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

2.1 Breeding *Brachiaria* for resistant to biotic and abiotic stresses

Highlights

- Implementation of a scheme of selection on testcross progeny performance has begun in the *Brachiaria* grass breeding program. This year was year 1 of the first, 3-year selection cycle.
- A total of 1,419 *Brachiaria* hybrid clones (series BR05) were established in space-planted field trials at CIAT-Quilichao and at the Matazul research field in the Llanos.
- Seed of 16 selections (14 BR02's and 2 MX02's) was delivered to Semillas Papalotla early this year and distributed for agronomic trials in Asia (Thailand and Lao) and Central America and Mexico.
- One hundred thirty-seven preselections (series BR04) were assessed for resistance to three Colombian spittlebug species. Thirteen promising resistant hybrids were identified.
- Promising new *Brachiaria* hybrids are being progeny tested to identify apomicts, i.e., candidates for cultivar release.

2.1.1 Screening and selection of sexual clones (SX05)

Contributors: J.W. Miles; C. Plazas; A. Betancourt; J. Muñoz; and D. Vergara (CIAT)

Rationale

A broad-based, synthetic tetraploid sexual *Brachiaria* population is the core of the breeding program. This population has been cyclically selected over six cycles (12 years) on "per se" performance, mainly on spittlebug resistance and general agronomic merit. Significant progress in spittlebug resistance has been achieved (Miles et al., In press).

Recently, selection pressure on Al tolerance, Rhizoctonia resistance, and nutritional quality is being incorporated in the selection process as screening protocols for these additional traits improve in capacity and speed.

A proposal to implement selection based on combining ability (rather than on "per se" performance) in the sexual synthetic *Brachiaria* grass breeding population was made a decade ago (Miles 1995).

This scheme requires increasing cycle time from two years to three. We expect that the greater accuracy in identifying sexual clones that combine well with our chosen tester (cv. Basilisk) will more than offset the longer cycle time.

Effective implementation has been delayed owing to logistical complications and to the fact that important genetic gains in spittlebug resistance continued to be achieved by the simpler "per se" selection.

However, the moment has been reached, now that very high levels of multiple spittlebug resistance have been achieved, to modify the selection scheme to take into account hybrid performance (i.e., combining ability).

Materials and Methods

A total of 565 sexual clones (508 SX05's produced by recombining 34 highly resistant SX03 clones, plus 57 highly spittlebug-resistant SX03 clones that were identified too late for inclusion in SX03 recombination block) were established as widely-spaced plants (approx. 5x5 m) in a field of *B. decumbens*, CIAT 606 at Popayán in April 2005. Seed harvest (OP seed) began last August, and will be terminated by 30NOV05. OP seed (resulting from crosses to CIAT 606) will be sown in 2006 to generate 200-300 testcross progenies for field evaluation.

Results and Discussion

All 565 clones were screened for reaction to three spittlebug species and 67 clones were culled on susceptibility to one or more species. Further clones will be culled on seed fill (information from testcross crossing block). Full, apparently viable seed harvested on the individual sexual plants ranges from zero up to 1,864.

We anticipate producing on the order of 200-300 testcross families (of 10 individuals each: 2,000 to 3,000 plants total) for field evaluation in 2006. Sexual clones selected on the basis of testcross performance will be recombined in 2007, and a new selection cycle initiated in 2008.

2.1.2 Screening a population of hybrid clones (BR05) resulting from crosses with *Brachiaria decumbens*

Contributors: J.W. Miles; A. Betancourt; J. Muñoz; C. Plazas; and D. Vergara (CIAT)

Rationale

The *Brachiaria* breeding project seeks to create, identify and propagate useful apomictic hybrids. Each cycle of selection in the synthetic sexual population identifies a small number of elite sexual clones. These are recombined and also are crossed (as females) to selected apomictic accessions to generate novel apomictic hybrids.

Materials and Methods

Thirty-four highly spittlebug-resistant SX03 clones were identified in mid-2004. These were propagated and established as widely-spaced (approx. 5x5 m) plants in a field of *B. decumbens* cv. Basilisk in August 2004. Open-pollinated seeds of these sexual plants (crosses with Basilisk) were scarified and germinated in early 2005.

A total of 1,419 hybrid clones (series BR05) were effectively established in space-planted field trials at CIAT-Quilichao and at the Matazul ranch in May.

Results and Discussion

Two hundred fourteen preselections (cultivar candidates) have been identified (as of late October) and successfully propagated to CIAT for evaluation of reactions to spittlebug, Rhizoctonia, and aluminum, and for quality assessment. Seed is being harvested at CIAT-Quilichao on these 214 plants for a progeny test, to be established during second semester, 2006 (to assess reproductive mode). Small quantities of seed of apomictic final selections may be available for distribution in early 2007, or in 2008.

2.1.3 Screening a population of hybrid clones (RZ05) resulting from crosses with *Brachiaria brizantha* CIAT 16320

Contributors: J.W. Miles; S. Kelemu; A. Betancourt; J. Muñoz; C. Plazas; and D. Vergara (CIAT)

Rationale

Our mainstream tetraploid sexual breeding population is generally very susceptible to Rhizoctonia foliar blight. The accession of *B. brizantha* CIAT 16320 shows exceptional resistance to Rhizoctonia foliar blight. In 2001, several selected clones from the sexual population were crossed with CIAT 16320. Several hundred of these hybrids were assessed for Rhizoctonia resistance by the detached-leaf assay. Seven apparently sexual hybrids with an intermediate level of resistance were identified. These were recombined to create a large segregating population in which we expected to identify tetraploid sexual clones with higher levels of resistance so that this resistance (originally derived from CIAT 16320) can be introgressed into the mainstream tetraploid sexual breeding population.

Materials and Methods

Seven apparently sexual (on progeny test), apparently more or less resistant to Rhizoctonia foliar blight (on detached-leaf assay) hybrids with the highly Rhizoctonia-resistant *B. brizantha* accession CIAT 16320 were identified in 2003, from crosses made in 2001. These seven clones were propagated vegetatively and a small isolated recombination block established early in 2004. Open pollinated seed harvested from this recombination block were germinated in early 2005, and 498 seedlings obtained. These were propagated and transplanted to two, space-planted field trials (Quilichao and Matazul).

