-selected" SX x AP (sexual-by-apomictic) hybrids received from the Breeding Program were evaluated in the glasshouse for resistance to *Aeneolamia varia, A. reducta*, and *Zulia carbonaria*. Test materials were compared with six checks fully characterized for resistance to one or more spittlebug species. Plants were infested with six eggs per plant of the respective spittlebug species and the infestation was allowed to proceed without interference until all nymphs reached the fifth instar stage or adult emergence occurred. Plants (5 per genotype) were scored for symptoms using the damage scale (1, no damage; 5, plant dead) developed in previous years. Percentage nymph survival was calculated. Materials were selected on the basis of low damage scores (<2.0 in the 1-5 scale) and reduced percentage survival (<30%). Those genotypes showing resistance to two or more spittlebug species were reconfirmed in replicated nurseries (10 replications per genotype per spittlebug species).

Results and Discussion

The preliminary screening revealed that 10 of the 64 hybrids showed acceptable levels of resistance to at least two spittlebug species (Table 8). As in previous occasions, fewer genotypes showed antibiosis resistance to *Z. carbonaria.*

Table 8. Sexual-by-apomicitic hybrids selected in 2003 for reconfirmation of resistance to three spittlebug species. Means of five replicates per genotype per spittlebug species

Genotype	Damage scores			Percentage nymph survival		
	Aeneolamia varia	*Aeneolamia reducta*	*Zulia carbonaria*	*Aeneolamia varia*	*Aeneolamia reducta*	*Zulia carbonaria*
			Hybrids			
BR02NO/0419	1.2	1.6	2.1	3.3	10.0	23.3
BR02NO/0465	1.8	1.1	1.4	30.0	0.0	20.0
BR02NO/0638	1.2	1.6	2.6	23.3	36.7	-
BR02NO/0643	1.1	1.9	3.2	3.3	40.0	-
BR02NO/0644	1.4	1.3	2.2	33.3	20.0	80.0
BR02NO/0649	1.4	1.2	1.6	16.7	3.3	53.3
BR02NO/0756	3.0	1.2	2.0	-	0.0	26.7
BR02NO/0812	2.3	1.6	2.4	-	16.7	56.7
BR02NO/1372	2.4	1.6	1.6	-	20.0	56.7
BR02NO/1485	1.3	1.7	2.5	13.3	20.0	66.7
			Checks[a]			
FM9503/4624 (T)	3.5	2.2	1.5	85.0	25.0	11.7
CIAT 6294 (R)	2.0	1.4	2.5	26.7	25.0	56.7
SX0NO/0102 (R)	1.0	1.1	1.1	0.0	0.0	0.0
CIAT 36062 (R)	1.1	1.2	1.4	1.7	0.0	16.7
CIAT 0606 (S)	5.0	4.3	4.1	95.0	80.0	68.3
BR4X/44-02 (S)	4.9	4.8	3.5	90.0	73.3	70.0
LSD 5%	1.21	1.23	0.94	28.8	22.2	23.7

[a] Classified according to their reaction to *Aeneolamia varia* (S, susceptible; R, resistant; T, tolerant).

Further testing with 10 replications per genotype per insect species allowed us to identify four hybrids combining antibiosis resistance to *A. varia*, *A. reducta*, and *Z. carbonaria* (Table 9). Levels of resistance in this case were comparable to those exhibited by the resistant checks CIAT 36062 and 'Marandú' (CIAT 6294).

Table 9. Sexual-by-apomictic hybrids selected in 2003 for high antibiosis resistance (<30% nymphal survival) to three spittlebug species. Means of 10 replicates per genotype per spittlebug species

Genotype	Damage scores			Percentage nymph survival		
	Aeneolamia varia	*Aeneolamia reducta*	*Zulia carbonaria*	*Aeneolamia varia*	*Aeneolamia reducta*	*Zulia carbonaria*
			Selected hybrids			
BR02NO/0419	1.0	1.1	1.8	0.0	0.0	5.0
BR02NO/0465	1.0	1.0	1.1	10.0	1.7	18.3
BR02NO/0756	1.4	1.2	1.8	18.3	3.3	21.7
BR02NO/0812	1.0	1.5	2.4	6.7	8.3	28.3
			Checks[a]			
FM9503/4624 (T)	1.6	1.8	1.9	51.7	21.7	30.0
CIAT 6294 (R)	1.2	1.9	2.7	25.0	21.7	63.3
SX01NO/0102 (R)	1.0	1.0	1.1	1.7	0.0	1.7
CIAT 36062 (R)	1.0	1.0	1.5	0.0	0.0	6.7
CIAT 0606 (S)	4.9	4.4	3.8	96.7	68.5	46.7
BRU4X/44-02 (S)	4.8	4.1	3.8	96.7	55.0	75.0
LSD 5%	0.36	0.65	0.68	16.8	17.8	26.3

[a] Classified according to their reaction to *Aeneolamia varia* (S, susceptible; R, resistant; T, tolerant).

2.2.2.2 Field screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species (*Aeneolamia varia, Zulia carbonaria, Z. pubescens, Mahanarva trifissa*)

Rationale

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

Materials and Methods

Using the experimental unit described in our 1998 Annual Report, the genotypes (usually 10 replicates) are initially infested in the greenhouse with an average of 10 eggs per stem. Once the infestation is well established, with all nymphs feeding on the roots, the units are transferred to the field and transplanted 10-15 days after infestation. The infestation is then allowed to proceed without interference until all nymphs have developed and adults emerge some 30-35 days thereafter. The plants are then scored for damage by means of the 1-5 visual scale utilized in greenhouse screenings. The number of stems per clump is counted before and after infestation and a tiller ratio (tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process) is then calculated. Using this methodology, eight major screening trials (six with *Zulia pubescens*, two with *Mahanarva trifissa*) were conducted in Caquetá in 2003.

Results and Discussion

In Table 10 we highlight the results of evaluating 32 hybrids for resistance to *Z. pubescens* in comparison with six checks well known for their reaction to *Aeneolamia varia*. As in previous

occasions, there was a significant negative correlation
($r = -0.452$; $P < 0.001$; $n = 2273$) between damage scores and tiller ratios. This means that damage scores are useful in predicting tiller losses resulting from intense insect damage. Selected hybrids in Table 10 showed significantly lower damage scores and significantly higher tiller ratios than the susceptible checks CIAT 0606 and BRUZ4X/44-02.

The same set of 32 hybrids was evaluated for field resistance to *Mahanarva trifissa.* Results are shown in Table 11. Resistant hybrids exposed to *M. trifissa* performed significantly better than the checks both in terms of damage scores and tiller ratios.

Table 10. Field resistance to *Zulia pubescens* in selected *Brachiaria* hybrids and checks. Means of six trials, 10 replicates per genotype per trial.

Genotype	Damage scores	Tiller ratio[a]
	Hybrids	
BR00NO/1494	2.0	1.04
BR00NO/0755	2.0	0.92
BR00NO/1392	2.0	0.91
BR00NO/1032	2.1	0.89
BR00NO/0604	2.1	0.88
BR00NO/1076	2.1	0.88
BR00NO/1295	2.0	0.88
BR00NO/0036	2.0	0.87
BR00NO/0042	2.1	0.96
BR00NO/0029	2.1	0.86
	Checks[b]	
FM9503/46/024 (T)	1.1	1.04
CIAT 6294 (R)	1.1	1.04
CIAT 36062 (R)	1.1	1.01
CIAT 6133 (T)	1.8	0.92
CIAT 0606 (S)	3.6	0.46
BRUZ4X/44-02 (S)	3.9	0.47
LSD 5%	0.13	0.12

[a] Tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process
[b] Classified according to their reaction to *Aeneolamia varia* (S, susceptible; R, resistant; T, tolerant).

Table 11. Field resistance to *Mahanarva trifissa* in selected *Brachiaria* hybrids and checks. Means of two trials, 10 replicates per genotype per trial

Genotype	Damage scores	Tiller ratio[a]
	Hybrids	
BR00NO/0587	2.1	1.16
BR00NO/1494	2.1	1.07
BR00NO/1392	2.0	1.06
BR00NO/0106	2.0	1.01
BR00NO/0078	2.1	1.00
BR00NO/0049	2.1	0.97
BR00NO/1733	2.1	0.96
BR00NO/0235	2.1	0.96
	Checks[b]	
FM9503/46/024 (T)	1.1	0.98
CIAT 6294 (R)	1.1	1.22
CIAT 36062 (R)	1.0	1.03
CIAT 6133 (T)	1.8	0.99
CIAT 0606 (S)	3.8	0.41
BRUZ4X/44-02 (S)	4.3	0.28
LSD 5%	0.19	0.16

[a] Tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process
[b] Classified according to their reaction to *Aeneolamia varia* (S, susceptible; R, resistant; T, tolerant).

2.3 Identification of host mechanisms for spittlebug resistance in *Brachiaria*

Highlights

- Finalized studies on mechanisms of resistance to five species of spittlebug: *Aeneolamia varia*, *A. reducta*, *Zulia carbonaria*, *Z. pubescens*, and *Mahanarva trifissa*
- Finalized studies on the effect of simultaneous infestation by two or more spittlebug species on resistance expressions in selected *Brachiaria* genotypes
- Initiated studies on interactions between strains of five spittlebug species and resistance expression in selected *Brachiaria* genotypes (genotype x species x strain interactions)
- Initiated studies on mechanisms of resistance to adults of five spittlebug species
- Initiated studies on possible sub-lethal effects of resistance on adults of *Aeneolamia varia*

2.3.1 Mechanisms of resistance of Brachiaria genotypes to five spittlebug species

Contributors: C. Cardona, G. Sotelo. A. Pabón, P. Fory, and J. W. Miles (CIAT)

Rationale

We have shown in previous reports that resistance to one spittlebug species does not necessarily apply to other species. We have also shown that the mechanisms of resistance vary. In 2003 we finalized the characterization of antibiosis and tolerance to *Aeneolamia reducta*, the most important species in the Caribbean zone. What follows is a summary of what we know about host plant resistance mechanisms to five major spittlebug species present in Colombia.

Materials and Methods

Several experiments were conducted and are reported herein. As test materials we used four germplasm accessions well known for their reaction to *Aeneolamia varia*: the susceptible checks CIAT 0606 and CIAT 0654 and the resistant checks CIAT 6294 (‘Marandú’) and CIAT 36062 (a hybrid-derived clone). These four host genotypes were also used to compare their resistance to other spittlebug species. CIAT 0654 and CIAT 36062, highly susceptible and resistant, respectively, were used in antibiosis studies. Tolerance studies were conducted with CIAT 0654, CIAT 6294, and CIAT 36062. *A. varia*, *A. reducta*, and *Z. carbonaria* were mass-reared on plants of CIAT 0654 in a screen-house. Mature eggs were used to infest test plants in the different experiments. In the case of *Z. pubescens* and *M. trifissa*, large numbers of adults were collected in the field with a sweep net and transferred to muslin cages in a screen-house to feed on potted plants of CIAT 0654. Adults were allowed to oviposit and eggs were separated from the soil. As with other spittlebug species, test plants were infested with mature eggs. All tests were conducted in a glasshouse at a mean temperature of 24°C (range, 19-27°C) and mean relative humidity of 75% (range, 70-90%).

To evaluate antibiotic effects, cohorts of no fewer than 900 individuals of each of the five species under study were established on each of two host genotypes well characterized for their reaction to *A. varia*: CIAT 0654 (highly susceptible) and CIAT 36062 (highly resistant). Cohorts were established by infesting 150 single-plant units with 6 eggs of the respective species per unit as described above. Following eclosion, a sample of two or three tubes per host genotype was examined daily to determine the fate of 12 or 18 individual insects. Nymphal instars and their duration were determined from measurement of the width of the head capsule of every nymph recovered (dead or alive). Survival rates were calculated. The dry weight of each nymph was recorded. Daily sampling continued until all surviving nymphs reached adulthood.

To study tolerance we initially compared the response of the susceptible CIAT 0654 and the *A. varia*-resistant CIAT 6294 ('Marandú') to increasing levels of infestation with nymphs of *A. varia*, *M. trifissa*, *Z. carbonaria*, and *Z. pubescens*. *A. reducta* was not included in these studies. Thirty-day-old plants of CIAT 0654 and CIAT 6294 were exposed to 0, 2, 3, 5, 7, or 10 nymphs per plant of each of the spittlebug species. The 48 host genotype- insect species-infestation level treatment combinations were randomly assigned to single-plant experimental units within 10 complete blocks. Plants were infested with mature eggs and the infestation was allowed to proceed until all nymphs were mature or adult emergence occurred. Plants were then scored for damage using the 5-point scale described above and the percentage nymphal survival recorded. Aboveground dry weight of plants was recorded following drying in an oven at 40°C. Percent weight loss was calculated (relative to the uninfested controls). We calculated functional plant loss indexes for each infestation level based on plant weight loss and damage response. Using the same general methodology, we conducted one more tolerance test in which plants of CIAT 0654 (susceptible) and CIAT 36062 (resistant to *A. varia*) were submitted to increasing levels of infestation (0, 2, 3, 5, 7, or 10 nymphs per plant) with each of the following species: *A. varia*, *A. reducta*, *M. trifissa*, *Z. carbonaria*, and *Z. pubescens*. We used a randomized complete block design with 10 repetitions per species-infestation level-host genotype combination. Damage scores, nymph survival, and above ground plant dry weights were recorded. Functional Plant Loss Indices were calculated.

All data were analyzed using the general linear model procedure. Means were separated by least significant difference (LSD: á = 0.05) only when the overall *F* test was significant (á = 0.05). Percentage nymph survival was transformed to arcsine square root of proportion; percentages of dry weight loss were transformed to square root. Means and standard errors of untransformed data are presented. Antibiotic effects for the different spittlebug species were assessed by comparing nymphal instar duration and nymph weight between the susceptible and resistant host genotypes by paired *t*-test. To compare survivorship of nymphs on susceptible and resistant host genotypes, median survival times were calculated using the Kaplan-Meier test. The Cox-Mantel survival test was used to compare survival distributions on susceptible and resistant host genotypes. Tolerance to the different spittlebug species was assessed by comparing mean percentage survival, mean damage scores, and mean percentage plant dry weight loss of five infestation levels between the susceptible and resistant host genotypes by paired *t*-test within spittlebug species.

Results and Discussion

Antibiosis tests. Relative to the susceptible control, CIAT 0654, there was a significant delay in development time of nymphs of *A. varia*, *A. reducta*, and *Z. pubescens* reared on CIAT 36062 (Figure 7). No such effect was found in the case of *Z. carbonaria*. Mortality of second instars of *M. trifissa* was so high, that we were unable to calculate developmental times for this species.

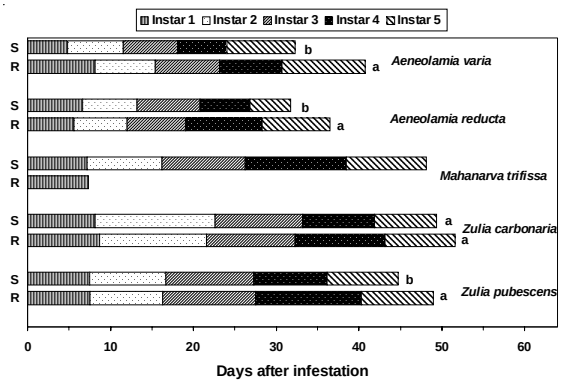


Figure 7. Duration of nymphal instars of five spittlebug species reared on susceptible (S, CIAT 0654) or resistant (R, CIAT 36062) *Brachiaria* genotypes. Bars with the same letter do not differ ($P < 0.05$). Pair-wise comparison by *t*-test within species. *Mahanarva trifissa* was not analyzed due to very high mortality of second instars.

Nymphal survival on the resistant CIAT 36062 was least for *M. trifissa* and greatest for *Z. carbonaria*. Survival of all species was less on CIAT 36062 than on the susceptible CIAT 0654. The Kaplan-Meier survival test revealed significant effects (no overlapping confidence intervals) of the resistant genotype on the median survival times of *A. varia*, *A. reducta*, and ***M. trifissa*** and, to a lesser extent, *Z. pubescens* populations. Survival time of *Z. carbonaria* was not affected by the resistant genotype. Calculation of the Cox-Mantel survival statistic showed differences at the 1% level of significance between CIAT 0654 and CIAT 36062 (Table 12) in terms of survival rates for *A. varia*, *A. reducta*, and *M. trifissa*, and at the 5% level of confidence for *Z. pubescens* (a lower level of antibiosis). No difference was found in the case of *Z. carbonaria* meaning that there is no antibiosis to this species in CIAT 36062. This was confirmed when survival rates of *Z. carbonaria* and *Z. pubescens* on CIAT 36062 were compared. The Cox-Mantel survival test statistic (2.8) was positive and significant at the 1% level, indicating that CIAT 36062 is more favorable to *Z. carbonaria* than to *Z. pubescens*.

Table 12. Survivorship parameters for nymphs of five spittlebug species reared on susceptible (CIAT 0654) or resistant (CIAT 36062) *Brachiaria* genotypes

Spittlebug species	Number tested		C[a]
	On CIAT 0654	On CIAT 36062	
Aeneolamia varia	480	480	4.8**
Aeneolamia reducta	420	480	6.4**
Mahanarva trifissa	708	246	9.7**
Zulia carbonaria	744	720	1.4ns
Zulia pubescens	648	648	2.2*

**, Significant at the 1% level; *, significant at the 5% level; ns, not significant

[a]C is the test statistic for the Cox-Mantel two-sample survival test (CIAT 0654 versus CIAT 36062).

Antibiosis to *A. varia*, *A. reducta*, and *Z. pubescens* in CIAT 36062 was also manifested by the reduced weight of surviving 4th and 5th instar nymphs, and adults. No effect on nymphal or adult weight of *Z. carbonaria* was detected. Other manifestations of antibiosis were the occurrence of minute 2nd, 3rd, and 4th instar nymphs, staggering of developmental times, and reduced spittle production by surviving nymphs. Also, we found that nymphs reared on CIAT 36062 usually leave the spittle and wander over the soil surface, eventually dying of dehydration. We found no deformation of nymphs or adults nor did we detect obvious disruptions in the molting process. The level of antibiosis resistance in CIAT 36062 clearly differs by spittlebug species and can be classified as follows: very high for *M. trifissa*, high for *A. varia* and *A. reducta*, moderate for *Z. pubescens*, and absent for *Z. carbonaria*.