Results and Discussion

Field observations revealed that one of the seven parental clones was in fact a facultative apomict (produced mostly uniform progeny). Hence, the population is "contaminated" with apomixis and not completely sexual as anticipated: This is a temporary, but not fatal, setback.

One hundred seventeen preselections were made, in the progenies of the six sexual parental clones. These preselections will need careful progeny testing in 2006 to discard any facultative or fully apomictic individuals. Fully sexual clones will be assessed for reaction to Rhizoctonia and spittlebugs. Depending on the levels of resistance to Rhizoctonia identified in fully sexual plants, these will be either introgressed directly into the main sexual brachiariagrass population (if high levels of resistance are found), or subjected to further selection and recombination to develop levels of Rhizoctonia resistance in sexual tetraploid germplasm adequate to be introgressed into the main sexual population.

2.1.4 Spittlebug reaction of selected *Brachiaria* hybrids (series BR04)

Contributors: J.W. Miles; C. Cardona; G. Sotelo; and J. Muñoz (CIAT)

Rationale

The current Brachiaria breeding scheme generates a cohort of sexual-by-apomictic hybrids every selection cycle. In 2003, two spittlebug-resistant sexual clones were identified, one of which was exceptionally resistant (antibiotic) to three Colombian species. The resistance of hybrids of these resistant sexual clones crossed with susceptible, but apomictic, pollen parents needs to be determined.

Materials and Methods

Two spittlebug-resistant sexual clones were identified in 2002 from the cohort of sexual plants produced in 2001. One of the two clones was exceptionally resistant (strong antibiosis) to three Colombian species. These two sexual clones were each crossed to four apomictic accessions of *B. brizantha*. Hybrid progenies were assessed in field trials during 2004, and preselections (137) tested for spittlebug reaction in replicated trials in the CIAT forage entomology glasshouse, using standard procedures.

Results and Discussion

Thirteen hybrids were considered "resistant" to the three Colombian spittlebug species used in glasshouse tests. For only five of the thirteen were enough progeny obtained for a progeny trial; observations to date (04NOV05) suggest that at least two of these five are apomicts. Additional open-pollinated seed of the remaining eight hybrids is being generated currently for progeny a trial in 2006.

2.1.5 Reproductive mode (by progeny test) of selected *Brachiaria* hybrids (series BR04)

Contributors: J.W. Miles; A. Betancourt; and J. Muñoz (CIAT)

Rationale

Individuals in sexual-by-apomictic hybrid populations segregate approximately 1:1 for reproductive mode (sexual:apomictic). When plants with promising attributes [vigor, leafiness, seed set, spittlebug resistance (where required), etc.] are identified, their reproductive behavior must be determined, as only apomicts are viable candidates for commercial release.

A simple progeny test is the most straightforward means to determine actual reproductive behavior while simultaneously achieving an initial seed multiplication of the apomicts that are identified, by harvesting seed from the (uniform) progeny rows.

Materials and Methods

We attempted to harvest open pollinated seed at Quilichao from individual spaced plants of over 300 BR04 hybrid clones preselected during the 2004 season. On the basis of seed yield and seed fill (caryopsis formation), the cohort of preselections was culled to 137.

Results and Discussion

Sufficient progeny seedlings to be included in the progeny test (at least 10) were obtained for only 42 of the 137 preselected hybrids. Seed of eight additional promising BR04 hybrids (spittlebug resistance) is being multiplied for progeny testing next year.

Preliminary results suggest that 23 of the 42 preselections are probably apomicts, the remainder appear to be either sexuals or facultative apomicts.

2.1.6 Fingerprinting selected *Brachiaria* hybrids with appropriate molecular markers (BR02 and MX02)

Contributors: J.W. Miles and J. Tohme (CIAT)

Rationale

With each selection cycle a new cohort of sexual-by-apomictic hybrids is formed. As these are assessed for a series of attributes, most are culled and a small group of promising apomictic pre-selections identified for distribution for wider evaluation. As a means of positive identification of these advanced hybrids (and potential cultivars) they need to be "fingerprinted" by reliable molecular markers prior to (or at least simultaneous with) wider distribution outside of CIAT, so as to avoid its unauthorized "escape" prior to formal release.

Materials and Methods

Greenhouse-grown plants of 42 selected genotypes were sampled in early December 2005. We anticipate having these plants "fingerprinted" for 25 microsatellite markers by the end of January 2006.

2.1.7 Distribution of seed of promising apomictic (BR02 and MX02) hybrids to SE Asia, Mexico, Costa Rica, and Brazil

Contributors: J.W. Miles (CIAT); E. Stern (Semillas Papalotla); M. Hare (Univ. Ubon Ratchathani, Thailand); and P. Horne (CIAT, Lao)

Rationale

Selected apomictic hybrids require regional evaluation before being considered for commercial release. This function is being coordinated and fully financed by Semillas Papalotla through a network of employees and collaborators throughout the tropical world.

Materials and Methods

Small quantities of seed of nine apomictic hybrids was produced on progeny test plots at CIAT during 2003, and delivered to CIAT-Lao in early 2004. These hybrids and seven more were multiplied in larger plots, at CIAT-Popayán during 2004, so that larger quantities of seed were available for distribution this year. Seed was sent to Semillas Papalotla, and from there distributed to collaborators in Asia (Thailand and Lao), Central America, Mexico, and Brazil. Agronomic trials are in progress.

Results and Discussion

Dr. Michael Hare has already tentatively identified a couple of "promising" hybrids, based on general vigor, upright growth habit (preferred for cut-and-carry systems in Asia), and regrowth following cutting. We have noticed two hybrids with growth habit very similar to cv. Basilisk. At least one of these prostrate lines has excellent aluminum tolerance and is more resistant to spittlebugs than cv. Basilisk.

2.2 Screening and selection of *Brachiaria* genotypes for spittlebug resistance

Highlights

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

2.2.1 Continuous mass rearing of spittlebug species

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

A permanent supply of insects is essential in the process of evaluating genotypes for resistance to spittlebug. At present, the progress made in mass rearing of nymphs and in obtaining eggs from adults collected in the field allows us to conduct simultaneous screening of large number of *Brachiaria* genotypes for resistance to all major spittlebug species present in Colombia. Insects produced in our mass rearing facilities are used for greenhouse evaluations in Palmira and field evaluations in Caquetá. Our mass rearing and mass screening techniques have proved to be successful in Brazil and Mexico.

2.2.2 Greenhouse screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species

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

Rationale

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

Materials and Methods

Screenings for resistance in the greenhouse were conducted with *Aeneolamia varia, A. reducta, Zulia carbonaria, Z. pubescens, Mahanarva trifissa* and *Prosapia simulans*. Test materials were usually compared with five checks fully characterized for resistance or susceptibility to *A. varia*. Plants were infested with six eggs per plant of the respective spittlebug species and the infestation was allowed to proceed without interference until all nymphs were mature (fifth instar stage) or adult emergence occurred. Plants (usually 5-10 per genotype) were scored for symptoms using a damage score scale (1, no visible damage; 5, plant dead) developed in previous years. Percentage nymph survival was

calculated. Materials were selected on the basis of low damage scores (<2.0 in a 1-5 scale) and reduced percentage nymph survival (<30%). All those rated as resistant or intermediate were reconfirmed. All susceptible hybrids were discarded.

Results and Discussion

In 2005, 119 pre-selected sexual (SX03) hybrids were simultaneously screened for resistance to *A. varia*, *A. reducta*, and *Z. carbonaria*. We used five replications per hybrid per insect species. For comparison, we used five well-known checks replicated 10 times per insect species. All but one of the hybrids were resistant to all three test species. To the extent that mean percentage nymph survival in the population did not differ from percentage survival in our most resistant check, the hybrid SX01NO/0102 (Table 11). These results clearly indicate that a very significant progress has been made in incorporating antibiosis resistance to all of the three test species in a relatively short period of time.

Table 11. Levels of resistance to three spittlebug species in 119 sexual *Brachiaria* hybrids and checks

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

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

Further proof of the rapid progress made in incorporating resistance to spittlebug was obtained when 565 new hybrids (SX05 series) were tested for resistance to three spittlebug species. As shown in Figure 4, 96.2%, 94,7% and 93.9% were rated as resistant to *A. varia*, *A. reducta*, and *Z. carbonaria*, respectively. 468 hybrids (82.8%) were classified as highly resistant to all three species tested. Progress was also detected when resistance reactions in two consecutive cycles were compared (Figure 5) It is valid to conclude that there has been a steady increase in the frequency of resistant genotypes as a result of recurrent selection through cycles.

In support of continuous breeding activities we screened a set of 141 apomictic BR04 hybrids. Most were susceptible but a handful of them showed acceptable levels of antibiosis resistance to all three tests species (Table 12). As in previous occasions, correlations between damage

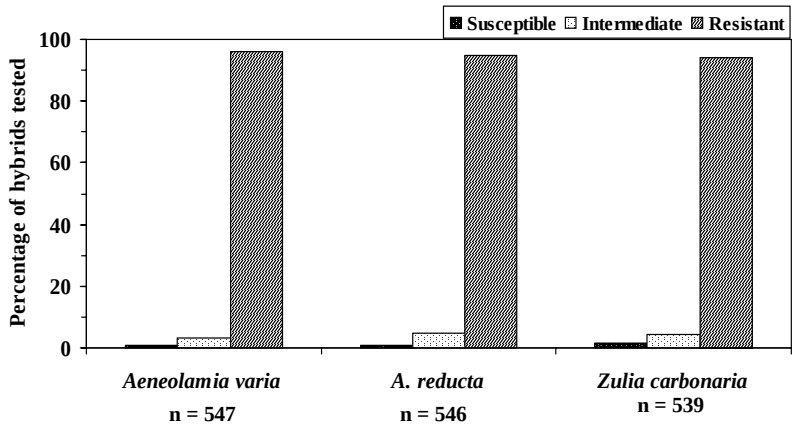

Figure 4. Frequency distribution of resistance reactions in a population of 565 sexual *Brachiaria* hybrids (SX05 series) tested for resistance to three major spittlebug species.

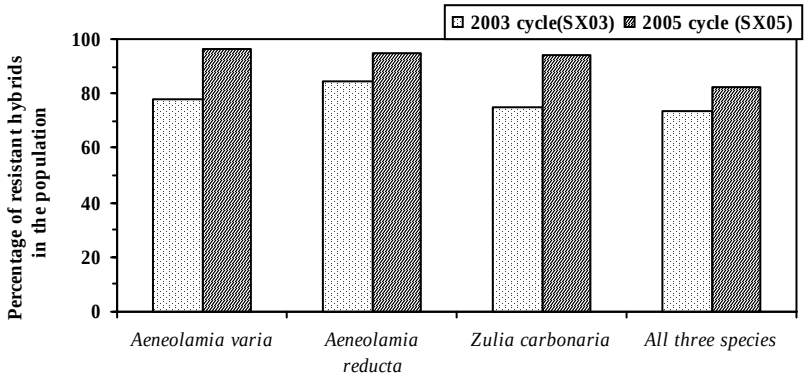

Figure 5. Frequency distribution of resistant reactions in two consecutive cycles of selection in *Brachiaria* for resistance to three major spittlebug species.

Table 12. Percentage nymph survival in selected *Brachiaria* genotypes screened for resistance to three major spittlebug species.

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

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

scores and percentage nymph survival were highly significant: 0.802** for *A. varia*, 0.924** for *A. reducta* and 0.840** for *Z. carbonaria*. In 2004 we reported on varying levels of resistance to *Prosapia simulans* (one of the most important species affecting *Brachiaria* in Mexico) in 34 apomictic hybrids (coded MX).

These hybrids had been pre-selected in Mexico for good adaptation and desirable agronomic characteristics. In 2005 we conducted a series of replicated tests to evaluate the resistance of these genotypes to four major species present in Colombia. Those showing multiple resistances are listed in Table 13.

Table 13. Percentage nymph survival in selected *Brachiaria* apomictic hybrids tested for resistance to five spittlebug species. Means ± SEM of five replications per genotype.

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

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

2.2.3 Field screening of *Brachiaria* accessions and hybrids for resistance to four spittlebug species

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

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, 20 major screening trials (seven with *A. varia*, six with *Zulia carbonaria,* five with *Z. pubescens*, and two with *Mahanarva trifissa*) were conducted in Caquetá in 2005. The main purpose of these trials was to reconfirm resistance in 36 apomictic hybrids (BR02) and 34 apomictic hybrids (MX) that had been previously evaluated in Palmira under greenhouse conditions.