Tolerance tests. CIAT 6294 expressed clear antibiosis to *A. varia* and *M. trifissa* as the mean nymphal survival of five infestation levels was significantly lower than the mean for the susceptible control CIAT 0654 (Figure 8). However, survival of *Z. carbonaria* or *Z. pubescens* nymphs was high on both genotypes at all levels of infestation, indicating lack of antibiosis in CIAT 6294 to these two species. These results were consistent with those obtained in resistance reconfirmation tests. CIAT 6294 plants suffered less damage and less plant dry weight loss than the susceptible control at all levels of infestation (Figure 9). As in previous studies, visual damage scores predicted biomass loss. Since survival of the *Zulia* spp. nymphs did not differ between the genotypes, we interpret the lower damage scores and lower plant dry weight losses caused by *Z. carbonaria* and *Z. pubescens* on CIAT 6294 as tolerance.

At all levels of infestation, survival of nymphs on CIAT 36062 was much less than on the susceptible control for *A. varia*, *A. reducta*, and *M. trifissa*, but only moderately less for *Z. pubescens*. *Z. carbonaria* nymphs survived equally well on the two genotypes (Fig. 3). Thus, expression of antibiosis in CIAT 36062 was dependent on spittlebug species. CIAT 36062 suffered significantly less damage (expressed as damage scores or plant weight loss) than the susceptible control at all levels of infestation with *Z. carbonaria* (Figure 9). Since *Z. carbonaria* nymphs survived equally well on both genotypes, we interpret the mechanism of resistance to *Z. carbonaria* in CIAT 36062 as tolerance.

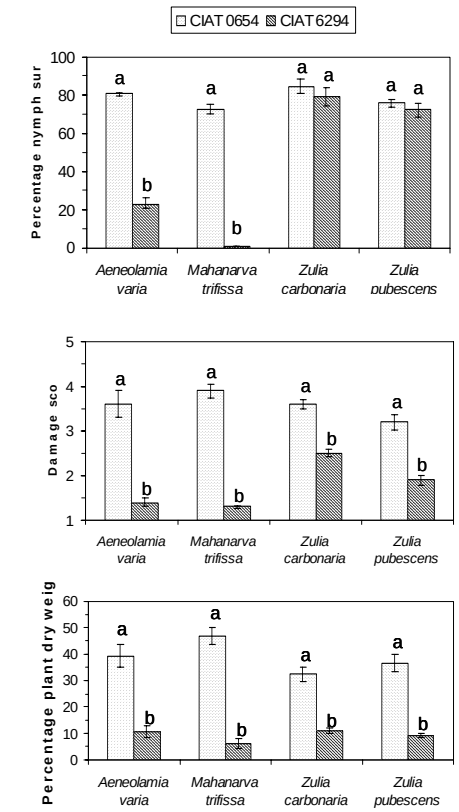


Figure 8. Response of susceptible (CIAT 0654) or resistant (CIAT 6294) *Brachiaria* genotypes to attack by nymphs of four spittlebug species. Means (± SEM) of five levels of infestation. Bars with the same letter do not differ ($P < 0.05$). Pair-wise comparison by *t*-test within spittlebug species

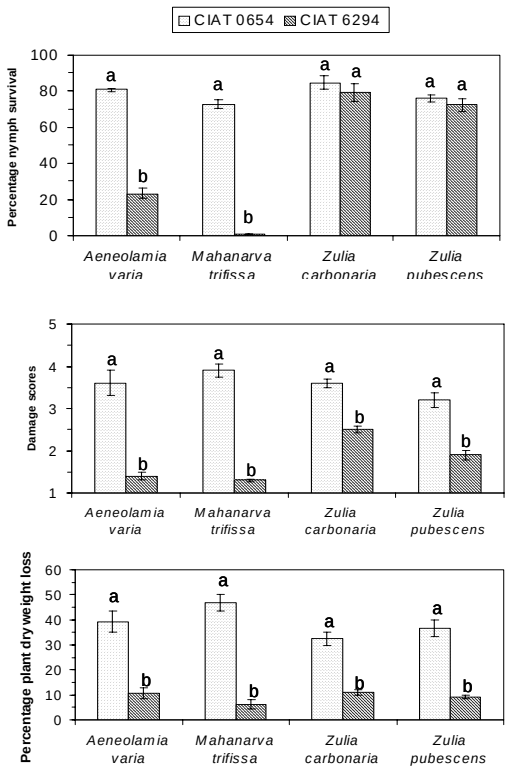


Figure 9. Response of susceptible (CIAT 0654) or resistant (CIAT 36062) *Brachiaria* genotypes to attack by nymphs of five spittlebug species. Means (± SEM) of five levels of infestation. Bars with the same letter do not differ ($P < 0.05$). Pair-wise comparison by *t*-test within spittlebug species

We also calculated a functional plant loss index to measure tolerance to both *Z. carbonaria* and *Z. pubescens*. Losses were highest for the susceptible control, CIAT 0654, at all levels of infestation (Figure 10). Losses caused by both species on CIAT 6294 and on CIAT 36062 were lower at all infestation levels. These results suggest the presence of true tolerance to *Z. carbonaria* in CIAT 6294 and CIAT 36062, true tolerance to *Z. pubescens* in CIAT 6294 and a combination of tolerance coupled with antibiosis as mechanisms of resistance to *Z. pubescens* in CIAT 36062.

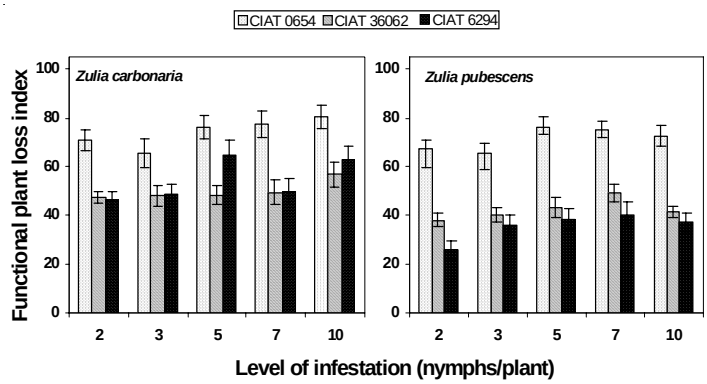


Figure 10. Functional plant loss indices (percentage) for susceptible (CIAT 0654) or resistant (CIAT 36062, CIAT 6294) *Brachiaria* genotypes exposed to five levels of infestation with each of two spittlebug species.

2.3.2 Effect of infestation with two species of spittlebug on resistance expression in selected *Brachiaria* genotypes

Contributors: A. Pabón, G. Sotelo, and C. Cardona (CIAT)

Rationale

As explained in the 2002 Annual Report, we can identify spittlebug nymphs with absolute precision by means of RAPD-PCR DNA analysis or by comparison of esterase banding patterns. Using these techniques we have been able to detect mixed infestations in commercial fields and to measure percentage survival of different species when mixed infestations by two or more species occur. This in turn has allowed us to study how different species combinations affect resistant expressions in selected resistant or susceptible genotypes.

Materials and Methods

In 2003 we measured the effect of single species infestation as opposed to mixed infestations by infesting plants with eggs of two or more spittlebug species in different proportions. The infestation was allowed to proceed until adult emergence occurred. Plants were then scored for damage and the surviving nymphs were collected and identified to species level by comparison of esterase banding patterns or, in some cases, by RAPDs-PCR analysis. Percentage survival was calculated for each spittlebug species.

Results and Discussion

We will highlight results of studies on the effect of mixed *Aeneolamia reducta* - *Zulia carbonaria* infestations. These are two of the most important spittlebug species present in Colombia. As shown in Figure 11, when the resistant genotype CIAT 36062 is exposed to *Zulia carbonaria* alone or when *Z. carbonaria* predominates in the mixture, damage scores increase so that the genotype is classified as intermediate resistant rather than resistant. This was not the case with the hybrid SX01NO/0102, the most resistant hybrid tested to date for resistance to five spittlebug species.

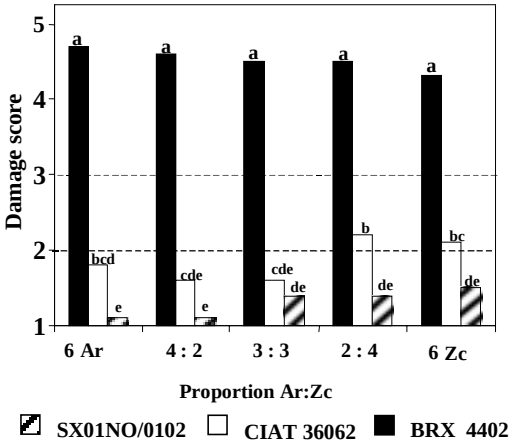


Figure 11. Damage scores recorded on susceptible (S) and resistant (R) *Brachiaria* genotypes exposed to individual or simultaneous attack by nymphs of *Aeneolamia reducta* (Ar) or *Zulia carbonaria* (Zc). Dotted lines represent cut-off points for resistance (< 2) and intermediate ratings (2 -3) in a 1 - 5 damage score scale. Bars accompanied by the same letter represent means that are not significantly different at the 5% level by LSD.

Most important, we detected significant and differential antibiosis effects (Figure 12) when mixed populations of *A. reducta* and *Z. carbonaria* in different proportions were used to infest plants of the resistant genotypes CIAT 36062 and SX01NO/0102 (Figure 12). At all levels of infestation, survival of *A. reducta* on both resistant genotypes was significantly reduced to levels below the cut-off point for resistance rating (< 30%). On the contrary, the survival of *Z. carbonaria* nymphs on CIAT 36062 was significantly higher, in some cases well above the 50% level used to classify genotypes as susceptible. The hybrid SX01NO/0102 showed intermediate resistance to *Z. carbonaria* at two of the levels of infestation tested. Again, these findings emphasize the need to characterize resistance to as many species as possible and illustrate the need to breed for multiple antibiosis resistance.

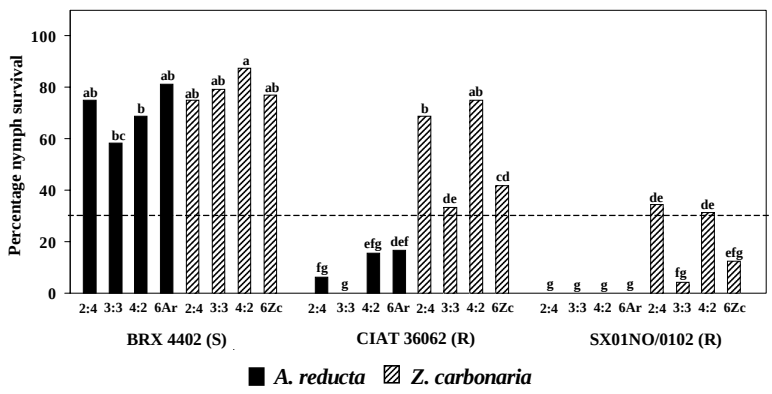


Figure 12. Levels of antibiosis (reduced percentage nymph survival) detected when plants of susceptible (S) or resistant (R) *Brachiaria* genotypes were infested with *Aeneolamia reducta* or *Zulia carbonaria* or combinations thereof (*A. varia*: *Z. carbonaria*). The dotted line represents the cut-off point for resistance rating (< 30% percentage survival). Bars accompanied by the same letter represent means that are not significantly different at the 5% level by LSD.

2.3.3 Interactions between strains of five spittlebug species and resistance expression in selected *Brachiaria* genotypes

Contributors: C. Cardona, G. Sotelo, and A. Pabón (CIAT)

Rationale

The protective properties of insect-resistant cultivars may be overcome by the development of resistance-breaking strains of a given insect species. These are insect populations that possess an inherent genetic capability to overcome plant resistance. Typically, biotypes develop as a result of selection from the parent population in response to exposure to the resistant cultivar. It may occur that the genetic capability of an insect to overcome resistance is so great that resistance is nullified before the resistance cultivar is grown in a large geographical area. This is why it is important to obtain information on the reaction of resistant genotypes to as many geographical strains of a given insect pest as it is possible. We initiated a series of experiments aimed at measuring the response of resistant cultivars to populations of *A. varia*, *A. reducta*, *Z. pubescens*, *Z. carbonaria*, and *M. trifissa* collected in several different areas of Colombia. For the first time, we also generated information on resistance to *Prosapia simulans* (Walker).

Materials and Methods

All trials were conducted using test materials well known for their reaction to *Aeneolamia varia*. We evaluated the susceptible checks CIAT 0606 and CIAT 0654, the resistant checks CIAT 6294 and CIAT 36062, and two new sexual hybrids SX01NO/0102 and SX01NO/0233 classified as highly resistant to *A. varia* in previous studies. The *A. varia*-CIAT colony combination was used as the standard check in all trials. Screening for resistance was conducted using standard methodologies. Plants were infested with six eggs per plant of the respective spittlebug species-geographical combination and the infestation was allowed to proceed without interference until all nymphs reached the fifth instar stage or adult emergence occurred. Plants (20 per genotype) were scored for symptoms using the damage scale (1, no damage; 5, plant dead) developed in previous years. Percentage nymph survival was calculated.

Results and Discussion

We have conducted four trials. At this point, we will highlight results obtained with geographical strains of *A. varia* and *Z. pubescens*. We will also report on our first-ever screening for resistance to *P. simulans*.

The reaction of six genotypes to attack by nymphs of three strains of *A. varia* is shown in Table 13. No significant genotype x strain interaction was detected for damage scores or percentage nymph survival, meaning that resistance ratings did not change when the genotypes were exposed to different strains of *A. varia*. Similarly, no significant genotype x strain interaction was detected when susceptible and resistant genotypes were exposed to attack by nymphs of *Z. pubescens* (Table 14).

Table 13. Reaction of selected *Brachiaria* genotypes to strains of *Aeneolamia varia* from two geographical areas of Colombia

Origin of strain	Genotypes[a]					
	BRUZ4X-44-02	CIAT 0606	CIAT 6294	CIAT 36062	SX01NO/ 0102	SX01NO/ 0233
	Damage scores					
Florencia, Caquetá	3.9b	3.8b	1.2a	1.3a	1.1a	2.0a
V/vicencio, Meta	4.2a	3.7b	1.3a	1.1a	1.0a	1.3b
CIAT colony	4.9a	4.6a	1.3a	1.1a	1.0a	2.2a
Mean	4.3A	4.0A	1.3C	1.2C	1.0C	1.8B
	Percentage nymph survival					
Florencia, Caquetá	75.8b	86.6a	17.5b	0.0c	2.5b	26.7b
V/vicencio, Meta	90.8a	71.1b	35.8a	9.2a	6.7a	14.2c
CIAT colony	86.1a	87.1a	19.8b	4.7b	6.3a	40.7a
Mean	84.2A	81.6A	24.4B	4.6C	5.2C	27.2B

Means of 20 replicates by genotype by insect strain. For each variable, means within a column followed by the same lowercase letter, and means within a row followed by the same uppercase letter are not significantly different at the 5% level by LSD.

Table 14. Reaction of selected *Brachiaria* genotypes to strains of *Zulia pubescens* from three geographical areas of Colombia

Origin of strain	Genotypes					
	BRX 44-02	CIAT 0606	CIAT 6294	CIAT 36062	SX01NO /0102	SX01NO /0233
	Damage scores					
Darién, Valle	3.9a	4.2a	2.0a	1.0b	1.1a	1.5b
Popayán, Cauca	3.8a	3.3b	2.1a	1.2ab	1.1a	1.3b
S. José de Fragua, Caquetá	4.3a	4.1a	2.1a	1.4a	1.3a	2.6a
Mean	4.0A	3.9A	2.1B	1.2C	1.2C	1.8B
	Percentage nymph survival					
Darién, Valle	71.6a	55.9ab	34.1b	2.8b	0.9c	4.6b
Popayán, Cauca	44.3b	45.5b	47.5a	11.6a	6.7b	5.0b
S. José de Fragua, Caquetá	74.1a	69.0a	34.2b	8.8a	21.6a	47.2a
Mean	63.3A	56.8A	38.6B	7.7D	9.7D	18.9C

Means of 20 replicates by genotype by insect strain. For each variable, means within a column followed by the same lowercase letter, and means within a row followed by the same uppercase letter are not significantly different at the 5% level by LSD.

In Table 15 we summarize results of our first screening for resistance to *Prosapia simulans*. Susceptible (CIAT 0606, BRX-44-02) and resistant genotypes (CIAT 6294, CIAT 36062, SX01NO/0102, and SX01NO/0233) differed for damage scores for all spittlebug species tested (Table 15). *P. simulans* caused more damage than *A. varia* and *M. trifissa* on the resistant genotype CIAT 6294 ('Marandú'). Using our resistance classification, CIAT 6294 would be classified as resistant to *A. varia* and *M. trifissa* (damage scores: 1-2) but intermediate to *P. simulans* (damage scores: 2.1-3.0). SX01NO/0233 was intermediate to all three species tested. Survival of nymphs of *A. varia* and *M. trifissa* was significantly lower on the *A. varia*-resistant genotypes than on the susceptible controls CIAT 0606 and BRX-44-02 (Table 15). Survival of *P. simulans* nymphs was significantly higher on CIAT 6294 than on the other resistant genotypes suggesting that antibiosis resistance to this species is absent in 'Marandú'. Using our resistance classification, CIAT 6294 would be classified as susceptible (> 50% survival) to *P. simulans*. The relatively low levels of damage caused by *P. simulans* on CIAT 6294 could be the result of tolerance to this species.

Table 15. Response of selected *Brachiaria* genotypes to attack by nymphs of three spittlebug species

Spittlebug species	Spittlebug species		
	Mahanarva trifissa	*Aeneolamia varia*	*Prosapia simulans*
	Damage scores		
BRX-44-02	4.2aA	4.9aA	4.3aA
CIAT 0606	3.5bB	4.6aA	4.3aA
CIAT 6294	1.2cB	1.3cB	2.4bA
CIAT 36062	1.1cA	1.1cA	1.3cA
SX01NO/0102	1.0cA	1.0cA	1.5cA
SX01NO/0233	2.1bcA	2.2bA	2.3bA
	Percentage nymph survival		
BRX-44-02	55.0aB	86.1aA	90.8aA
CIAT 0606	36.0aC	87.1aA	79.2bA
CIAT 6294	0.0bC	19.8cB	65.0bA
CIAT 36062	0.0bB	4.7dA	14.2cA
SX01NO/0102	0.0bA	6.3dA	6.7dA
SX01NO/0233	34.5aA	40.7bA	5.0dB

Means of 20 replicates by genotype by insect species. For each variable, means within a column followed by the same lowercase letter, and means within a row followed by the same uppercase letter are not significantly different at the 5% level by LSD.