Results and Discussion

Using tiller ratios (the ratio between tillers per plant at the beginning of the infestation process and tillers per plant at the end of the infestation

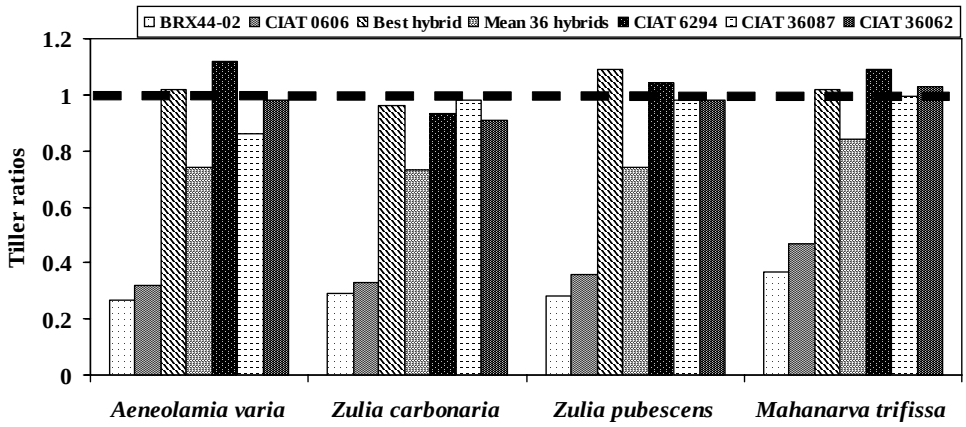

Figure 6. Resistance to four spittlebug species in selected *Brachiaria* apomictic (BR02) hybrids and checks tested under field conditions. The dotted line represents the cut-off point for resistance rating and selection.

process) as the main selection criterion, we found that most of the BR02 hybrids tested were susceptible to spittlebug (Figure 6). A handful, listed in Table 14, showed a more or less acceptable level of field resistance due to the relatively high levels of antibiosis resistance present in these hybrids. The mechanism protected the plants from intense insect damage, allowing the plants to grow and lose less tillers than the susceptible checks. One of the commercial checks (CIAT 36087, 'Mulato 2') was resistant. Better results were obtained when 33 apomictic hybrids coded MX were tested for resistance to *A. varia*, *Z. carbonaria* and *M. trifissa*. Most of the genotypes were

Table 14. Tiller ratios (tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process) in selected *Brachiaria* genotypes tested for resistance to four spittlebug species under field conditions in Caquetá, Colombia.

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

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

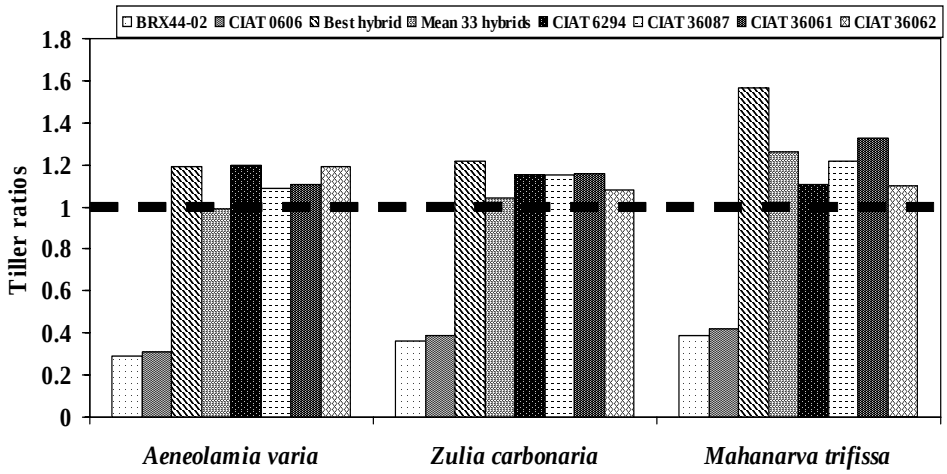

Figure 7. Resistance to four spittlebug species in selected *Brachiaria* apomictic (MX02) hybrids and checks tested under field conditions. The dotted line represents the cut-off point for resistance rating and selection.

classified as resistant both in terms of damage scores (data not shown) and tiller ratios (Figure 7). The mean of selected genotypes did not differ from the mean of the resistant checks. The two commercial checks, 'Mulato' and 'Mulato II' showed a high level of field resistance (Table 15).

Table 15. Tiller ratios (tillers per plant at the end of the infestation process/tiller per plant at the beginning of the infestation process) in selected *Brachiaria* genotypes tested for resistance to three spittlebug species under field conditions in Caquetá, Colombia.

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

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

2.3 Host mechanisms for spittlebug resistance in *Brachiaria*

Highlights

- Antibiosis resistance in a Brachiaria hybrid had a significant effect on the demography of *Zulia carbonaria*.
- No correlation was found between amino acid content in xylem of Brachiaria hybrids and resistance ratings to spittlebug measured as nymph survival.
- No signs of antibiosis to adults of spittlebug (*A. varia*) has been detected.

2.3.1 Effect of host plant resistance on the demography of *Zulia carbonaria*

Contributors: M. F. Miller, C. Cardona, and G. Sotelo (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 was known about possible direct effects of antibiotic genotypes on the biology of adults. Even less was known about sub-lethal effects (i. e., reduced oviposition rates, reduced longevity, prolonged generation times, reduced rates of growth, etc.) on adults resulting from nymphs feeding on antibiotic genotypes. In 2004 we initiated a series of studies aimed at measuring how antibiotic genotypes may directly or indirectly (through sub-lethal effects) affect the biology of adults of *A. varia*.

In 2005, similar studies were conducted with another major species, *Z. carbonaria*. We used the life-table technique, which is widely recognized as one of the most effective means of teasing apart the subtle, interrelated aspects of changes in population density. Longevity, age-specific fecundity, sex ratio and generation time can be examined and compared among treatments as they relate to the most important demographic parameter, the intrinsic rate of natural increase.

Materials and Methods

A comprehensive series of experiments aimed at determining whether antibiosis to nymphs has an adverse effect on the demography of *Z. carbonaria* were conducted. For this, 8 life tables (four fecundity, four complete) were constructed. Treatment combinations are shown in Table 16.