2.3.4 Mechanisms of resistance in Brachiaria to adults of five spittlebug species and sub-lethal effects of antibiosis on adults of spittlebug

Contributors: P. Sotelo, G. Sotelo, and C. Cardona (CIAT)

Rationale

Varying levels of antibiosis resistance to nymphs of several spittlebug species have been well characterized in a number of resistant *Brachiaria* genotypes. The effects of antibiosis on the biology of nymphs have also been studied. Not much is known about possible direct effects of antibiotic genotypes on the biology of adults. Even less is known about sub-lethal effects (i. e. reduced oviposition rates, reduced longevity, prolonged generation times, reduced rates of growth, etc.) on adults resulting from nymphs feeding on antibiotic

genotypes. We initiated a series of studies aimed at measuring how antibiotic genotypes may directly or indirectly (through sub-lethal effects) affect the biology of adults of *A. varia*.

Materials and Methods

Initially, we conducted two experiments aimed at measuring how feeding on an antibiotic genotype affects the biology of adults of *A. varia*. Later on, we initiated a comprehensive series of experiments aimed at determining whether antibiosis to nymphs has an adverse effect on the biology of resulting adults. For this, a number of life tables will be constructed. Treatment combinations are shown in Table 16.

This work is in progress. Results will be presented in full in 2004.

Table 16. Treatment combinations to study possible sub-lethal effects of intermediate and high levels of nymphal antibiosis on adults of *Aeneolamia varia.*

Nymphs reared on:	Adults feeding on:	Null hypothesis
BRX 44-02[a]	BRX 44-02	Absolute check
BRX 44-02	CIAT 06294	A genotype that is moderately antibiotic to nymphs does not affect adults
BRX 44-02	CIAT 36062	A genotype that is highly antibiotic to nymphs does not affect adults
CIAT 06294	BRX 44-02	Intermediate antibiosis to nymphs does not affect resulting adults
CIAT 06294	CIAT 06294	Intermediate antibiosis to nymphs does not affect resulting adults even when these are feeding on a moderately antibiotic genotype
CIAT 06294	CIAT 36062	Intermediate antibiosis to nymphs does not affect resulting adults even when these are feeding on a highly antibiotic genotype
CIAT 36062	BRX 44-02	High antibiosis to nymphs does not affect resulting adults
CIAT 36062	CIAT 06294	High antibiosis to nymphs does not affect resulting adults even when these are feeding on a moderately antibiotic genotype
CIAT 36062	CIAT 36062	High antibiosis to nymphs does not affect resulting adults even when these are feeding on a highly antibiotic genotype

[a] BRX44-02 is susceptible to *A. varia*. CIAT 6294 and CIAT 36062 show intermediate and high levels of antibiosis resistance to nymphs of *A. varia*, respectively.

2.4 Selection of Brachiaria hybrids for resistance to *Rhizoctonia* foliar blight disease

Highlights

- Detection and identification of a nitrogen fixing/growth promoting bacteria associated with *Brachiaria*.
- Development of a quick inoculation method in *Brachiaria* for *Rhizoctonia solani*.

2.4.1 Development of a new inoculation method for *Rhizoctonia solani* in *Brachiaria*

Contributors: C. Zuleta, S. Kelemu, J. Miles, I. Rao (CIAT)

Rationale

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

Rhizoctonia solani is the most widely known species of *Rhizoctonia* with a wide host range. The fungus is a basidiomycete and does not

produce any asexual spores (conidia). Occasionally the fungus produces sexual basidiospores. Out in nature *R. solani* reproduces mainly asexually and exists as vegetative mycelia and/or dense sclerotia. The pathogen primarily infects below ground plant parts in a number of plant species, but can also infect above ground plant parts such as pods, fruits, and leaves and stems as is the case with *Brachiaria*. In *Brachiaria*, infected leaves first appear water-soaked, then darken, and finally turn to a light brown color (see symptom Photo 1). Lesions may coalesce quickly during periods of prolonged leaf wetness and temperatures between 21 and 32 ºC.

The pathogen's sclerotia can survive in soil and on plant debris for several years. These sclerotia can germinate and produce hyphae that can infect a wide range of host plants. The infection process is enhanced by the production of many different extracellular enzymes that degrade components of plant cell walls. As the plant cells die due to infection, the hyphae continue to grow and colonize dead tissue, eventually forming sclerotia (eg. see Photo 1). New inocula are produced on or in host tissues, and a new cycle is repeated when plant hosts or other substrates become available.

The ability to uniformly induce disease and measure resistance accurately is crucial in a breeding program for developing resistant cultivars. The objectives of this study are to: 1) develop a rapid and reliable artificial inoculation method that allows uniform disease development in genetically susceptible genotypes of *Brachiaria*, 2) accurately measure resistance for subsequent use in identifications of resistant materials among *Brachiaria* genotypes.

Photo 1. Foliar blight symptoms caused by *Rhizoctonia solani* on leaves of *Brachiaria*. Note mature brown and young white sclerotia on the leaves.

Materials and Methods

Plant materials: Two *Brachiaria* genotypes CIAT 16320 (resistant to *R. solani*) and a susceptible CIAT 36061 (cv. Mulato) were used for developing a new inoculation method.

Inoculum preparation: Sclerotia of *R. solani* originally isolated from *B. brizantha* CIAT 6780 was germinated on potato dextrose agar (PDA) at 28 ºC. Mycelial discs (0.5 mm in diameter) were cut out of the actively growing 2 days old PDA culture.

New inoculation method: Mature leaves were detached and cut to 12 cm in length. Two leaves (one from CIAT 16320 and another of CIAT 36061) were placed in each plastic Petri dish of 15 cm in diameter containing a 2-mm- thick filter paper (see Photo 3). Ten-ml sterile distilled water was applied to the filter paper to keep it moist and to create humidity. A mycelial disc was placed in the middle of each leaf. The Perti dish was closed with its lid and sealed with a parafilm tape. All plates containing inoculated leaves were placed on a laboratory bench with access to sunlight through a large glass window and incubated at room temperature that ranged 26-29 °C. Control leaves were inoculated with pure agar discs free from *R. solani*. Three replications were made for each treatment.

For comparison, whole plants were inoculated in the greenhouse using previously described methods (IP-5 Annual Report 2002) that were slightly modified. CIAT 16320 and CIAT 36061 (each 20 plants) generated from tillers were inoculated with mycelial discs of *R. solani*. A mycelial disc was placed in contact of each plants stem just 2 cm above the soil level and wrapped with parafilm to secure the contact. Each inoculated plant was kept inside a plastic column

to keep them separated from each other (see Photo 3). The inoculated plants were kept in a humidity chamber with 2 hours of mist application per day for 10 days. They were then evaluated for resistance using visual estimation based on Harsfall-Barratt rating system (1-11, where 1 = 1-3%, 2 = 4-6%, 3 = 7-12%, 4 = 13-25%, 5 = 26-50%, 6 = 51-75%, 7 = 76-87%, 8 = 88-93%, 9 = 94-97%, 10 = 98-99%, and 11 = 100%; as well as using measurements of chlorophyll content.

Photo 2. Plastic columns separating individual plants after inoculation with *Rhizoctonia solani* in greenhouse tests.

Evaluation of resistance: Disease reaction was assessed at 0, 120, 144 and 168 hr after inoculation. Three different measurements were taken. 1) disease lesion size (both width and length). These measurements were presented as percent of the total leaf size (both width and length); 2) chlorophyll content of each leaf. Thirty measurements were taken across each leaf with chlorophyll meter SPAD 502 (Minolta), and an average of these 30 measurements recorded which gives a good indication of the chlorophyll content of the leaf being evaluated (the values are calculated based on the amount of light transmitted by the leaf in 2 wavelength regions in which the chlorophyll absorption is different); 3) Chlorophyll fluorescence. Chlorophyll fluorescence transients emitted from leaves were measured by a plant efficiency analyzer) PEA; Hansatech Ltd., King's Lynn, Northfolk, UK). Light was provided by an array of six high intensity light-emitting diodes (650 nm wavelength) that were focused onto the leaf surface to be evaluated (providing a red light of a peak 650 nm wavelength which is absorbed by the chloroplasts of the leaf). The values recorded correspond to a ratio of the variable fluorescence divided by the maximum fluorescence that is automatically calculated during measurement.

Results and Discussion

The appearances of disease symptoms on susceptible leaves start 48 hr after inoculation. Symptoms fully develop throughout the inoculated leaf of the susceptible genotype (CIAT 36061) 120 hr after inoculation (see Photo 3). The experiment was repeated three times and the data obtained were consistent in all the three experiments.

There are clear distinctions in values (lesion size, chlorophyll content and chlorophyll fluorescence) between CIAT 16320 (highly resistant) and the susceptible CIAT 36061 (Table 17). In addition, there is strong correlations (r=0.88) between measurements of lesion size and chlorophyll content as well as those of lesion size and photosynthesis efficiency, indicating that either one of the measurements can be used to measure resistance (see Figures 13 and 14). There was also a high correlation (r=0.88) between the chlorophyll content and fluorescence measurements.

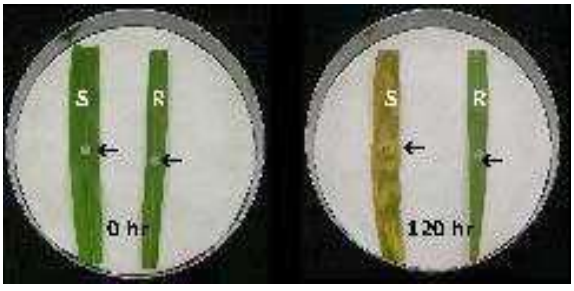


Photo 3. Reactions of detached *Brachiaria* leaves of CIAT 36061 (S; susceptible) and CIAT 16320(R; resistant) to *Rhizoctonia solani*, at the time of inoculation (0 hr) and 120 hr. after inoculation. The arrows indicate the inoculation point where a potato dextrose agar (PDA) mycelial disc was placed.

Table 17. Disease reaction measurements for two contrasting genotypes of *Brachiaria* inoculated with *Rhizoctonia solani* at various hours after inoculation.

Brachiaria CIAT No.	Hour after inoculation	Lesion size (% of total leaf area)	Chlorophyll content (SPAD)	Chlorophyll fluorescence Fv/Fm
16320	0	0.0	48.1	0.84
36061	0	0.0	43.8	0.84
16320	120	1.1	39.2	0.82
36061	120	46.4	9.0	0.40
16320	144	1.5	32.0	0.79
36061	144	53.9	6.7	0.08
16320	168	2.5	24.6	0.74
36061	168	60.1	5.8	0.00

Lesion size measurements are time consuming and provide no additional advantage in resistance measurement. Measuring fluorescence values is more time consuming than chlorophyll content measurement. Because these values are highly correlated with each other, we selected chlorophyll content values as a measure of resistance to foliar blight disease.

Disease reaction data on live plants in greenhouse tests are comparable to those on detached leaves in Petri dishes. Chlorophyll content measurements of one of the middle infected leaves and the visual rating system were correlated (r=0.70).

This new inoculation and resistance evaluation method we developed has several advantages: 1) it is rapid: disease reactions can e evaluated within 120 h (5 days) after inoculation as compared to 10-15 days in GH whole plant inoculations; 2) reproducible: results are consistent among repeated experiments; 3) highly uniform among replications: there is little or no variation among leaf replications of the same genotype; 4) very little space on a laboratory bench, compared to a whole green house room for whole plant assays, is needed for the same number of materials to be evaluated; 5) less labor and material cost since no soil preparation, planting pots, transplanting, watering and maintaining plants are needed, 6) leaf tissues can be cut directly from the breeding population for evaluation without the need to propagate tillers; 7) chlorophyll content measurement for the evaluation of resistance is faster than disease lesion measurement and yet provides more consistent quantification than commonly used visual rating methods which can vary from person to person and can be subjective to individual judgments; 8) comparable to disease reaction data on whole plants in green house tests.

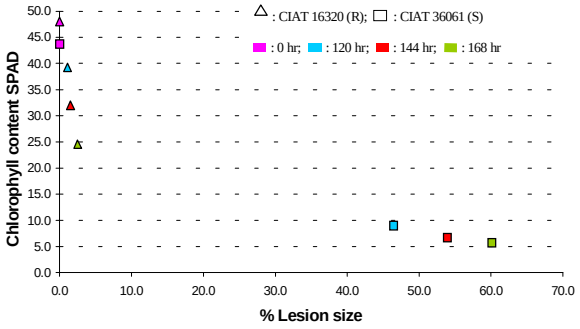


Figure 13. Chlorophyll content and percentage foliar blight lesion size of *Brachiaria* CIAT 16320 (resistant) and CIAT 36061 (susceptible) detached leaves inoculated with *Rhizoctonia solani*

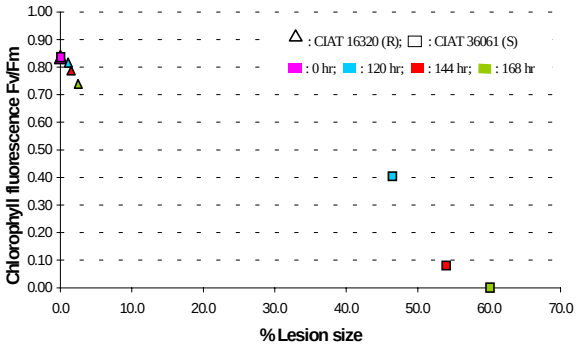


Figure 14. Chlorophyll fluorescence values and percentage foliar blight lesion size of *Brachiaria* CIAT 16320 (resistant) and CIAT 36061 (susceptible) detached leaves inoculated with *Rhizoctonia solani*

2.4.2 Characterization of pathogen associated with bacterial wilt disease in Brachiaria

Contributors: M. Rodriguez and S. Kelemu (CIAT)

Rationale

We have previously reported a bacterial wilt disease of *Brachiaria* and its casual agent, (Zuleta et al., 2002. Manejo Integrado de Plagas y Agroecologia 64:41-47). *Xanthomonas campestris* pv. *graminis* infects a number of cultivated forage grasses. Some of the first symptoms are chlorotic/ necrotic stripes along the leaves. As the disease advances, the whole leaf may die. Under severe conditions, the whole plant may turn yellow and die. Another typical symptom is wilting and curling of leaves without any discoloration or lesions, which result in quick plant death.

We have demonstrated that the pathogen is seed transmitted and is also transmitted vegetatively (AR-2001). Although the disease is not economically important to date, it is important for quarantine purposes.

Sixty-seven isolates of *X. campestris* pv. *graminis* have been collected from sites in Colombia from various genotypes of *Brachiaria* in order to determine pathogenicity and genetic diversity.

Families of repetitive DNA sequences such as repetitive extragenic palindromic (REP), enterobacterial repetitive intergenic consensus (ERIC), and box elements (BOX), which are present in all prokaryotes can be used for bacterial fingerprinting. Polymerase chain reactions (PCRs) based on these repetitive sequences, collectively designated as rep-PCR, have been used to assess variation among pathovars as well as to differentiate strains of the same pathotype of *Xanthomonas* species. In this study, we used rep-PCR with REP, BOX and ERIC primers to evaluate the genetic diversity of *X. campestris* pv. *graminis* isolates.

Materials and Methods

Bacterial isolates: A total of 67 independent colonies of X*anthomonas campestris* pv. *graminis* were collected from naturally infected species of *Brachiaria* at Carimagua, Santander de Quilichao, Popayán, and Palmira. Leaves were cut into small pieces (approximately 1 $cm^{2)}$ and surface-sterilized in1% NaOCl solution for 2 min and in 70 % ethanol for 1 min. They were then rinsed with sterile deionized water, and macerated in sterile water. A dilution series of the macerated suspension was plated on nutrient agar for selection of independent bacterial colonies. Their pathogenicity was confirmed by inoculating a susceptible material (hybrid *Brachiaria* CIAT 36062). Selected colonies were grown in nutrient broth with shaking (200 rpm) at 28 ^{o}C. They were stored in 30% glycerol at -20 ^{o}C for use in further studies. Two isolates (CIAT 46 and CIAT 469) of *X. axonopodis* pv. *manihotis* were included as control.

DNA isolations: Bacterial cells were grown overnight in Luria broth medium in a shaker (200 rpm) at 28^{o}C. Cells were collected in microcentrifuge tubes by centrifugation (8000 rpm for 10 min) and discarding the supernatant. The cells were re-suspended in 600-µl TE (50mM Tris, pH 8 and 50mM de EDTA) and stored at –20 ^{o}C for 10 min, and subsequently thawed at room temperature. Sixty-µl of a freshly prepared lysozyme (10 mg/mL in 25 mM Tris pH 8) and a 6-µl RNase (stock concentration 10mg/ml) were added to the cell suspension and incubated 15 min at room temperature and transferred to ice for another 15 min. A 120-µl STEP solution (0.5% SDS, 50mM Tris pH 7.5 and 280-ìg of proteinase) was added and incubated at 37 ^{o}C for an hour. Subsequently, 216-µl of ammonium acetate solution (7.5 M concentration) was added and mixed well. The solution was precipitated with

phenol: chloroform: isoamyl alcohol (25:24:1) The supernatant was treated twice with equal volume of chloroform: isoamyl alcohol (24:1). The DNA was then precipitated with isopropanol and centrifuged. The DNA pellet was washed with 70% ethanol, air-dried and re-suspended in 100-ìl sterile distilled water.

DNA amplifications: The following primers were used: 1) ERIC (enterobacterial repetitive intergenic consensus sequence) ERIC-1R: 5'ATG TAA GCT CCT GGG GAT TCA C 3', ERIC –2: 5'AAG TAA GTG ACT GGG GTG AGC G 3'; 2) BOXA1R (Box element sequence): 5' CTA CGG CAA GGC GAC GCT GAC GCT GAC G 3'; 3) REP (repetitive extragenic palindromic sequence) REP1R-I: 5' IIII CGI CGI CAT CIG GC 3', REP2-I : 5' ICG ICT TAT CIG GCC TAC 3'

Each 25-µ reaction mixture contained 30 ng template DNA, 3mM $MgCl_2$, 1.2 (for BOX) and 2 (for ERIC and REP) pmol each primer, 200-ìM each of the four dNTPs, 1 U of Taq-DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Tritón X-100 and 10% (v/v) DMSO (dimethyl sulfoxide).