For each of these treatments we established cohorts of 105 pairs of spittlebug and the fate and reproductive rate of individuals were recorded until death occurred. From these data the following life-table statistics were derived: net reproductive rate (R_o) [net contribution per female to the next generation]; mean generation time (T) [mean time span between the birth of individuals of a generation and that of the next generation]; doubling time (D) [time span necessary to double the initial population]; finite rate of population increase (ë) [multiplication factor of the original population at each time period]; and intrinsic rate of natural increase (r_m) [innate capacity of the population to increase in numbers]. Life-table statistics were analyzed using the SAS program based on jackknife estimates of demographic parameters. Other variables recorded were sex ratios, percentage egg fertility and adult dry weights. These data were submitted to analysis of variance and when the *F* test was significant, we performed mean separation by LSD.

Table 16. Treatment combinations to study possible sub-lethal effects of high levels of nymphal antibiosis on adults of *Zulia carbonaria.*

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

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

Results and Discussion

A. Sub-lethal effects of resistance on adults of *Zulia carbonaria*: The impact of antibiosis to nymphs on the reproductive biology of resulting adults

The resistant genotype SX01NO/0102 caused significant effects on the demography of *Z. carbonaria*. In general, rearing of nymphs of *Z. carbonaria* on the resistant genotype had a deleterious effect on the weight of resulting males and on the number and fertility of eggs laid per female (Table 17).

Age-specific survival and age-specific fecundity curves for *Z. carbonaria* adults are presented in Figure 8. Mean survival times for the four treatment combinations did not differ at the 5% level, meaning that there was not a major impact of nymphal antibiosis on the survival of resulting males or females. On the contrary, rearing of the insect on the resistant genotype SX01NO/0102 did have a pronounced effect on the ability of resulting females to lay eggs. Independently of the food substrate used to feed the adults, females obtained from rearing the nymphs on the resistant genotype laid fewer eggs, for a slightly shorter period of time, than those obtained from rearing the insect on the susceptible genotype. This can be interpreted as a sub-lethal effect of nymphal antibiosis on the reproductive capacity of the insect.

All demographic parameters of *Z. carbonaria* adults were significantly affected by the antibiotic effect of SX01NO/0102 on the nymphs (Table 18). Females originating from nymphs reared on the resistant genotype had lower net reproductive rates, lower intrinsic rates of natural increase, and lower finite rates of increase than those obtained from rearing the insect on the susceptible genotype. We conclude that antibiosis to nymphs in the resistant *Brachiaria* hybrid SX01NO/0102 causes significant sub-lethal effects on the reproductive biology of resulting adults.

Table 17. Life history parameters of *Zulia carbonaria* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes.

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

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

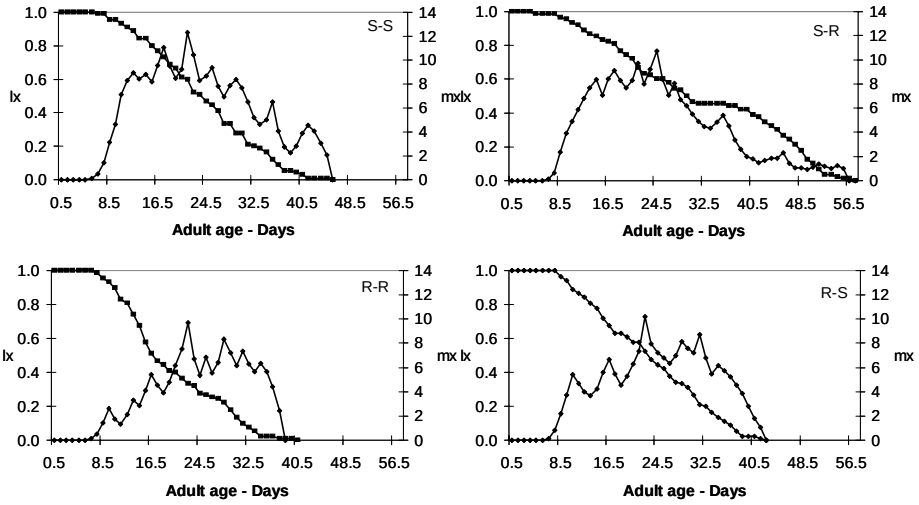

Figure 8. Age-specific survival (l_x) (%) and age-specific fecundity (m_x) (f&) curves for adults of *Zulia carbonaria* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (CIAT 0654); R, resistant genotype (SX01NO/0102).

Table 18. Fecundity life-table statistics for *Zulia carbonaria* adults as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes.

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

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

B. Total effects of resistance on the demography of *Zulia carbonaria*

To measure the total impact of antibiosis resistance on the demography of *Z. carbonaria*, we took into account the rates of immature mortality caused by both the resistant and the susceptible genotypes. Age-specific survival curves for nymphs and adults, as well as age-specific fecundity curves for *Z. carbonaria* adults are presented in Figure 9. The antibiosis to nymphs present in the resistant genotype SX01NO/0102 had a significant deleterious effect on the biology of the insect, which reflected in very high levels of immature mortality. As a result, survival curves were very low as compared to those obtained with the susceptible genotype. Rearing of the insect on the resistant genotype caused a delay in the emergence of adults. Antibiosis also had a significant effect on the ability of resulting females to lay eggs. Independently of the food substrate used to feed the adults, females obtained from rearing the nymphs on the resistant genotype laid less eggs than those obtained from rearing the insect on the susceptible genotype.