Amplifications were performed in an automated thermocycler (MJ Research Inc, MA) with an initial denaturation (3 min at 94 °C), followed by 35 cycles of denaturation (30 s at 92 °C, annealing ((1 min at 50 °C for ERIC and BOX; at 40 °C for REP), and extension (8 min at 65°C), with a final extension (10 min at 65°C).

RAPD-PCR: Amplifications were carried out with 7 primers from Operon Technologies, Inc. with codes and sequences as follows OPA-01 (5'-CAGGCCCTTC –3'), OPA-02 (5'-TGCCGAGCTG –3'), OPA-03 (5'-AGTCAGCCAC-3'), OPA-04 (5'-AATCGGGCTG-3´), OPAJ-11 (5´-GAACGCTGCC-3´), OPC-02 (5´-GTGAGGCGTC-3´), OPD- 03 (5´-GTCGCCGTCA-3´). The reaction had a total volume of 20-µl with 30 ng DNA, 3-mM MgCl2, 0.5-µM primer, 0.26-mM of mixture of dNTPs, 50-mM KCl, 10-mM Tris-HCl (pH 8.8), 0.1% Tritón X-100 and 1 U Taq-DNA polymerase.

Amplifications were performed in an automated thermocycler (MJ Research Inc, MA) with an initial denaturation (2 min at 94 °C), annealing (5 min at 28 °C), denaturation (1 min at 94 °C) followed by 45 cycles of denaturation (20 s at 92 °C), annealing (1 min at 35 °C), and extension (1 min at 72 °C), with a final extension (7 min at 72 °C).

***Data analysis*:** rep-PCR fingerprints were converted to binary form (presence =1; absence = 0) and similarity coefficients for pairs of strains were calculated NTSYS (Numerical Taxonomy and multivariate Analysis system) version 2.02 (Exeter Software), using SIMQUAL with the Dice coefficient and were subjected to unweighted pair group method (UPGMA) cluster analysis. The same data matrix was subjected to multiple correspondence analysis (MCA) and analyzed using CORRESP Procedure of SAS/STAT Software.

Results and Discussion

Multiple correspondence analysis of the combined data matrix generated using REP-PCR, ERIC-PCR and BOX-PCR resulted in 3 groups with an average similarity index of 78% (Figure 15). Isolates *Xc* 44 and *Xc* 45 that were collected in Carimagua appear to be clonal. The same was true with isolates *Xc* 49 and *Xc* 50 that were collected in Palmira.

Group 1 consists of 30 isolates with an average similarity index of 52%. Group 2 had 15 isolates with a 50% similarity index. The two control isolates from cassava were clustered within group 2. Group 3 consisted of 24 isolates and had a high similarity index of 89%.

Multiple correspondence analysis of the RAPD data set resulted in three groups of isolates as well (Figure 16). The first dimension clearly separated group 2 from groups 1 and 3, whereas dimensions 2 and 3 did not contained 40 isolates with 84% similarity index. Group 2 consisted of 25 isolates that were all collected in Palmira. Group 3 had only 4 isolates.

Both RAPD and rep-PCR generated multiple bands. However, correlation between these two techniques was low (r = 0.36). RAPD data did not

differentiate the two isolates of *X. axonopodis* pv. *manihotis*. The same was the case with ERIC-PCR. However, with BOX primers, as well as the combined data of BOX and REP-PCR separated the two distinct isolates from those of *X. campestris* pv. *graminis*. These results hint that rep-PCR can be used to develop rapid diagnostic tools for *Xanthomonas* pathovars. In addition, both RAPD and rep-PCR revealed genetic diversity among isolates of *X. campestris* pv. *graminis*. In light of this, caution has to be taken in germplasm movement in order not to transfer new isolates of this seed-borne pathogen from one location to another.

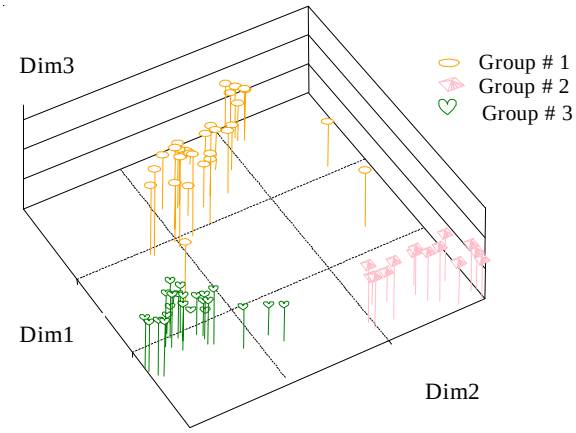


Figure 15. Multiple Correspondence Analysis (MCS) by combining the rep-PCR fingerprints of 69 isolates of *Xanthomonas* obtained with each of the two primers REP and ERIC, and BOX primer.

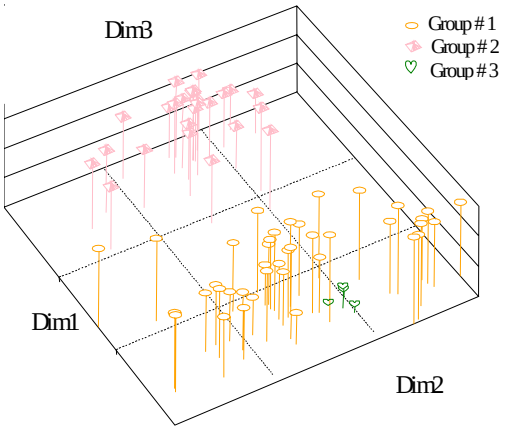


Figure 16. Multiple Correspondence Analysis (MCS) of RAPD-PCR data conducted with 69 *Xanthomonas* isolates.

2.4.3 Resistance in Brachiaria to *Xanthomonas campestris* pv. *graminis* (*Xcg*)

Contributors: M. Rodriguez and S. Kelemu (CIAT)

Rationale

Breeding host plants for disease resistance is the most important, cheapest and practical method of disease control. The financial, environmental and social benefits of using resistant cultivars of important crops are big. By combining the genes for resistance from various different genotypes, a formidable host resistance should be built against evolving races of the pathogen. The serious obstacle to this could be the variability encountered in the pathogen.

Although, there is currently no breeding program in IP-5 to combat this disease, we examined some genotypes of *Brachiaria* for their reaction to *X. campestris* pv. *graminis*. The objective of this study is to identify some sources of resistance as well as to evaluate important genotypes of *Brachiaria*.

Materials and Methods

Inoculum preparation and plant inoculation: Bacterial cells from a single colony of each isolate were grown in tubes containing freshly prepared nutrient broth (Difco), and incubated with shaking at 200 rpm, 28 C, overnight. Bacterial cells were collected by centrifugation at 4,000 rpm for 20 minutes. The medium was removed and bacterial cells re-suspended in sterile distilled water and adjusted to an optical density of OD_{600} = 0.1. Sterilized scissors were immersed in the bacterial suspension and used to cut leaves of *Brachiaria* plants. Leaves of control plants were cut with scissors immersed in sterile distilled water. All plants were placed in humidity chambers maintained at 27 C and RH of 70% for 48 hours. They were then moved to a growth chamber at 28-30 C and photo- period of 12 hours, or in the green house until symptoms were expressed.

Plant evaluation: Selected *Brachiaria* accessions and hybrids were evaluated for their reactions to *X. campestris* pv. *graminis*. Plants that showed any visible wilt symptoms within 15 days after inoculations were rated as susceptible (S), and those that maintained "healthy" appearance were rated resistant (R).

Results and Discussion

Thirteen *Brachiaria* genotypes, BRO-02-193, BRO-02-415, BRO-02-445, BRO-02-465, BRO-02-968, BRO-02-1045, BRO-02-1405, BRO-02-1474, CIAT 16322, CIAT 26110, CIAT 26990, CIAT 36061, 36062, were tested for their reaction to *X. campestris* pv. *graminis*. Seventeen isolates of the pathogen were used to inoculate each of the genotypes. Three genotypes, CIAT #16322, 26110 and 26990, showed no disease symptoms after inoculations with each of the 17 isolates. CIAT 36062 was the most susceptible of the genotypes evaluated, being infected with 16 of the isolates. Isolates of *X. campestris* pv. *graminis* that infect *Brachiaria* exhibit a wide range of genetic diversity. Pathogenic variation reveal that a wide range of pathotypes exist within the pathogen population. It is encouraging to note that high levels of resistance exist in *Brachiaria*, and it is possible to combine the available resistance in a breeding program.

2.5 Elucidate the role of endophytes in tropical grasses

Highlights

- First evidence that endophyte infection can improve dry season performance under field condition by improving the uptake of N, P and K acquisition by two accessions of Brachiaria brizantha.

2.5.1 Endophyte seed transmission studies in *Brachiaria*

Contributors: H. Dongyi (South China University) and S. Kelemu (CIAT)

Rationale

Brachiaria is a pan-tropical genus of grasses with about 100 species. The fungus *Acremonium implicatum* can develop an endophytic association that is mutually beneficial with *Brachiaria* species.

DNA from isolates of *A. implicatum* was amplified using 10-base random primers. Primer OPAK 10 (Operon Technology Inc.) amplified bands including a 500-bp product common to all of the isolates tested. This fragment has been cloned

and sequenced. Based on this sequence data, several primers were designed and synthesized. A primer pair designated P1 (5'-TTCGAATGATAAGGCAGATC-3' and P4 (5'-ACGCATCCACTGTATGCTAC-3') amplified a 500-bp product with template DNA from isolates of *A. implicatum* in pure cultures and in tissues of *Brachiaria* infected with *A. implicatum*. No amplification product was detected in plants free from *A. implicatum* or using DNA of non-endophytic fungi or the bacterium *Xanthomonas campestris* pv. *graminis*, a pathogen of species of *Brachiaria* (Kelemu et al., 2003. Molecular Plant Pathology 4: 115-118).

This primer pair was used to conduct seed transmission studies in plants with and without *A. implicatum*. We report here the results of *A. implicatum* transmission studies in seedsand seedlings of *Brachiaria*. Preliminary data have been reported in IP-5 annual report 2002. The primer pair amplified a 500-bp product with template DNA of seeds harvested from *A. implicatum* infected *Brachiaria* plants, but no amplified products were observed with DNA of seeds from endophyte-free plants.

Materials and Methods

Endophyte elimination: The fungicide Folicur® was used to generate endophyte-free *Brachiaria* clones. Twenty or more plantlets were propagated from a mother plant naturally or artificially infected with the endophyte. Half of these plantlets were soaked in a solution of 0.6 mL/L of Folicur® (250 g a.i./L) for 6 h to eliminate the endophyte, and the other half were left untreated to serve as controls. All plantlets were individually planted in small pots and placed in the greenhouse. Plants were examined 4-6 weeks after treatment for the presence or absence of *A. implicatum*.
DNA isolations: Fresh mycelia of endophyte isolates cultured on PDA plates, endophyte-infected or endophyte-free plant leaves, or seeds were collected and macerated in liquid nitrogen for genomic DNA isolation. Genomic DNA was extracted using the DNeasy™ Plant Mini Kits (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

PCR Amplifications: Specific primers P1 (5'-TTCGAATGATAAGGCAGATC-3') and P4 (5'-ACGCATCCACTGTATGCTAC-3') were used in the PCR reactions. Amplifications were carried out in a Programmable Thermal Controller (MJ Research, Inc.), programmed with 44 cycles for genomic DNA of endophyte pure cultures or plant leaves, and 54 cycles for DNA from *Brachiaria* seeds, of a 30 sec denaturation step at 94°C (3 min for the first cycle), follwed by 1 min at 65°C, and primer extension for 1 min (10 min in the final cycle) at 72°C. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad), stained with ethidium bromide and photographed under UV lighting.

Seed samples were collected from plants confirmed to be endophyte-infected or endophyte-free using the PCR tests with template DNA isolated from plant tissues, and fungal endophyte isolation on culture media.

Results and Discussion

Acremonium implicatum forms a symbiotic endophytic association with at least some of the economically important *Brachiaria* species. We sought to ascertain whether endophytic *A. implicatum* could be seed-transmitted in *Brachiaria*. Twenty tillers were vegetatively propagated from a single, endophyte-infected mother plant. Ten tillers were treated with the fungicide Folicur® to eliminate the endophyte while the remaining ten tillers were untreated. Seeds were harvested individually from these genetically identical plants, with or without the endophyte. Some of the seeds were germinated and seedlings grown in the glasshouse. A polymerase chain reaction (PCR)-based method developed previously uses a pair of endophyte-specific primers to amplify a single DNA fragment of about 500 bp. DNA both from remnant seeds and from 2-month-old seedlings was amplified with these primers to detect presence of the endophyte. The diagnostic DNA fragment was consistently amplified in DNA of seeds harvested from the endophyte-infected plants and DNA from seedlings grown from seeds harvested from endophyte-infected plants, but not from seeds or seedlings originating from fungicide

treated endophyte-free plants. We conclude that *A. implicatum* can be transmitted through seeds.

The primer pair, P1/P4, allows the precise and rapid detection of *A. implicatum* in *Brachiaria* plants and permits a differentiation between endophytic and non-endophytic fungi (Kelemu et al., 2003. Molecular Plant Pathology 4: 115-118). A single band of about 500-bp in all examined isolates of *A. implicatum* was amplified. Endophyte-containing and endophyte-free plants were also consistently differentiated using this primer combination (data not shown). Seeds were collected from plants whose tissue samples were used as well as other plants. All seed DNA from endophyte-containing plants had a 500-bp amplified product. No amplification product was detected with seed DNA from endophyte-free plants (Figure 17).

Seedlings generated from seed samples of endophyte-containing and endophyte-free plants had consistently tested positive or negative, respectively, for the diagnostic 500-bp amplified product (Figure 18). From these results, we concluded that *A. implicatum* maintains its symbiotic association with species of *Brachiaria* through seed transmission.

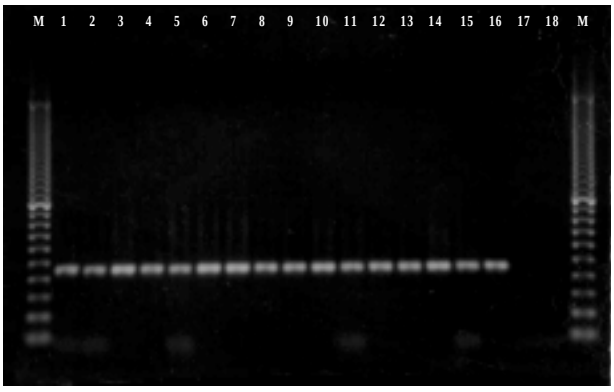


Figure 17. Specific detection of *Acremonium implicatum* in seeds harvested from endophyte-infected *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. **Lanes 1-16**, template DNA extracted from seeds of endophyte infected *Brachiaria* hybrids SX99/3488 (8), SX99/0275 (14), BR99NO/4132 (22), FM9201/1873 (29), BR99NO/4015 (37), BR99NO/4132 (39), *B. decumbens* CIAT 606 (42), BRUZ4X/4402 (44), FM9201/1873 (48), SX99/0731 (52), *B. brizantha* CIAT 16320 (32a), FM9503/S046/024 (45), *B. brizantha* CIAT 26110 (15), *B. brizantha* CIAT 6780 (56), *B. brizantha* CIAT 6780 (68), and *B. brizantha* CIAT 6780 (111), respectively. **Lanes 17,18**, DNA extracted from seeds of endophyte-free plants of *B. brizantha* CIAT 16320 (32-25) and *B. brizantha* CIAT 16320 (32-29); **lanes M**, 100-bp ladders. *B. brizantha* CIAT 26110 (15), *B. brizantha* CIAT 6780 (56), *B. brizantha* CIAT 6780 (68), *B. brizantha* CIAT 6780 (111) were artificially infected. All others were naturally infected.

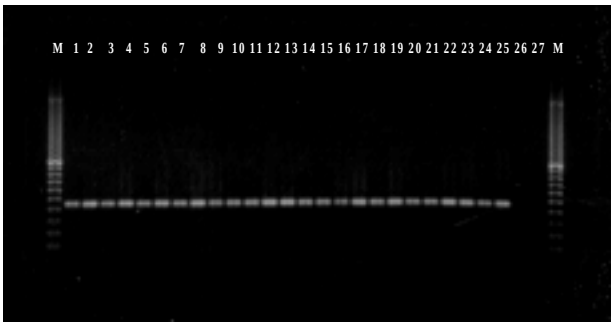


Figure 18. Specific detection of *Acremonium implicatum* in seedlings generated from seeds of endophyte-infected and endophyte-free *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. **Lanes 1-7**, seedlings from seeds harvested from naturally endophyte-infected plants SX99/3488 (8), BRN99NO/4132 (22), BRN99NO/4132 (39), *B. decumbens* accession CIAT 606 (42), BRUZ4X/4402 (44), FM9201/1873 (48), SX99/073 (52), respectively; **lanes 8-17**, seedlings generated from seeds of ten artificially infected *B. brizantha* CIAT 26110 (15) plants; **lanes 18-25**, seedlings generated from seeds of eight naturally infected *B. brizantha* CIAT 16320 (32) plants; **lanes 26 & 27**, seedlings generated from seeds of two endophyte-free *B. brizantha* CIAT 16320 (32-25) plants; **lanes M**, 100-bp ladders.

2.5.2 Distribution of endophytes in different plant parts of Brachiaria

Contributors: H. Dongyi (South China University), T. Sakai (JICA), and S. Kelemu (CIAT)

Rationale

Endophytic fungi often develop a systemic association with their hosts. Several reports demonstrated that endophytic fungi, such as *Epichloë* and *Neotyphodium*, could be distributed in leaf sheaths, leaf blades, stems, roots, seeds and embryos of their grass hosts.

Although endophytes infect their hosts systemically, the concentration of hyphae is not uniform throughout parts of infected plants. Some parts of endophyte-infected plants can even be endophyte-free. Using tissue staining and culturing methods, endophytic fungus *A. implicatum* was observed in leaf sheaths and seeds of *Brachiaria*. These two methods, however, are time consuming and unreliable for endophyte distribution studies in different parts of the plant, especially where fungal mycelia are sparsely distributed. We have developed a rapid and sensitive PCR-based method for specific detection of *A. implicatum* in tissues of *Brachiaria* (Kelemu et al., 2003. Molecular Plant Pathology 4: 115-118). We used this method to determine the distribution of *A. implicatum* in various parts of *Brachiaria* plants.

Materials and Methods

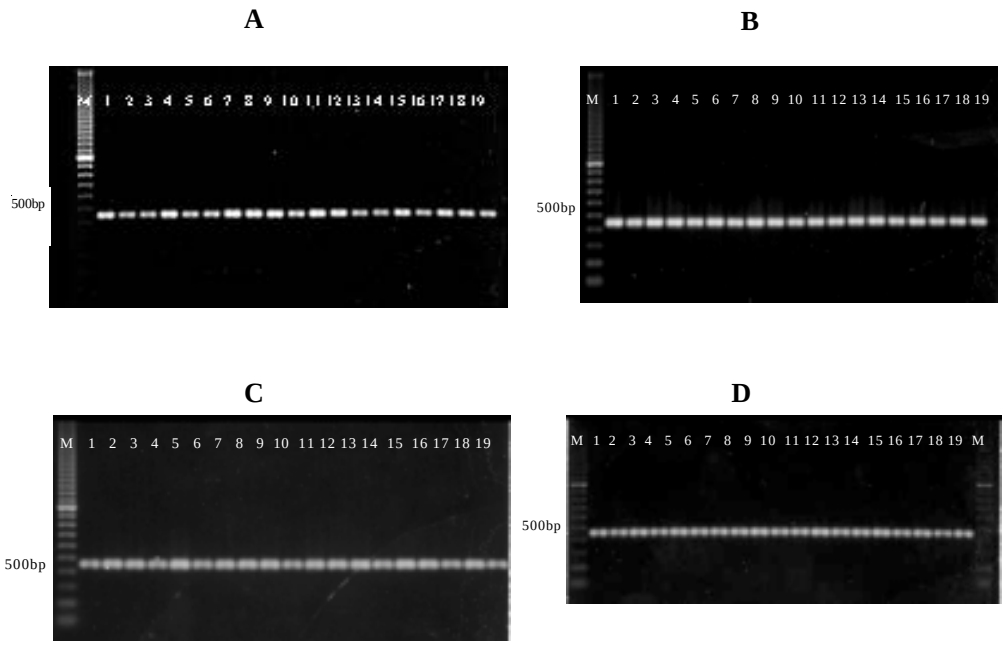
DNA isolation: Leaf sheaths, leaf blades, stems, roots, seeds, embryo and endosperm of seeds were collected from endophyte-infected or endophyte-free plants and macerated separately in liquid nitrogen for genomic DNA isolation. Genomic DNA was extracted using the DNeasy™ Plant Mini Kits (QIAGEN, Valencia, CA).

PCR amplifications: Composition of PCR reactions (20 mL) were 1x PCR buffer, 3mM $MgCl_2$, 0.25mM dNTPs, 0.5 ìM primer P1 and P4, 1U Taq DNA polymerase, and 30ng template DNA. Amplification cycles were programmed in a Programmable Thermal Controller (MJ Research, Inc.) as follows: step 1, 94°C 3min; step 2, 94°C 30 sec; step 3, 65°C 1 min; step 4, 72°C 1 min; step 5, go to step 2 for 44 cycles (for genomic DNA of leaf sheaths and leaf blades) or 54 cycles (for genomic DNA from stems, roots, seeds, embryos and endosperms); then 72°C 10min. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad), stained with ethidium bromide and photographed under UV lighting.

The distribution of *A. implicatum* in plant tissues was determined by the presence or absence of a diagnostic 500-bp amplification product.

Results and Discussions

A diagnostic 500-bp amplification product was observed in all examined leaf sheaths, leaf blades, stems, and roots of *Brachiaria* plants infected with *A. implicatum* (Figures 19 A, B, C, D). The amplification product was also detected in whole seeds, embryo, and endosperm of seeds (data not shown). These results indicate that *A. implicatum* is distributed in the plant parts described above. Johnson et al. (1985, Plant Disease 69:200-202) described the concentration and distribution of Epi*chloë typhina* in tall fescue individual plants with decreasing order in leaf sheaths, seeds, crows, stems, leaf blades, and roots. Because amplifications with template DNA from leaf sheaths, leaf blades and fresh seeds generate the diagnostic 500-bp product with just 45 cycles, as opposed to 55 cycles with DNA from roots and stems, it is likely that mycelial concentrations and distributions in *A. implicatum*/*Brachiaria* associations have a similar trend as those reported in Epi*chloë typhina*/ tall fescue. There was no obvious difference in sensitivity with genomic DNA from leaf blades and sheaths, although isolations on culture media is more routinely and successfully done from leaf sheaths than leaf blades.



Figures 19 A, B, C, D. Specific detection of *Acremonium implicatum* in leaf blades (Figure A), leaf sheaths (Figure B), stems (Figure C), and roots (Figure D) collected from endophyte-infected *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. **Lanes 1~19**: *Brachiaria* hybrid plants SX99/3488 (8), SX99/0275(14), BR99NO/4132 (22), SX99/1513 (23), FM9201/1873 (29), BR99NO/4015 (37), BR99NO/4132 (39); *B. decumbens* accession CIAT 606 (42); *B.* hybrids BRUZ4X/4402 (44), FM9201/1873 (48), SX99/0731(52), FM9503/S046/024 (19); *B. brizantha* accession CIAT 16320 (32a); *Brachiaria* hybrid FM9503/S046/024 (45), SX99/2341(47); *B. brizantha* accession CIAT 26110 (15), *B. brizantha* 6780 (56), *B. brizantha* 6780 (63), *B. brizantha* 6780 (111), respectively. *Brachiaria* hybrid FM9503/S046/024 (19), *B. brizantha* CIAT 16320 (32a), *Brachiaria* hybrid FM9503/S046/024 (45), *Brachiaria* hybrid SX99/2341(47) were naturally infected with isolates we have previously characterized. Plant *B. brizantha* CIAT 26110 (15), *B. brizantha* CIAT 6780 (56), *B. brizantha* CIAT 6780 (63), and *B. brizantha* CIAT 6780 (111) were artificially infected with an isolate (EB 6780(201) of *A. implicatum*. All remaining plants were naturally infected with yet to be isolated and characterized strains.

2.5.3 Effect of fungal endophytes on pathogens *in planta*

Contributors: H. Dongyi (South China University) and S. Kelemu (CIAT)

Rationale

Several *in vitro* studies have demonstrated that *Acremonium* endophytes and *Epichloë typhina* cultures exhibit antifungal activity. White and Cole (1985, Mycologia 77:487-489; 1986, Mycologia 78:102-107) reported that an *Acremonium* spp. from *Festuca, A. coenophialum* (now renamed *Neotyphodium coenophialum*) from tall fescue, and *A. lolii* (renamed *N. lolii*) from perennial ryegrass inhibited mycelial growth of seven different fungi including *Rhizoctonia* spp. in culture. Siegel and Latch (1991, Mycologia 83:529-537) examined the effect of a series of isolates of A*cremonium sp., E. typhina, Phialophora*-like sp. and *Gliocladium*-like sp. on mycelial growth of several grass pathogens in agar culture. Their results indicate that individual isolates of the same species differed in their growth inhibition activities of grass pathogens.

Although many endophyte isolates show antifungal activities *in vitro,* there are only a few reports on resistance to pathogens conferred by endophytes *in planta*. Reduction of tall fescue seedling density due to *Rhizoctonia zeae* was inversely correlated with endophyte (*N. coenophialum)* infestation level of the seed lot (Gwinn and Gavin, 1992, Plant Disease 76:911-914). Plant protection by *E. typhina*

against *Cladosporium phlei*, the causing pathogen of purple leaf spot of timothy grass, was reported (Greulich et al. 1999, Ann. Phytopathol. Soc. Jpn. 65:454-459).

Apart from providing direct resistance to fungal pathogens, endophytes can reduce the spread of viral diseases by deterring insect vectors such as the aphid *Rhopalosiphum padi.*

Drechslera sp. and *Rhizoctonia soloni* are the most important pathogens of species of *Brachiaria*. Our earlier results showed that *A. implicatum* infected plants had fewer and smaller disease lesions caused by *Drechslera* sp. than did genetically identical endophyte-free plants (Kelemu et al., 2001, Canadian Journal of Microbiology 47:55-62). Some genotypes of *Brachiaria* are resistant to *R. solani*. We speculate that *A. implicatum* may contribute to some of this resistance to foliar blight disease caused by *R. solani*.

Materials and Methods

Culture maintenance: All endophytic or pathogenic fungi were cultured and maintained as described by Kelemu et al. (2001, Canadian Journal of Microbiology 47:55-62).

Antifungal extractions from endophyte cultures: Mycelia/conidia were collected from 27 colonies (about 20 mm in size) of *A. implicatum* isolate EH32a grown on potato dextrose agar (PDA). This was macerated in 50 mL sterile distilled water and centrifuged at 12000 rpm for 30 minutes. The supernatant was lyophilised and re-suspended in 9 mL sterile distilled water. This extract was then filter sterilized using 0.22 ìm pore-size nylon membranes. The 9 mL filtrate was then divided into 3 parts of 3 mL each. The first part was heat treated at 100 ºC for 20 minutes. The second portion was treated with pronase (2.0 mg/mL final concentration) and incubated at 37 ºC for 4 hours. The third portion was left in its natural state as control.

Antifungal activity tests: Filter paper discs were soaked with 400-ìl endophyte mycelial/conidial extract prepared as described above. These were placed on PDA-containing petri dishes individually inoculated with *Drechslera* sp. and *R. solani* as shown in Photo 4. These were incubated at 28 ºC in the dark for 3 –5 days.

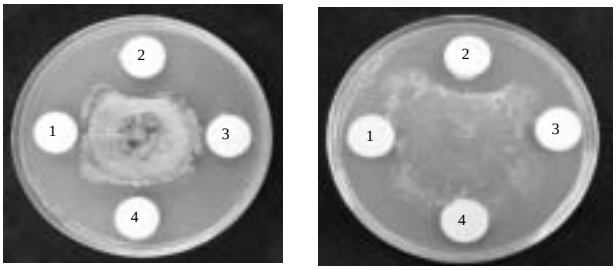
Plant inoculation and disease evaluations: Young tillers from genetically identical endophyte-infected and endophyte-free *B. brizantha* CIAT 6780 or CIAT 16320 were transplanted individually in pots. Plants were inoculated with mycelial agar discs removed from actively growing *R. solani* cultures, by placing the discs in contact with the plant stems just above the soil level and wrapping them with parafilm to secure the contact. Inoculated plants were maintained at high relative humidity in the greenhouse. The upward progression of disease spread and symptoms was measured as distance from the inoculation point.

Results and Discussion

In *in vitro* inhibition tests, most of the 11 *A. implicatum* strains showed antifungal activities although they differ in the inhibition zone area they generated (data not shown). Strains EB 6780(501) and EH 32a showed strong inhibition to both *Drechslera sp.* and *R. solani.*

With *in vivo* tests, endophyte-infected *B. brizantha* CIAT 6780 and CIAT 16320 plants showed more resistance (exhibited as slower upward disease progression) to foliar blight disease than their endophyte-free counterparts at the early stages of infection (7 days after inoculation). Using the Harsfall-Barratt visual rating system (1945. Phytopathology 35:655), disease severity was 4% and 25% on CIAT 16320 and *B. brizantha* CIAT 6780, respectively; as opposed to 25% and 39% on their endophyte-free counterparts, respectively. We concluded that *A. implicatum* contributes to *Rhizoctonia* foliar blight resistance in these two genotypes of *Brachiaria*. It is also important to note that those isolates that exhibited strong inhibitory activities in *vitro* contributed to *in planta* resistance.

Extracts from *A. implicatum* strain EH32 showed strong inhibition to *Drechslera sp* and *R. solani* (Photos 4a and 4b). Extracts treated with heat or pronase lost their antifungal activity. Further extensive studies are needed to determine the nature of the antifungal activity in *A. implicatum*.



Photos 4. Growth inhibition of *Drechslera* spp. (a) and *Rhizoctonia solani* (b) by cell-free culture extracts of *Acremonium implicatum* strain EH32a. Filter paper discs 1-3 were soaked with cell-free extracts of *Acremonium implicatum* strain EH32a. Filter paper discs # 1, #2, #3, and # 4 were soaked with heat-treated extracts, extracts in their natural state, extracts treated with pronase, and sterile distilled water, respectively.

2.5.4 Drought tolerance in endophyte-infected Brachiaria accessions under field conditions

Contributors: S. Kelemu, X. Bonilla, Carolina Zuleta, C. Plazas, J. Ricaurte, R. García and I. M. Rao (CIAT)

Rationale

Previous research conducted in the greenhouse with soil-grown plants showed that endophyte- infected plants under severe drought stress conditions could maintain better leaf expansion and produce significantly greater leaf biomass (IP-5 Annual Report, 1999; 2000). Last year, to validate the findings from the greenhouse study, we initiated a field study in the Llanos of Colombia to quantify the impact of endophytes in improving drought tolerance and persistence in *Brachiaria*. This year we report the preliminary results from that trial based on measurements conducted in the rainy season and dry season.

Materials and Methods

A field trial was established at Matazul farm in May of 2002. The trial included 2 accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110). Plantlets were propagated from the original mother plant containing the endophyte *Acremonium implicatum* (J. Gilman and E. V. Abbott) W. Gams. Half of these plants were treated with the fungicide (Folicur) to eliminate the endophyte (method described in Kelemu et al. 2001. Canadian Journal of Microbiology 47:55-62) while the remaining half was left untreated. The trial was planted as a randomized block in split-plot arrangement with the presence or absence of endophytes as main plots and two accessions as subplots with 3 replications. Each plot included 3 rows with 8 plants per row (24 plants/plot). The plot size was 5 x 1.5 m. The trial was established with low levels of initial fertilizer application (kg/ha: 20 P, 20 K, 33 Ca, 14 Mg, 10 S) that are recommended for establishment of grass alone pastures. A number of plant attributes including forage yield, green leaf production, dry matter distribution and green forage nutrient uptake were measured at the end of wet season (November 2002) and dry season (March 2003).

Results and Discussion

At 6 months after establishment, i.e., at the end of rainy season, the endophyte-infected plants (E+) showed significantly lower values of leaf biomass (Figure 20a) in both accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110). Between these two accessions, CIAT 26110 was more productive. Results on shoot nutrient uptake also showed that uptake of N, P, K, Ca and Mg was greater with uninfected (E-) plants than that of endophyte-infected (E+) plants (Figure 20b).

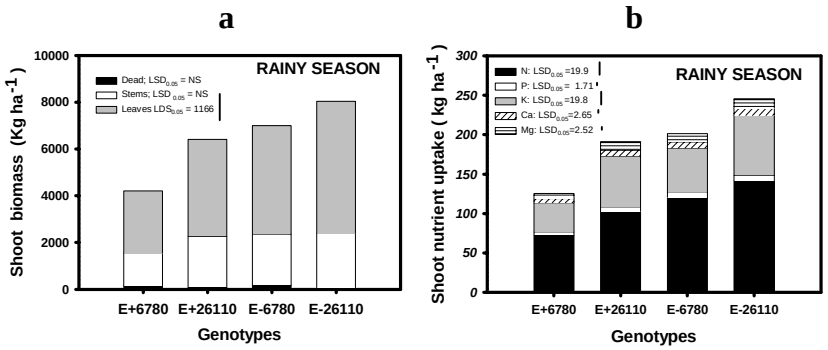


Figure 20. Influence of endophyte infection on (a) shoot biomass production and (b) nutrient uptake of two accessions of *Brachiaria brizantha* CIAT 6780 and CIAT 26110 at 6 months after establishment (at the end of rainy season). E+ are endophyte-infected plants while E- are endophyte-free plants.

In contrast to the results at the end of rainy season, at 10 months after establishment, i.e., at the end of dry season, the endophyte infected plants showed significantly greater values of green leaf biomass (Figure 21a) in both accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110). This observation is consistent with the results from the greenhouse study where the benefits of endophyte infection were noted only under severe drought stress. Results on nutrient uptake at the end of dry season also showed that the uptake of N, P and K was greater with endophyte-infected plants than that of uninfected plants (Figure 21b).

This on-going field study indicated that endophyte infection could improve dry season performance by improving the uptake of nutrients by two accessions of *Brachiaria brizantha*. Further field evaluations for the next two years are needed to cofirm the role of endophytes in improving dry season tolerance of *Brachiaria* grasses.

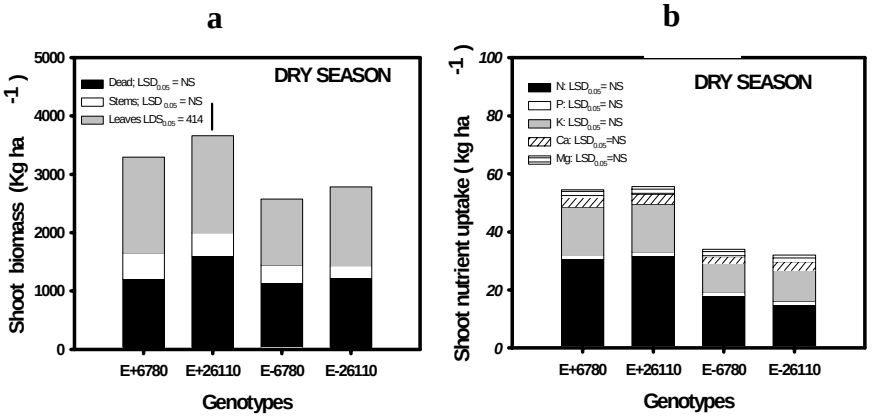


Figure 21. Influence of endophyte infection on (a) shoot biomass production and (b) nutrient uptake of two accessions of *Brachiaria brizantha* CIAT 6780 and CIAT 26110 at 10 months after establishment (at the end of dry season). E+ are endophyte-infected plants while E- are endophyte-free plants.