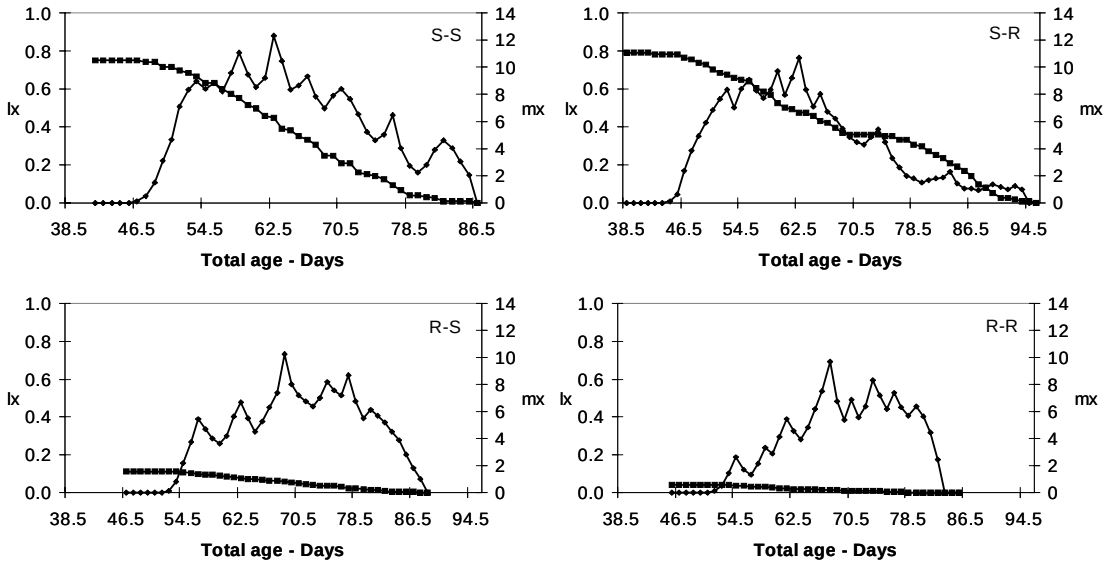

Figure 9. Age-specific survival (l_x) (%) and age-specific fecundity (m_x) (f&) curves for *Zulia carbonaria* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (CIAT 0654); R, resistant genotype (SX01NO/0102).

As a result of high immature mortality and sub-lethal effects on resulting adults, all demographic statistics of the *Z. carbonaria* population tested were significantly affected by the antibiosis present in SX01NO/0102 (Table 19). Populations derived from the resistant genotype had lower net reproductive rates, lower intrinsic rates of natural increase, lower finite rates of increase and longer generation times than those obtained from rearing the insect on the susceptible genotype. The finite rate of increase is a parameter that describes deleterious effects on a given population. It is defined as a multiplication factor of the original population at each time period. The decimal part of the finite rate of increase corresponds to the daily rate of increase expressed as a percentage. This means that populations reared on the susceptible genotype would grow by 8% whereas those on the resistant genotype would grow by 2.3-4.2% (Table 19). We conclude that high immature mortality caused by the resistant *Brachiaria* hybrid SX01NO/0102 and sub-lethal effects of antibiosis on resulting adults have a very major impact on the demography of *Z. carbonaria*.

Table 19. Life-table statistics for *Zulia carbonaria* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes.

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

[a] S, susceptible; R, resistant

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

2.3.2 Studies on possible biochemical factors associated with antibiosis resistance to spittlebug

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

Rationale

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

Materials and Methods

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

Results and Discussion

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

2.3.3 Studies on tolerance to adult feeding damage as a component of resistance to spittlebug

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

Rationale

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

Materials and Methods

To study tolerance to adult feeding we initially compared the response of the susceptible accession CIAT 0654 and the resistant hybrid SX01NO/0102 to increasing levels of infestation with adults of *A. varia*. Thirty-day old plants of CIAT 0654 and SX01NO/0102 were exposed to 0, 2, 3, 5, 7, 9, 12, and 15 adults per plant. The 16 host genotype-infestation level treatment combinations were randomly assigned to single-plant experimental units with 10 replications per treatment combination. Plants were infested with neonate adults and the infestation was allowed to proceed until all adults died. Percentage adult survival was calculated. Damage scores in a 1-5 visual damage score scale were taken 5 and 10 days after infestation. To measure chlorophyll loss as a result of adult feeding, we used a SPAD-502 chlorophyll meter 5 and 10 days after infestation. Four representative readings per plant were taken and their averages were recorded. SPAD index values were then calculated with respect to the uninfested checks. At the end of the trial, when all insects had died, plants were cut at soil level and dried in an oven at 40° C. Percentage biomass losses were calculated with respect to the uninfested checks. Damage scores and percentage biomass losses were used to calculate functional plan loss indices.

Results and Discussion

Adult survival was not affected by the genotype when plants were infested with 2, 3 or 5 adults per plant. At higher infestation levels (7, 9, 12, and 15 adults per plant) adult survival on the susceptible genotype was significantly lower possibly due to depletion of food and increased competition among insects. This means that a 5-6 level of infestation could be used in future studies. The resistant sexual hybrid SX01NO/0102 plants suffered significantly less damage than susceptible (CIAT 0654) plants at all levels of infestation (Figure 10). In addition, at all levels of infestation, SX01NO/0102 plants suffered significantly less damage (expressed as percentage chlorophyll loss and percentage biomass loss) than susceptible CIAT 0654 plants (Figure 11).

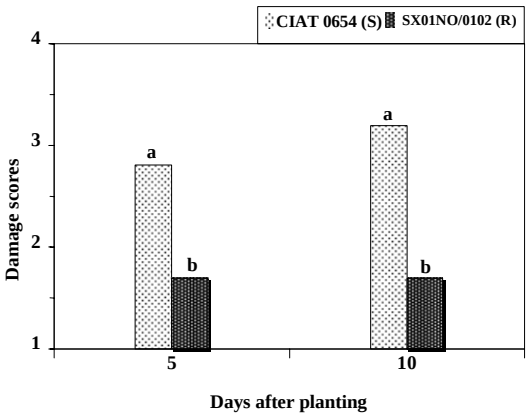

Figure 10. Response of susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes to attack by adults of *Aeneolamia varia*. Means of eight levels of infestation. For each scoring date, bars with the same letter do not differ at the 5% level of significance by LSD.