2.6 Association of bacteria with Brachiaria genotypes

Highlight

- Identification of a nitrogen-fixing bacteria associated with Brachiaria hybrid CIAT 36062
- Isolated an endophytic bacteria in the Brachiaria hybrid CIAT 36062

2.6.1. Search for nitrogen fixing bacteria associated with species of *Brachiaria*

Contributors: C. Zuleta, R. Sedano and S. Kelemu (CIAT)

Rationale

Nitrogen fixation is conducted by phylogenetically diverse groups of prokaryotes. Evidence on nitrogen fixation by rhizospheric bacteria associated with grass roots was first presented in the tropics (Döbereiner and Day, 1976. Associated symbioses in tropical grasses: characterization of microorganisms and nirogen-fixing sites. In: W. E. Newton and C. J. Nyman ed. Proc. of the 1st International Symposium on nitrogen fixation, Washington State Univ. Press, Pullman, pp. 518-538). Tropical forage grasses and grasslands could be ideal for investigating associations with nitrogen fixing bacteria because of their perennial nature and low chemical inputs including fertilizers. The main objectives of this initiative are to: 1) look for endophytic and rhizospheric bacteria responsible for nitrogen fixation in association with species of *Brachiaria*, 2) identify and characterize both plant growth promoting and nitrogen-fixing bacteria that also result in healthier plants.

Because nitrogen fixation is performed by diverse groups of prokaryotic organisms, detection of a marker gene which is unique and is required for nitrogen fixation may be useful to conduct our studies. The *nifH* gene (encodes nitrogenase reductase) has been used with a number of PCR primers that amplify the gene from microbes and other samples by a number of researchers.

Materials and Methods

Bacterial isolates: Isolates of the genera *Rhizobium* of *Bradyrhizobium* were used as positive controls. A bacterium which was consistently isolated from *Brachiaria* CIAT 36062 in 1999, and which we suspected might have a role in fixing nitrogen was included in the test. An isolate of *Xanthomonas campestris* pv. *graminis* (isolate 1015), the causal agent of bacterial wilt of species of *Brachiaria*, was used as a negative control. Bacterial isolates include the following: 1) *Bradyrhizobium* 3101 isolated from forage legume *Centrosema* (Colombia), 2) *Bradyrhizobium* 2469 isolated from forage legume *Desmodium* (Colombia), 3) BR97-155 CBT, unidentified bacterium isolated from *Brachiaria* BR97-155 (Colombia), 4) 16445 CBT, unidentified bacterium isolated from *Brachiaria* CIAT 16445 (Colombia), 5) 16497 CBH, unidentified bacterium isolated from *Brachiaria* CIAT 16497 (Colombia), 6) FM97-383 CACT, a bacterium isolated from *Brachiaria* FM97-383 (Colombia), 7) *Rhizobium* 668 isolated from *Phaseolus vulgaris*, 8) BR97-1371, a bacterium isolated from *Brachiaria* CIAT 36062 (Colombia), 9) *Xanthomonas campestris* pv. *graminis* isolated from *Brachiaria* 1015.

DNA extractions from bacteria: 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 2mg/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.

DNA isolations from soil samples: A protocol described by Porteous et al. (1997. An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. Technical note. Molecular Ecology. 6: 787-791) was used to isolate DNA from soil. The method in general involves lysis of microbial cells, sonication, precipitation, and various steps of cleaning.

Plant DNA extraction: A method described by Dellaporta et al (1983. A plant DNA mini-preparation: version II. Plant Molecular Biology Reporter 1: 19-21)

Nested PCR Amplification: Three primers were used, which were originally designed by Zehr and McReynolds (1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. 55: 2522-2526) and Ueda, et al. (1995. Remarkable N2-fixing bacterial diversity detected in rice roots by molecular evolutionary análysis of *nifH* gene sequences. J. Bacteriol. 177: 1414-1417), to amplify fragments of *nifH* genes. Amplification steps described by Widmer et al (1999. Analysis of *nifH* gene pool complexity in soil and litter at a douglas fir forest site in the Oregon cascade mountain range. Applied and Environmental Microbiology 65:374-380) were adopted.

Results and Discussion

DNA extraction from bacterial cells: DNA extracted from bacterial cells is shown in Figure 22.

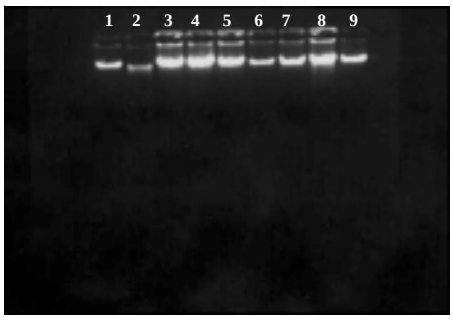


Figure 22. DNA isolated from: 1) *Bradyrhizobium* 3101, 2) *Bradyrhizobium* 2469, 3) unidentified bacterium BR97-155 CBT, 4) unidentified bacterium16445 CBT, 5) unidentified bacterium 16497 CBH, 6) unidentified bacterium FM97-383 CACT, 7) *Rhizobium* 668, unidentified bacterium. BR97-1371, 9) *Xanthomonas campestris* pv. *graminis* 1015.

Nested PCR amplifications:.Amplified products of approximately 370-bp size were amplified with template DNA from nitrogen-fixing bacteria *Rhizobium* and *Bradyrhizobium*, as well as from those randomly picked bacterial colonies isolated from *Brachiaria* CIAT 16445, *Brachiaria* CIAT 16497, and *Brachiaria* FM97-383 (Figure 23). Template DNA from a randomly picked bacterial colony from *Brachiaria* CIAT 36062 amplified a product with approximately 210-bp size (Figure 23). No amplification products were observed with DNA from the pathogen *X. campestris* pv. *graminis* (Figure 23; lane 9) and with that of a bacterium isolated and selected from *Brachiaria* BR97-155 (Figure 23; lane 3).

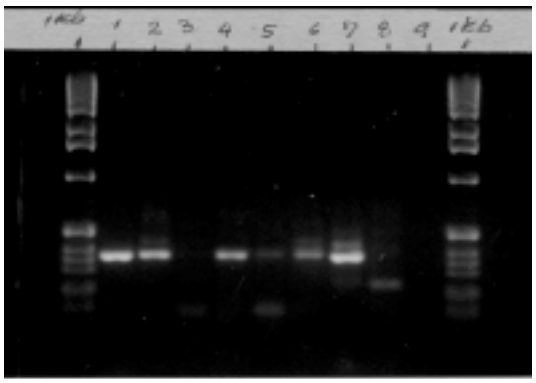


Figure 23. Nested PCR amplification products with three primers of sequences of *nifH* gene. Lanes 1-9, *Bradyrhizobium* 3101, *Bradyrhizobium* 2469, BR97-155 CBT, 16445 CBT, 16497 CBH, FM97-383 CACT, *Rhizobium* 668, BR97-1371, *Xanthomonas campestris* pv. *graminis* 1015 (negative control), respectively. Size markers 1kb ladder.

Brachiaria hybrid CIAT 36062 (BR97-1371) is of particular interest because of its maintenance of green color in the absence of nitrogen input. We have plants of this hybrid in pots in the glasshouse for the last 4 years with no application of nitrogen fertilizer, but are still green. We, therefore, concentrated on this hybrid and isolated independent bacterial colonies from roots, leaves, stems, and soil around the plant roots. Roots were sectioned into three parts: superficial (next to stems), middle and bottom parts. Pieces plant tissues were surface sterilized, macerated in sterile distilled water and plated on nutrient agar for bacterial isolations. Cells from individual bacterial colonies (random colony selection was based on colony color and morphology) were transferred to fresh nutrient agar for further increment. Nested PCR amplification with DNA of these colonies resulted in various size products ranging between 200-1000 bp sizes (Figure 24). Two colonies (Figure 24; lanes 7, 15) gave no amplification products. Some bacterial colonies isolated from the bottom part of the root, the leaf and stem generated strong amplification products with the same size as that produced by nitrogen-fixing bacteria used as positive controls (Figure 24).

In this study, the application of nested PCR amplifications of the *nifH* gene provided us with the first clue that there are bacteria associated with *Brachiaria* hybrid CIAT 36062 involved in nitrogen fixation. These bacteria exist in higher concentration around/in the roots and in the soil around the plant roots than in the leaves and stems. Using these preliminary results as a basis, we intend to conduct more detailed studies to understand the association and to exploit its field application.

DNA isolated from soil samples taken from the surface, middle and bottom part of the potted plants all generated strong amplification products with the same size as those produced by nitrogen-fixing bacteria used as controls (Figure 25).

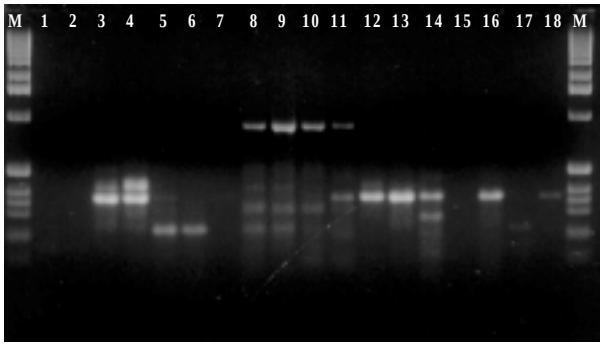


Figure 24. Nested PCR amplification products with three primers of sequences of *nifH* gene. Lane 1) negative control with no template DNA, 2) *Xanthomonas campestris* pv. *graminis* (negative control), 3) *Bradyrhizobium* 3101 (positive control), 4) *Rhizobium* 668 (positive control); lanes 5-7, bacteria isolated from the top part of the root (next to the stem) of *Brachiaria* CIAT 36062 – colonies 1, 2, 3, respectively1; lanes 8-10, bacteria isolated from the middle part of the root – colonies 1, 2,3, respectively; lanes 11-13, bacteria isolated from the bottom tip of the root – colonies 1, 2, 3, respectively; lanes 14-18, bacteria isolated from leaf -1, stem -B1, stem -A2, stem -A3, stem –C, respectively; M=1Kb

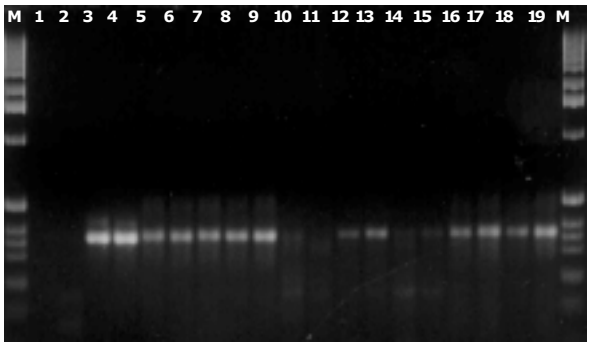


Figure 25. Nested amplifications of the *nifH* gene from soil samples of pots, where *Brachiaria* CIAT 36062 plants have been growing for four years, and bacterial cultures. Lane 1, control with no template DNA; lane 2) *Xanthomonas campestris* pv. *graminis* 1015 (negative control); lane 3) *Bradyrhizobium* 3101 (positive control), 4) *Rhizobium* 668 (positive control); lanes 5-19, surface soil-1, surface soil-2, middle-level soil-1, soil from bottom part of pot-1, soil from bottom-part of pot-2, leaf of CIAT 36062 plant 1, leaf of CIAT 36062 plant 1, leaf of CIAT 36062 plant 2, leaf of CIAT 36062 plant 2, leaf of CIAT 36062 plant 3, leaf of CIAT 36062 plant 3, root of CIAT 36062 plant 1, root of CIAT 36062 plant 3, root of CIAT 36062 plant 3, respectively. Total microbial and plant DNA was extracted from the plant tissues for amplification. M=1Kb ladder.

2.6.2 Bacterial endophytes isolated from *Brachiaria*

Contributors: C. Zuleta, R. Sedano, and S. Kelemu (CIAT)

Rationale

Endophytic bacteria are bacteria that reside in plant tissues without causing any visible harm to the plant. These bacteria can be isolated from surface-sterilized plant tissue or extracted from internal plant tissue. Different bacterial species have been isolated from a single plant. Although the primary point of entry for many of these bacteria is the root zone, aerial plant parts like flowers and stems may also be entries. Once inside a plant, they may be localized at the point of entry or spread throughout. They have been reported to live within cells, in the intercellular spaces or in the vascular system.

Soil bacteria of the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* are of great agricultural importance, because of their ability to fix atmospheric nitrogen in a symbiosis association with legumes. Populations of rhizobia can survive in the soil as saprophytes in the absence of legumes. In recent years, the natural habitat of rhizobia was extended to the roots of gramineous plants. *Rhizobium leguminosarum* bv. *trifolii* was reported to exist inside the roots of rice plants grown in rotation with clover in Egypt (Yanni et al. 1997. Plant Soil 194:99-114), without forming root nodules or nodule-like structures. Various other N_2-fixing endophytic bacteria, known as plant growth-promoting rhizobacteria (PGPR), such as *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in sugarcane, *Azoarcus* spp. in Kallar grass (*Leptochloa fusca*), and *Azospirillum* spp. in maize and rice have been reported. *Herbaspirillum seropedicae* has also been found in association with maize, sorghum and other gramineous plants. Sugarcane plants inoculated with a wild type strain of *A. diazotrophicus* had a higher nitrogen content those inoculated with a *nif* mutant strain or uninoculated controls in nitrogen-deficient conditions.

The objectives of this study were: 1) to isolate nitrogen-fixing endophytic bacteria associated with *Brachiaria*; 2) to identify these bacteria; 3) to characterize them using *nif* gene primers.

Materials and Methods

Bacterial isolation: Leaf, stem and root tissues of *Brachiaria* CIAT 36062 (grown in pots in the green house) were collected and cut into 3-5- cm long sections (roots were first washed in tap water before sectioning them). They were then surface sterilized in 1% NaOCl for 2 min, in 70% ethanol for 1 min, and rinsed 3 times in sterile distilled water. The tissues wee separately macerated in 1-mL sterile distilled water in mortar and pestle. Fifty-µl of the macerated solution was spread uniformly on agar nutrient medium (Difco Lab., Detroit, MI) and incubated at 28 ºC until bacterial colonies appeared.

DNA isolation: 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 2mg/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. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. 55: 2522-2526) and Ueda, et al. (1995. Remarkable N2-fixing bacterial diversity detected in rice roots by molecular evolutionary análysis of *nifH* gene

sequences. J. Bacteriol. 177: 1414-1417), to amplify fragments of *nifH* genes. Amplification steps described by Widmer et al (1999. Analysis of *nifH* gene pool complexity in soil and litter at a douglas fir forest site in the Oregon cascade mountain range. Applied and Environmental Microbiology 65:374-380) were adopted.

Bacterial identification: Three bacterial colonies (codes 36062-H4 [isolated from leaf]; 36062-R2 [isolated from root]; 36062-V2 [isolated from stem]) that tested positive for *nif* were sent to Microbial ID, Newark, DE for identification. The company's identification is based on Similarity Index which expresses how closely the fatty acid composition of the unidentified sample compares with the mean fatty acid composition of the strains used to create the library entry. An exact match of the fatty acid composition results in a similarity index of 1.000.

Results and Discussion

The three bacterial isolates 36062-R2, 36062-H4, and 36062-V2 consistently isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively, tested positive in nested PCR amplifications (Figure 26. The fatty acid analysis matched the bacterium coded 03-36062-V2 with *Flavimonas oryzihabitans* at 0.887 similarity index. *F. oryzihabitans* has been described as a plant growth promoting rhizobacterium in graminicolous plants (Luz, W.C., http://www.ag.auburn.edu/argentina/pdfmanuscripts/luz.pdf, accessed on 05 August, 2003). The analysis matched isolate 02-36062-H4 with *Agrobacterium rubi* at 0.845 similarity index. The name *A. rubi* is synonymous to *Rhizobium rubi* (Young et al. 2001. Int. J. Syst. Evol. Microbiol. 51:89-103). The match using fatty acid data of the isolate 01-36062-R2, however, was not conclusive, matching it with *Leclercia adecarboxylata*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, at 0.879, 0.841, and 0.820 similarity index, respectively. Of these, *E. cloacae* has been described as one of the dominant endophytic bacteria isolated from citrus plants (Araújo et al.2002. Applied and Environmental Microbiology 68:4906-4914).

Future research will include: 1) isolation and characterization of more endophytic bacteria and identifications based not only on fatty acid composition, but also on morphology and DNA based; and 2) the role of these bacteria in plant development.

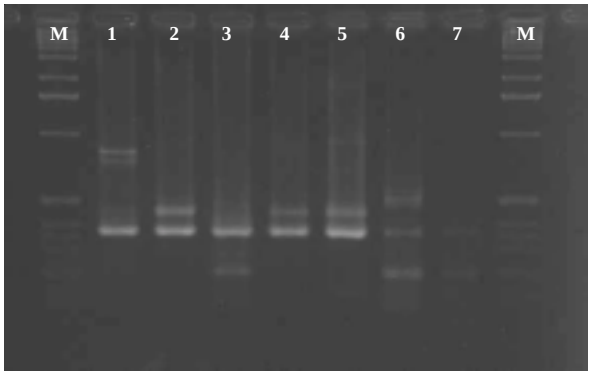


Figure 26 Nested PCR amplifications, using *nif* gene primers, with template DNA of bacterial isolates. Lanes1-3 bacterial isolates 36062-H4, 36062-R2, 36062-V2, isolated from leaf, root and leaf sheath tissues of *Brachiaria* CIAT 36062, respectively. Lane 4, positive control *Bradyrhizobium* isolate 2469; lane 5, positive control *Rhizobium* isolate 49; lane 6, negative control *Xanthomonas campestris* isolate 1015; lane 7, nested PCR cocktail control; lanes M, 1 kb-ladder.

2.7 Search for useful genes in tropical forage

Highlights

- Isolated a protein (Fenotin) from a tropical forage legume (*Clitorea ternatea*) that has antifungal, antibacterial and insecticides properties

2.7.1 Isolation of an antifungal protein in seeds of a tropical legume

Contributors: G. Segura, C. Cardona, and S. Kelemu (CIAT)

Rationale

An array of plant defense mechanisms can be triggered upon wounding or perception of microorganisms, including the synthesis of proteins and peptides that have antifungal activity. Like plants, bacteria, insects, fungi, and mammals synthesize a number of antifungal proteins and peptides (small proteins). Plant seeds use strategies to germinate and survive in the soil that is densely inhabited with a wide range of microfauna and microflora. Various antifungal and/or antibacterial proteins such as chitinases, β-glucanases, thionins, ribosome-inactivating proteins and permatins have been detected in seeds.

In this study, we examined a number of tropical forage legume seeds for antifungal properties. Among those examined, *Clitoria ternatea* (L.) seeds exhibited strong antifungal activity on the test fungus *Rhizoctonia solani* in vitro. Among other traits, *C. ternatea* is: 1) adapted to a wide range of soil conditions; 2) drought resistant; 3) practically free of diseases and pests.

We report here the isolation, purification, and characterization of a peptide from seeds of C. ternatea with antifungal, antibacterial and insecticidal properties.

Materials and Methods

Biological materials: *C. ternatea* CIAT 20692 samples were initially obtained from germplasm collection maintained by CIAT's genetic resources unit. Once antifungus activity was determined, we planted the remaining seeds on field plots at CIAT headquarters in Palmira for large quantities of seed production. The test fungus *R. solani* originally isolated from Centrosema pubescens CIAT 5596 was maintained as air-dried sclerotia produced on potato dextrose agar (PDA).

Seed extraction: Seeds (3 g) of *C. ternatea* CIAT 20692 were surface sterilized in 70% ethanol (4 min), in 2.5% NaOCl solution for 15 min, and rinsed 6 times with sterile distilled water. The seeds were left in sterile distilled water overnight to facilitate maceration. The imbibed seeds were then macerated in 30 mL sterile distilled water with sterile mortar and pestle. The macerated solution was filtered through several layers of cheese cloth. The filtrate was then centrifuged in Eppendorf tubes (1 mL) at 13,000 x g for 30 min. The supernatant was used to determine antifungal activity bioassay.

Antifungal activity bioassay: Three thick filter (#7) paper discs were placed on PDA containing petri dishes. A 300-µl seed extract filtrate was carefully applied onto one of the filter paper discs, where as sterile distilled water (300-µl) was pipetted onto the second filter paper. A single sclerotium of R. solani was then placed in the center of the plate and incubated at 28 ºC. Evaluations were made after two days of incubation.

Determination of the nature of active extract: The extract was either treated with pronase (2 mg/mL final concentration) and incubated at 37 ºC for 2 h; or heat treated at 100 ºC for 5 min.

Protein gel electrophoresis (IEF and SDS-PAGE): Samples were cleaned and concentrated (typically 10-fold) by ultrafiltration with Centricon-3 membrane tubes (3,000-molecular-weight cutoff; Amicon). They were then analyzed by isoelectric focusing (IEF) in ultra-thin-layer polyacrylamide gels (Serva Fein-biochemica GmbH & Co). The samples were loaded in triplicates on the same gel, leaving enough space between them for cutting the gel in three equal parts once the run was complete. One was stained with Coomassie Brilliant Blue R250 to visualize the proteins; the second was neutralized in a buffer and then lightly coated with PDA by pouring a warm PDA before it solidified; the third was neutralized and then over-imposed on the Coomassie-stained triplicate and gel areas corresponding to individual stained protein bands were cut out for further antifungal activity tests.

Samples were also analyzed by SDS-PAGE (separating gel: 12% total acrylamide, 0.3% bis-acrylamide; stacking gel: 4% total acrylamide, 0.2% bis-acrylamide).

Isolation and purification of antifungal protein: Proteins were extracted from10 g seeds macerated in 100 mL sterile distilled water for protein purification. The macerated suspension was filtered through several layers of cheese cloth and centrifuged at 13,000 x g for 30 minutes. The supernatant was deprived of low-molecular-weight solutes by ultrafiltration with Centricon-3 and then concentrated by lyophilization. The lyophilized powder was re-suspended in ($1/10^{th}$ of the original volume) sterile distilled water. The sample was resolved by preparative granulated bed isoelectric focusing (Bio-Rad Laboratories) with pH range of 3.5-9.5, and according to the manufacturer's instructions. The gel was divided into approximately 0.7 cm wide sections, which were scooped out and placed in microcentrifuge tubes. Proteins were eluted by centrifuging the fractions in microcentrifuge tubes.

Insect rearing and feeding tests: Tests were conducted with two species of bruchids that are key pests of stored beans around the world: the Mexican bean weevil, Zabrotes subfasciatus (Boheman), and the bean weevil, *Acanthoscelides obtectus* (Say). Techniques to maintain insect cultures of the bruchids were identical to those described by Cardona et al. (1989. J. Econ. Entomol. 82: 310-315). All experiments were conducted at 27°C and 70% RH in a controlled environment chamber.

To test for possible insecticidal effects of the protein on both bruchid species, "artificial" seeds were prepared with flour of the commercial, highly susceptible bean variety 'ICA Pijao'. Artificial seeds were prepared by following, without modifications, the technique devised by Shade et al. (1986. Environ. Entomol. 15: 1286-1291) for the cowpea weevil, *Callosobruchus maculatus* (F.). Briefly, beans were soaked, the testae were removed and the flour was dried and milled. The flour was then reconstituted in Teflon molds, lyophilized, then hydrated at room temperature. Artificial seeds were coated with gelatin and infested as if they were intact.

The purified protein was mixed with flour of 'ICA Pijao' at different concentrations (0, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, and 5% w/w). Infestation procedures were as follows: for Z. subfasciatus, seeds were infested with at least eight pairs of bruchids per seed. After five days, seeds were examined under a dissecting microscope and 5-6 eggs were left per seed by destroying the excess with a needle. For A. obtectus, seeds for each protein concentration were infested with 5-6 neonate larvae per seed. Larval penetration was subsequently checked to guarantee correct mortality counts. All artificial seeds were individually evaluated in glass vials. Percent adult emergence and days to adult emergence until the last insect emerged were the parameters recorded, although insect survival has been expressed in terms of percent mortality. At the end of the trial, when no more adult emergence occurred, the instar of dead larvae within the seed was determined by measuring the width of the head capsule after dissection of the seeds.

Statistical analysis: Concentration-mortality responses were estimated by means of probit regression analysis (SAS Institute1989). The Statistix package (Analytical Software 2000) was

used for analysis of variance performed with data on days to adult emergence.

Results and Discussion

Antifungal activity: The crude extract from seeds of *C. ternatea* CIAT 20692 showed strong antifungal activity on the test fungus *R. solani* (Photo 5). This activity could be eliminated by treatment with Pronase E (Photo 6), indicating that the active compound is a protein. The activity was heat stable (Photo 7). Seeds release this heat stable proteinaceous antifungal compound after mechanical disruption of their seed coat (Photo 8) or after germination (data not shown).

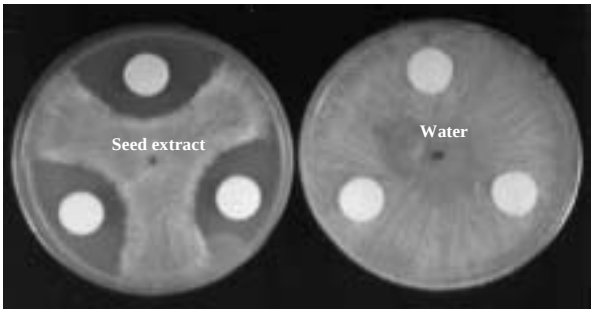


Photo 5.Growth inhibition of *Rhizoctonia solani* by seed extract from *Clitoria ternatea* CIAT 20692 on potato detrose agar plates incubated at 28 ºC for 2 days. Seed extract filtrate (300-µl) was applied on each of the three filter paper discs, whereas the control plate had discs with equal volumes of sterile distilled water. A single sclerotium of *R. solani* was placed on the center each plate.

Photo 6. Elimination of antifungal activity of extracts from seeds of *Clitoria ternatea* CIAT 20692 after treatment with pronase E. A single sclerotium of *R. solani* was placed on the center each plate containing potato dextrose agar and incubated at 28 ºC for 2 days. Seed extract filtrate (300-µl), either treated with pronase E or untreated, was applied on each of the three filter paper discs, whereas the control plate had discs with equal volumes of sterile distilled water.

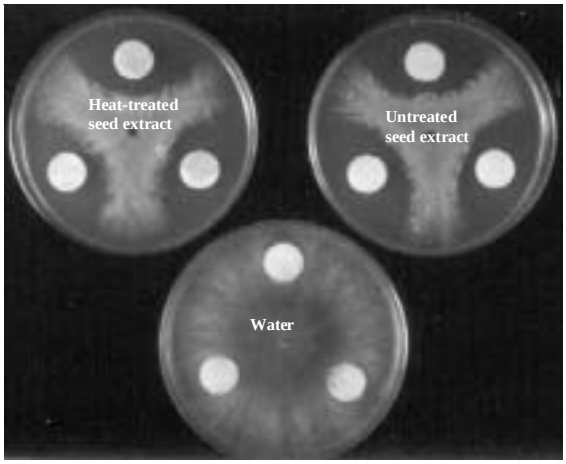


Photo 7. Heat stability of antifungal activity of extracts from *Clitoria ternatea* CIAT 20692 seeds after boiling for 5 min. A single sclerotium of *R. solani* was placed on the center each plate containing potato dextrose agar and incubated at 28 ºC for 2 days. Seed extract filtrate (300-µl), either boiled or untreated, was applied on each of the three filter paper discs, whereas the control plate had discs with equal volumes of sterile distilled water.

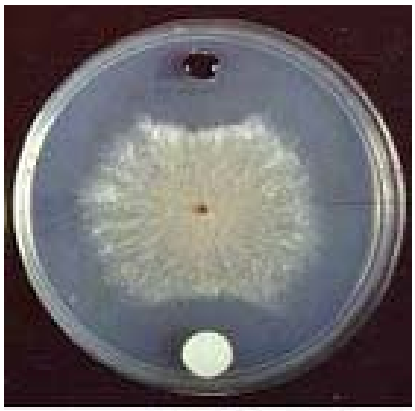
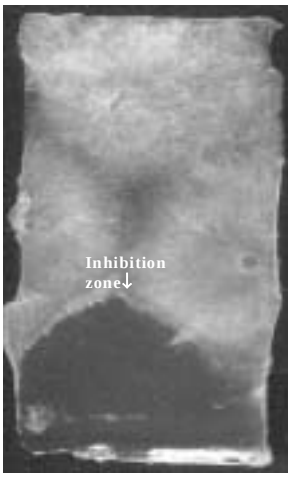
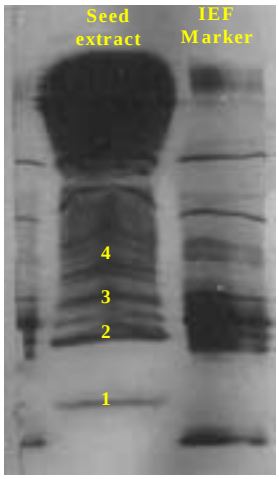


Photo 8. Seeds release an antifungal compound after mechanical disruption of their seed coat. Note that a single seed can release a compound with substantial antifungal activity against the test fungus *Rhizoctonia solani.*

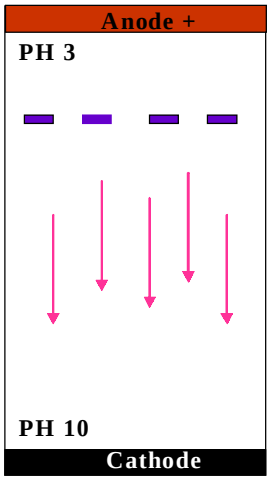
Identification of antifungal protein and purification: Resolving the seed extract by isoelectric focusing gel revealed a number of proteins. Thus, identifying the specific protein (s) responsible for the antifungal activity seemed a daunting task. To reduce the complexity of that task and to facilitate the identification of the specific protein of interest, we created a new protocol which involves: 1) resolving the seed extract by an IEF gel, 2) neutralizing the gel to eliminate the pH gradient, 3) lightly and uniformly coating the gel with warm PDA, 4) inoculating the gel/PDA composition with R. solani sclerotia, 5) wrapping it with Saran wrap to avoid loss of moisture and incubating it at 28 ºC for 2 days. This protocol, in fact, had greatly facilitated our task. R. solani grew uninhibited in the large portion of the gel/PDA composition, but was inhibited in the area where proteins with alkaline pI run (Photo 9).The specific antifungal protein was identified by cutting out ultra-thin-layer polyacrylamide gel areas corresponding to individual protein bands in a duplicate Coomassie-stained gel. The sliced gels were each macerated in 100-µl sterile distilled water in Eppendorf tubes and used for antifungal activity. The results of these tests show that a highly basic protein (numbered 1 in Photo 9) was responsible for the antifungal activity (Photo 10).



IEF gel replica coated with PDA and inoculated with *Rhizoctonia solani*



Coomassie blue-stained IEF gel for selection of protein bands



IEF gel pH gradient

Photo 9. Extracts of *Clitoria ternatea* seeds resolved by isoelectric focusing (IEF) gel. *Rhizoctonia solani* growth inhibition zone indicated that the protein (s) responsible for antifungal activity were in the alkaline part of the gel. A triplicate IEF gel was superimposed on an identical Coomassie-stained IEF gel. Gel areas corresponding to the stained bands 1-4 were cut out for identification of the antifungal protein.

The peptide was well separated from the other proteins on IEF gels, making the purification procedure using the preparative granulated bed isoelectric focusing a relatively easy task. Five of the fractions showed activity with decreasing intensity starting from the highly alkaline pI (isoelectric point) [Photo 11]. The active protein which was mostly recovered in fraction 1 was named Finotin. Both SDS-PAGE (Photo 12) and IEF gels (data not shown) showed that fraction 1 is pure and free of other proteins.

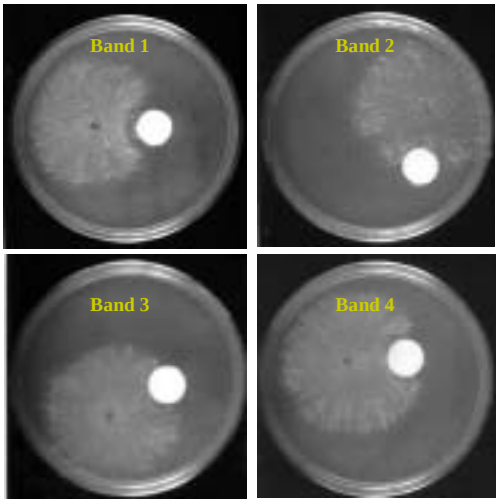


Photo 10. Identification of specific antifungal protein band. The protein band with the most alkaline pI (isoelectric point) numbered 1 in Figure Finotin 4 demonstrated growth inhibition of the test fungus *Rhizoctonia solani*.

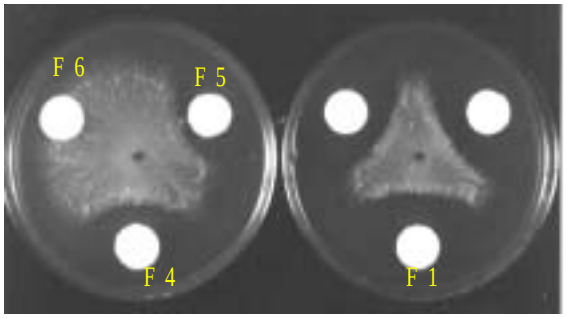


Photo 11. Purification of an antifungal protein from seeds of *Clitoria ternatea* using preparative granulated bed isoelectric focusing. Five fractions scooped from the gel starting from the alkaline part of the gel as # F1 demonstrated antifungal activities, with F5 having the least growth inhibitory activity against *Rhizoctonia solani*.

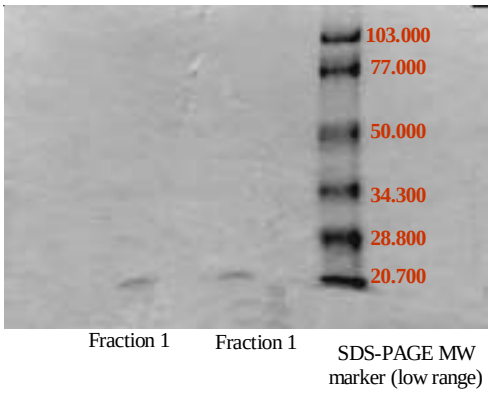


Photo 12. SDS-PAGE analysis of purified protein fraction from *Clitoria ternatea* seeds.

Antifungal/antibacterial activity: Finotin was active against a number of fungi and a bacterium pathogenic on common beans. It inhibits fungi pathogenic on a number of plants, including rice, beans, *Brachiaria*, and *Stylosanthes*, that we tested so far. *Colletotrichum lindemuthianum* and *Xanthomonas axonopodis* pv. *phaseoli* from common beans; *Lasiodiplodia theobromae* and *Colletotrichum gloeosporioides* from *Stylosanthes* spp; *Helminthosporium* spp. and *Pyricularia grisea* from rice; and *Rhizoctonia solani* from Brachiaria were all inhibited in growth by Finotin in culture. We have yet to determine the mode of action of Finotin.

Insecticidal activity: Mortality on artificial seeds prepared with the susceptible background bean flour ('ICA Pijao') was very low (less than 3%) for both *Z. subfasciatus* and *A. obtectus*. Enrichment of artificial seeds with increasing levels of the test protein led to an increase in mortality reaching maximal levels (100% larval mortality) at the dosage of 5% in the case of *Z. subfasciatus* and 1% in the case of *A. obtectus*. Probit analysis (Table 17) showed that the protein is highly toxic to both bruchid species with LC_{50} values that can be considered low (less than 2%). The LC_{50} value for *A. obtectus* (0.36%) was ca. four times lower than that for *Z. subfasciatus* (1.21) meaning that the protein is more toxic to *A. obtectus*. The protein is very toxic to first instar larvae of both bruchid species: dissection of infested seeds revealed that up to 75% of the larvae did not reach the second instar stage.

Table 17. Toxicological responses of bean bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus* to a purified protein (Finotin) isolated from seeds of *Clitoria ternatea*

No. tested	LC_{50} (95% FL)[a]	LC_{95} (95% FL)	Slope ± SEM	χ^2
		Zabrotes subfasciatus		
147	1.21 (0.99 - 1.47)	2.88 (2.17 - 5.21)	4.3 ± 0.88	2.78
		Acanthoscelides obtectus		
155	0.36 (0.28 - 0.43)	0.77 (0.61 - 1.28)	4.9 ± 0.96	0.84

[a] FL, fiducial limits

Concentration responses in terms of days to adult emergence are shown in Table 18. Developmental times of those few insects that survived the different protein concentrations were prolonged. There was a definite dosage response: the higher the dosage the longer the developmental time. This is further proof of the toxicity of the protein to both bruchid species.