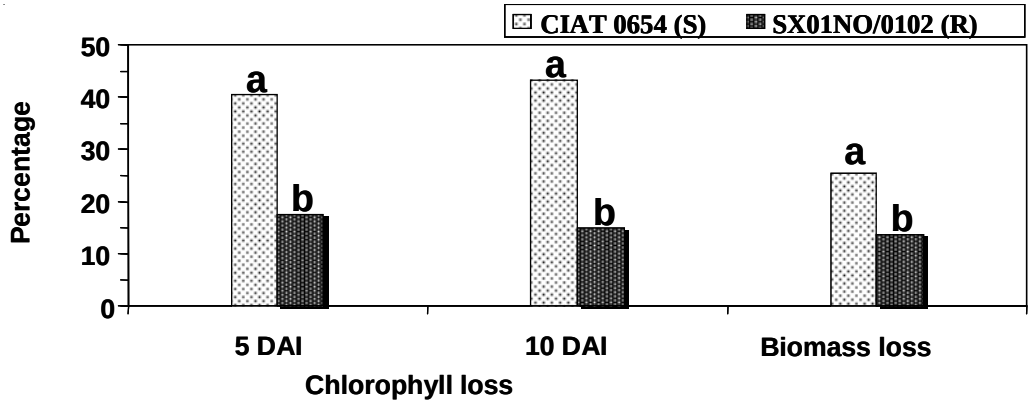

Figure 11. Chlorophyll and biomass losses due to adult *Aeneolamia varia* feeding on susceptible (CIAT 0654) or resistant SX01NO/0102 *Brachiaria* genotypes. DAI, days after infestation. Means of eight levels of infestation. For each variable, bars with the same letter do not differ at the 5% level of significance by LSD.

Significant correlations were found between damage scores and percentage chlorophyll losses ($r = 0.858$; $P<0.001$), between damage scores and percentage biomass losses (0.473; $P<0.001$) and between percentage chlorophyll losses and percentage biomass losses ($r = 0.891$; $P<0.001$) indicating that damage scores are useful in predicting losses and that SPAD units are useful in measuring insect damage. Furthermore, when a Functional Plant Loss Index (combining damage scores and percentage biomass losses) was calculated, we found that at all levels of infestation losses were highest for the susceptible genotype CIAT 0654 (Figure 12). Since no obvious signs of antibiosis to adults were found in this experiment, we interpret lower damage scores, lower chlorophyll and biomass losses, and lower functional plant losses as the expression of tolerance to adult feeding damage in the resistant genotype. Further results on this line of research, aimed at developing a mass screening procedure for adult spittlebug damage, will be reported in 2006.

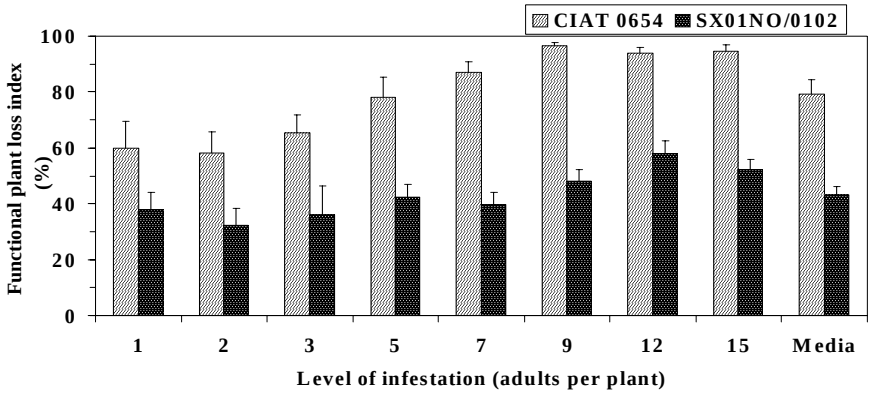

Figure 12. Functional plant loss indices (percentage) for susceptible (CIAT 0654) or resistant (SX01NO/0102) *Brachiaria* genotypes exposed to eight levels of infestation with adults of *Aeneolamia varia*.

2.4 Define interactions between host and pathogen in *Brachiaria*

Highlight

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

2.4.1 Evaluation of *Brachiaria* hybrids for resistance to *Rhizoctonia solani* under field conditions in Caqueta

Contributors: G. Segura, W., X. Bonilla, J. Miles, and S. Kelemu (CIAT)

Rationale

Rhizoctonia foliar blight, caused by *Rhizoctonia solani* Kühn, is a disease of increasing importance on a number of crops. The disease can be very destructive when environmental conditions are particularly conducive (high relative humidity, dense foliar growth, high nitrogen fertilization, and extended wet periods). *Rhizoctonia solani* is the most widely known species of *Rhizoctonia* with a wide host range. In nature *R. solani* reproduces mainly asexually and exists as vegetative mycelia and/or dense sclerotia. These sclerotia can survive in soil and on plant debris for several years, and can germinate and produce hyphae that can infect a wide range of host plants. The pathogen primarily infects below ground plant parts in a number of plant species, but can also infect above ground

plant parts such as pods, fruits, and leaves and stems as is the case with *Brachiaria*. In *Brachiaria*, infected leaves first appear water-soaked, then darken, and finally turn to a light brown color. Lesions may coalesce quickly during periods of prolonged leaf wetness and temperatures between 21 and 32°C.

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

Materials and Methods

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

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

Results and Discussion

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

The resistant control CIAT 16320 was consistently evaluated at scale 2. Four genotypes, BR04-2577, BR04-2557, BR04-2983, and BR04-1214 were evaluated at an average between 2.0 and 2.5. Twenty-four others, 1685, 1950, 1963, 3077, 1119, 1252, 1347, 1349, 1824, 1886, 1896, 2060, 2200, 2201, 2265, 3025, 3207, CIAT 36087, 1928, 1941, 2040, 2069, 3066, 3217, scored with an average rating scale of 3.0-3.3 (in the rating scale, this corresponds to a 6% - 9% overall plant tissue damage). The remaining 111 materials, 2429, 2518, 2539, 3051, 3175, 1061, 1219, 1796, 1845, 2774, 2793, 2874, 3214, 1021, 1073, 1141, 1819, 2404, 2405, 2457, 2475, 2515, 2532, 2841, 2940, 3056, 222, 1265, 1311, 1358, 1377, 1592, 1648, 1956, 2110, 2118, 2128, 2179, 2208, 2275, 2403, 2938, 2987, 3069, 3119, 3221, 36061, 1081, 1097, 1113, 1197, 1281, 1296, 1503, 1633, 1697, 2007, 2226, 2235, 2389, 2414, 2969, 1018, 1026, 1060, 1273, 1309, 1360, 1374, 1570, 1629, 1883, 1889, 2093, 2163, 2290, 2338, 2346, 2670, 2681, 2792, 2833, 2849, 2872, 2954, 3068, 3128, 1003, 1058, 1494, 1754, 2109, 2285, 2344, 1428, 1596, 1601, 1751, 1846, 1900, 2156, 2166, 2455, 2863, 2871, 3058, 3134, 2360, 2396, 1552, 3130, scored between 3.5- 5.0.