The protein Finotin showed growth inhibitory effects against fungi, bacteria and insects. This wide range of activity strongly suggests that this peptide is an important component of the natural defense system of *C. ternatea*. The preferential release of this peptide during seed germination or seed damage can contribute to the protection of the emerging seedlings from soil-borne pathogens. Interestingly, the expression of this peptide is not restricted to seeds, but it also occurs in other parts of *C. ternatea* such as roots upon drought stress in greenhouse tests (data not shown). However, it is not clear whether this peptide plays any role in drought tolerance, a trait which *C. ternatea* has. This and other potential roles played by Finotin are yet to be determined once the gene encoding Finotin is isolated.

Table 18. Effect of increasing concentrations of a purified protein isolated from seeds of *Clitoria ternatea* on the biology (days to adult emergence) of the bean bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus*

Protein concentration (% w/w)	Days to adult emergence	
	Zabrotes subfasciatus	*Acanthoscelides obtectus*
0.0[1]	43.1e	34.4c
0.0625	45.0e	33.8c
0.125	51.5d	35.2c
0.25	55.6c	49.4b
0.5	57.3c	63.4a
1.0	72.7b	N.E.
2.0	80.0a	N.E.
5.0	N.E[2].	N.E.

Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD. ANOVA on data testing for differences among dosages (protein concentrations). For *Z. subfasciatus*: $F = 106.9$; df = 6,21; $P < 0.001$; for *A. obtectus*: $F = 184.9$; df = 4,16; $P < 0.001$.
[1] Background flour prepared with 'ICA Pijao'
[2] N.E., no adult emergence, 100% larval mortality.

2.8 Genetic control and molecular markers for spittlebug and reproductive mode in *Brachiaria*

Highlight:

- Found two putative effector proteins that may be contribute to the antibiotic action of the resistant plants to spittlebug

2.8.1 Reproductive mode of new *Brachiaria* hybrids by progeny trial

Contributors: J.W. Miles, F. Feijoo, and A. Betancourt (CIAT)

SX x AP *Brachiaria* hybrid populations ought to segregate approximately 1:1 for reproductive mode. Only apomictic clones are candidates for development to commercial cultivar status. A reliable means of assessing reproductive mode is by progeny test. The progeny of an apomictic

clone will be uniform and provide a first seed increase of promising genotypes.
Over 2,000 SX x AP seedlings were produced in 2002. These were propagated and established in two un-replicated field nurseries. They were culled down to 64 genotypes on the basis of periodic visual assessment in the field. OP seed was hand-harvested from the 64 "pre-selected" plants in late 2002. This seed was germinated in mid-2003 and 20 random seedlings per progeny were transplanted to the field on August. Where 20 seedlings were not obtained, all available seedlings were transplanted.

2.8.2 Identification of genes induced during the defense response of *Brachiaria* to spittlebug

Contributors: C. Romero, I. F. Acosta, J. Miles, C. Cardon,a and J. Tohme (CIAT- SB2 Project)

Rationale

The molecular basis of plant defense responses to insects is a challenging area whose understanding should make feasible the use of natural immunity in economically important plants. Although molecular biology has recently been incorporated in the exploration of these defense mechanisms, it has been mainly limited to studying the interaction between dicotyledonous plants and herbivorous chewing insects. Herein we focus in the defense responses of a monocot that exhibits a conclusive resistance to a xylem-sucking insect, an interaction that is poorly understood at the molecular level given the peculiarity of this feeding habitat. One of the possible approaches to get closer to such a system without previous molecular data is the characterization of transcriptional changes during the plant response to the insect attack.

Materials and Methods

The subtractive hybridization was performed as described in the 2002 Annual Report, where we also showed the results of two 96-well pilot libraries (Annual Report, 2002). Then, we decided to construct two additional libraries of 384 wells in order to expand the coverage of the subtractive product and to avoid the RNA ribosomal artifacts. Four bands corresponding to rRNA generated in the cDNA synthesis were identified by size when the product of the subtraction was run in an acrylamide gel. The rest of the smear observed in this gel was excised and cloned to create the new libraries.

Similarity searches were performed in the GenBank using the BLASTX algorithm. The matching sequences were searched in annotated databases such as TAIR (The Arabidopsis Research Institute), GRAMENE, SWISS-PROT, and ENZYME in order to determine their putative functions. More specific information was obtained in secondary databases as InterPro (*Integrated Resource of Protein Families*), Pfam (*Protein Families Database*), PRINTS (*Protein Motif Fingerprint Database*), AraCyc (*AraCyc: Arabidopsis thaliana Biochemical Pathways*), and CDD (*Conserved Domain Database*, at NCBI). In these databases we found functional information such as precise biochemical roles, metabolic pathways and redundant proteins (other proteins with the same function). Furthermore, we found structural information such as protein motifs and domains contained in the predicted *Brachiaria* proteins. Finally, in some cases we performed two pair alignments of amino acid sequences using the pair BLAST algorithm to confirm the structural relationship between elements isolated from *Brachiaria* and the proteins previously reported in resistance studies in other species.

A macroarray experiment was carried out in order to test the differential expression of the isolated transcripts. The clones of the four libraries were arrayed using a 384-well pin replicator on duplicated nylon membranes and grown on LB-agar medium overnight. The bacterial colonies were denaturalized and the free DNA was UV-crosslinked. These filters were hybridized with

radioactivity labeled cDNA from infested plants and from non-infested plants and exposed to autoradiograph films.

Results and Discussion

The cloning strategy to avoid rRNA sequences in the new libraries -which have been partially screened at this point- was successful since the proportion of these artifacts decreased from 40% to 5%. Bringing back together the results of last year's pilot libraries, a total of 240 clones yielded readable sequences, which corresponded to 74 unique transcripts. No match was found for 26 (35%) of them in the GenBank; these may constitute a reservoir of new genes, absents in model species. Seven more sequences were not considered further (e-values over 10^{-7}). Fifteen out of the remaining 41 sequences are similar to genes that have been shown to be part of defense responses in other plant-insect or plant-pathogen interactions (see Table 19). These sequences can be divided in six main groups according to their presumed roles in defense (see Figure 27)

Table 19. Putative functions assigned to the transcripts isolated. Sequences related with defense responses in other systems are shown in bold

Putative Function	E value	Score
O-Methyltransferase	3.00E-89	329
Hypothetical Protein 1	1.00E-71	269
Ornithine carbamoyltransferase (OCTase)	**5.00E-67**	**233**
Cysteine Proteinase	**7.00E-63**	**241**
Phospholipase C (A)	**8.00E-58**	**213**
Hypothetical Protein 3	1.00E-52	206
dTDP-glucose 4-6-dehydratase	4.00E-53	206
Chlorophyll a/b-binding protein (CAB)	5.00E-50	196
Phospholipase C (B)	**1.00E-49**	**195**
Omega-3 fatty acid desatura	**4.00E-47**	**187**
CBL-interacting protein kinase (CIPK)	**1.00E-41**	**168**
Tubulin alpha	2.00E-41	168
Caffeoyl Coenzyme A 3-O-Methyltransferase 1	**4.00E-39**	**161**
MADS Box	**1.00E-37**	**148**
Glutathione S-conjugate ABC transporter	3.00E-35	147
Xyloglucan endotransglycolase	**2.00E-34**	**145**
Lypoxigenase A (LOX)	**3.00E-34**	**145**
Lypoxigenase B (LOX)	**3.00E-34**	**144**
Unknown protein 3 (TMS1d)	2.00E-31	132
Fructose bisphosphate aldolaseÊ	1.00E-27	122

Putative Function	E value	Score
Ca dependent mitochondiral carrier protein	7.00E-27	119
NAD+ dependent isocitrate dehydrogenase subunit 1	1.00E-25	116
Sequence associated to Pi2 (RG64 RFLP marker)	3.00E-24	119
Developmental Protein	3.00E-22	104
Elongation factor 1-alpha	6.00E-21	100
Unknown protein 2	2.00E-20	98
Pur-alpha 1Ê	**3.00E-17**	**88**
SCARECROW	**3.00E-17**	**88**
Putative replication proteinÊ	4.00E-16	84
Extracellular lipase 3 (A)	2.00E-15	55
Cold acclimation protein	8.00E-14	77
60s ribosomal protein L13	9.00E-14	76
Hypothetical Protein 6	7.00E-12	71
S-adenosylmethionine synthetaseÊ	**2.00E-11**	**69**
Hypothetical Protein 4	2.00E-10	66
Caffeic acid O-methyltransferase	**1.00E-09**	**62**
Carbonic Anhydrase	3.00E-09	146
Metalloprotease	**1.00E-08**	**57**
Unknown protein 1	1.00E-07	56
Steroid 22-alpha-hydroxylase (cytochrome p450)	**2.00E-07**	**56**
Extracellular lipase 3Ê(B)	2.00E-07	57

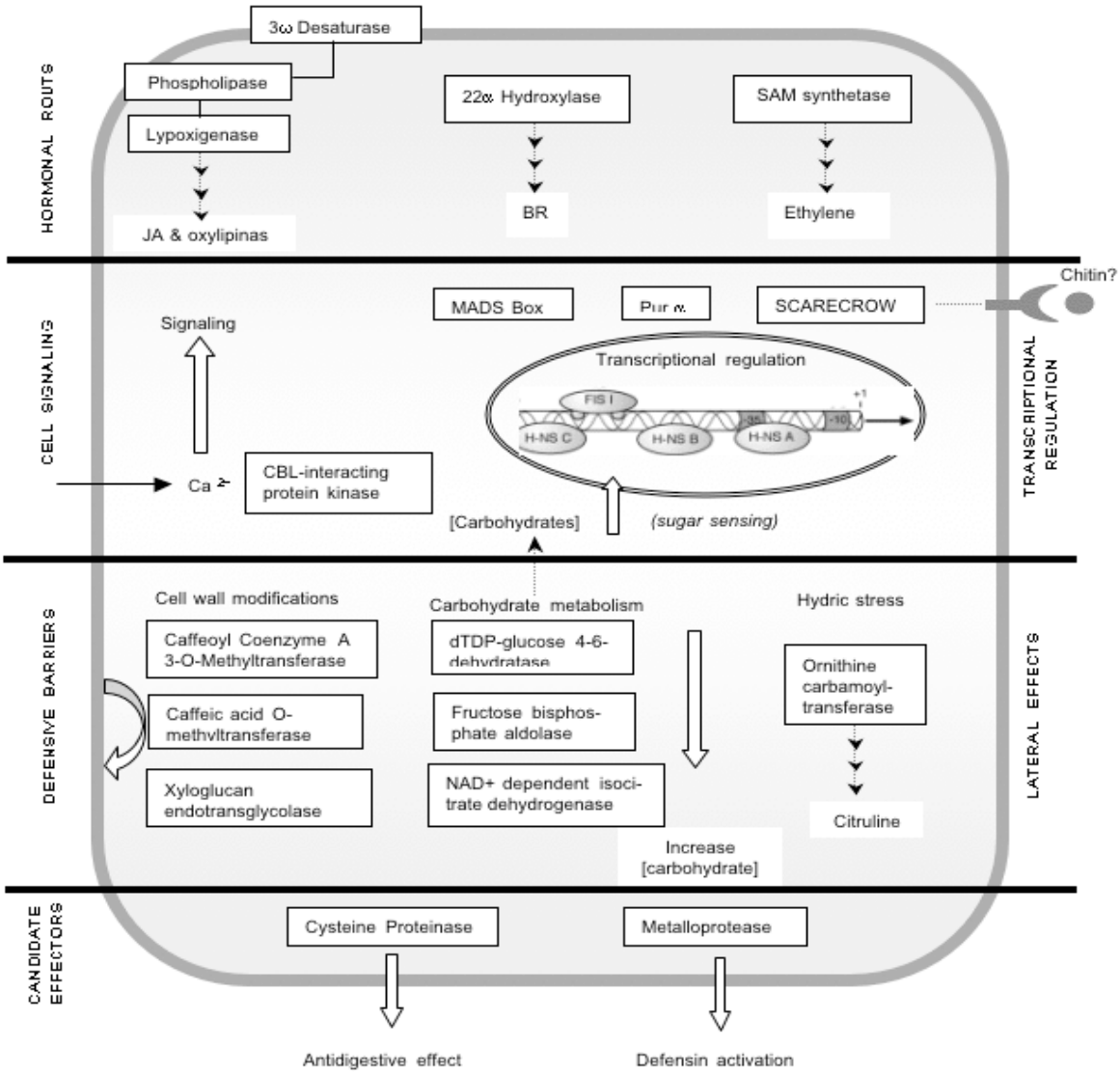


Figure 27. Summary of the candidate defense-related genes and cellular processes found in a *Brachiaria* resistant line in response to the interaction with the spittlebug (*Aeneolamia varia*). They can be divided in 6 main functional groups discussed in the text.

1. **Hormone Biosynthesis Pathways:** The first group contains sequences coding for putative enzymes that catalyze important steps in the biosynthesis of oxylipins, brassinosteroids and ethylene.

   Oxylipins are the most important hormones in the systemic response to wounding and insects. Plants deprived of Phospholipase (Turner *et al.*, 2002), Fatty acid desaturase (Martin *et al.*, 1999) and Lypoxigenase (Bell *et al.*, 1995) are not able to produce Jasmonic Acid (JA) and are incompetent to activate defense mechanisms such as proteinase inhibitors, defensins and thionins.

   Brassinosteroids have been mainly studied in cell division and plant development but recently they were assigned a role as systemic defense hormones in responses to virus, bacteria and fungi in both dicots and monocots (Nakashita *et al.*, 2003).

Although the role of **Ethylene** is widely recognized in plant-pathogen interactions, its effects can differ in different situations. The emission of ethylene has been proved as a mechanism of communication between organisms that attracts natural enemies of herbivores and causes unwounded leaves to initiate ethylene biosynthesis as a positive feedback loop (Arimura *et al.*, 2002).

2. **Cell signaling.** The presence of a putative CIP kinase can be explained by two different processes: a) this protein may interact with CBL, a plant calcium sensor, in the signaling cascade activated under a spittlebug attack. Ca+ signaling in responses to wounding and pathogens has been well documented. b) CIP kinases contain a SNF1 domain, suggesting a possible role in gene regulation controlled by cytoplasmic carbohydrate concentration (sugar sensing), an activity that may be related with the rapid change in metabolism that should occur to supply the energy requirements of the defense responses (Rolland *et al.*, 2002).

3. **Transcriptional Regulation.** SCARECROW is a transcription factor that is rapidly induced upon perception of the elicitor N-acetylchitooligosaccharide. Moreover, its mRNA is induced in rice upon fungal infection but not in the presence of bacterial pathogens (Day *et al.*, 2003). Both evidences suggest that this transcription factor may be involved in responses to enemies that contain chitin, which could be a way to regulate defenses against a broad but specific range of organisms (fungi and arthropods).

4. **Lateral effects (water stress).** The ornithine carbamoyltransferase participates in the synthesis of citrulline, a precursor amino acid of arginine. Some plants accumulate citrulline under water stress in order to increase the concentration of compatible solutes (those that do not alter the electrostatic equilibrium and thus do not disrupt the catalytic properties of enzymes). In this way, the plant enhances its capacity to absorb water decreasing its hydric potential. The production of citrulline in *Brachiaria* in its interaction with the spittlebug may be due to the dramatic alteration of the hydric state caused by this sucking insect on the xylem vessels.

5. **Defensive barriers.** Transcripts for 3 putative enzymes implicated in lignin biosynthesis and cell wall modification were detected in this study. This phenomenon is frequently found in response to pathogens to confine them to the site of infection and avoid their dispersion (Ye *et al.*, 2001), an action whose importance in the defense against an insect seems less obvious. The expression patterns of these elements agree with previous evidences because they have shown to be induced by wounding, ethylene and brassinosteroids.

6. **Effector mechanisms.** Finally, we found two sequences that encode putative proteins that may participate more directly in the reduced survival of the insect in resistant varieties (antibiosis): A metalloprotease with a domain similar to that of proteases involved in the activation of defensins (Liu *et al.*, 2001), this, in turn, may have been previously induced by the oxylipins.
   A cysteine protease highly homologous to one in maize that induces the disruption of the peritrophic matrix in the gut of a caterpillar. Maize callus transformed with the gene encoding this protein reduce growth of the insect as the resistant plant does. The effect of the cysteine proteinase is probably the perforation of the digestive tube (Pechan *et al.*, 2002), demonstrated by electron microscopy, an event that decreases nutrient absorption and facilitates invasion by pathogens.

The macroarray experiment ran to confirm the differential expression of the transcripts isolated showed an encouraging percentage of 5% of false negatives. Consistently the putative functions of these non-differential sequences

were unrelated with defense mechanisms, therefore they have not been taken in to account for the discussion.

In summary, in this work we show the results of isolation of differentially expressed sequences in the resistant *Brachiaria* hybrid CIAT 36062 when challenged with *Aeneolamia varia* nymphs. This was achieved by a subtractive hybridization technique and a rigorous sequence analysis to identify putative functions of the isolated transcripts. Sequencing analysis of ~240 clones from the subtractive library revealed that they corresponded to 74 unique expressed genes. Putative functions were assigned to 41 transcripts through sequence similarity searches and the predicted proteins were classified in eight functional groups. These functional groups fall into three biosynthetic pathways of important plant signaling hormones: cell signaling; transcriptional regulation; cell wall modification and the homeostasis of the plant during the water stress caused by the insect. Finally, we found two putative effector proteins that may be contribute to the antibiotic action of the resistant plants on the insect.

Conclusions

Our results shed the first lights on the molecular mechanisms that determine resistance of a monocotyledonous plant to a xylem-sucking insect.

New and exciting experiments should be designed to complement our findings and to test the assumptions that transcript sequence information has provided. In the short-term these are our goals:

- Exogenous applications of jasmonic acid and brassinolide hormone to susceptible varieties in order to determine phenotypic changes in defense capacities
- Real Time PCR amplification to quantify expression of these transcripts between plants infested by different spittlebug species in order to detect common central defense mechanisms.
- Use of Virus Induced Gene Silencing (VIGS) to evaluate the function in defense responses of the differentially expressed genes.
- A new subtractive hybridization to detect constitutive mechanisms of resistance by comparing gene expression of susceptible and resistant plants both exposed to the insect. A more subtle infestation method will be used and the experiment will span the first 24 hours post-infestation.