Table 20. Correlations between disease reaction data collected at various days after inoculations using Pearson's Correlation.

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

Figure 13 shows a graphical representation of the results using data from representative genotypes from each of these three groups.

The disease evaluation data taken 38 days after inoculations represented well-developed disease symptoms. The resistant control CIAT 16320 and the four *Brachiaria* hybrids BR04- 2577, BR04-2557, BR04-2983, BR04-1214 showed less than 6% overall plant tissue damage, and thus, a high-level of resistance (Photo 1). The second group of 24 genotypes including CIAT 36087 listed above still had an acceptable level of resistance. All the plants in this trial will be maintained in the field to further observe the level of disease at an extended period of time.

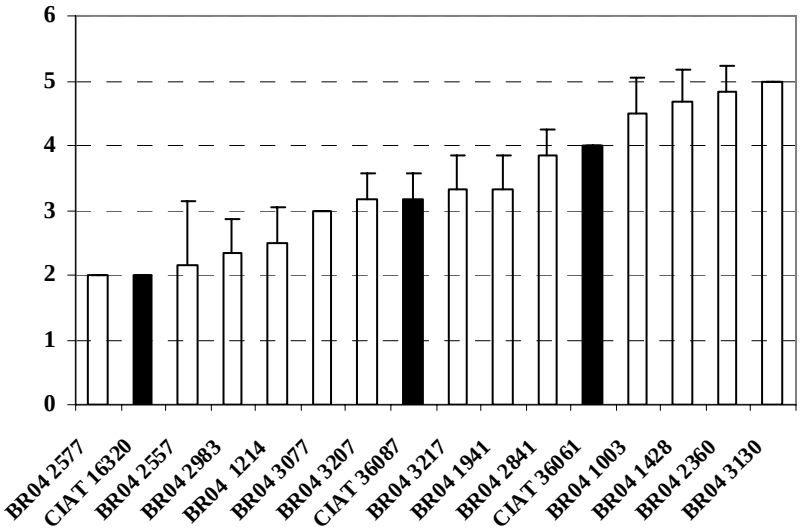

Figure 13. Ratings of *Brachiaria* genotypes for foliar blight disease reaction on a 1-5 scale 38 days after inoculations with sclerotia of *Rhizoctonia solani* under field conditions, Caquetá, Colombia. Bars indicate standard deviation.

Photo 1. Rhizoctonia foliar blight disease symptoms 34 days after inoculations under field conditions in Caquetá, Colombia. **A**: BR04-1214; **B**: BR04-2577: **C**: CIAT 36061; **D**: BR04-1754

2.5 Elucidate the role of endophytes in tropical grasses

Highlights

- The fungal endophyte *Acremonium implicatum* forms strong association with *Brachiaria* genotypes, demonstrated by its re-establishment within plants after elimination using systemic fungicides.

- An efficient transformation system was developed for *Acremonium implicatum* for the first time.

2.5.1 Drought tolerance in endophyte-infected plants under field conditions

Contributors: S. Kelemu, G. Segura, C. Plazas, J. Ricaurte, R. García and I. M. Rao (CIAT)

Rationale

Earlier experiments with potted plants in the greenhouse demonstrated 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). In order to validate the greenhouse results, in 2002 we initiated a field study in the Llanos of Colombia to quantify the impact of endophytes in improving drought tolerance and persistence in *Brachiaria*. In 2003, we reported preliminary results from this field trial that indicated that endophyte infection could improve dry season performance by improving the uptake of nutrients by two accessions of *Brachiaria brizantha*. In 2004, we conducted further field evaluations to confirm the role of endophytes in improving dry season tolerance of *Brachiaria* grasses. This on-going field study indicated beneficial effects of endophyte infection on drought tolerance in the first year (2003) and almost no effect in the second year (2004). We speculated that this might be due to the re-growth of the endophyte in the endophyte-free plants. There is evidence for this possibility in greenhouse grown plants. This year, we have tested the persistence of the endophytes in these plants in order to see if the lack of effects was due to the re-appearance of the endophytes in the endophyte-free plants.

Materials and Methods

Field set up: A field trial consisting of 2 accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110) was established at Matazul farm in Colombia in May, 2002. Genetically identical endopyte-free and endophyte-infected plants were generated from an original mother plant containing the endophyte *Acremonium implicatum* (J. Gilman and E. V. Abbott) W. Gams, using methods described in Kelemu et al. 2001. Canadian Journal of Microbiology 47:55-62.The trial was established 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 composition, leaf and stem total nonstructural carbohydrate (TNC) content, leaf and stem ash (mineral) content, and shoot nutrient uptake were measured at the end of wet season (November 2003) and dry season (March 2004).

DNA isolation: Leaf blades were collected from *Brachiaria* hybrids and known endophyte-infected or endophyte-free plants and macerated separately in liquid nitrogen for genomic DNA isolation. DNA was extracted using an improved CTAB (Hexadecyltrimethylammonium bromide) method. Extraction buffer [2% CTAB, 100mM Tris-HCl (pH8.0), 20mM EDTA (pH8.0), 1.4mM

NaCl and 1% PVP40) and 1/50 volume of Rnase A (10 mg / ml)] was added to macerated plant tissue, and incubated at 65°C for 30 min. An equal volume of Chloroform: Isoamylalcohol (24:1) was added and mixed well by vortexing. The mixture was then centrifuged at 13,200 rpm for 10 min. The supernatant was transferred to a new tube. About 0.8 ~ 1 volume of ice-cold isopropanol was added to the supernatant and kept at room temperature for 15 minutes to precipitate the DNA. DNA pellet was generated after centrifugation at 13,200 rpm for 20 min. The pellet was washed with 70 % ethanol and re-suspended in 50-ìl Tris-EDTA (TE) buffer (10 mM Tris-HCl (pH7.5), 1 mM EDTA (pH8.0)).

Polymerase chain reaction (PCR) analysis: PCR was carried out using the *A. implicatum*-specific primer pairs, P1 (5-TTCGAATGATAAGGCAGATC-3') and P4 (5'-ACGCATCCACTGTATGCTAC-3'). The PCR reaction volume was 20-ìl, and composition was as follow: 1x PCR buffer (QIAGEN); 3mM $MgCl_2$; 0.26mM each deoxynucleotide triphosphate (dNTPs); 1.25-ìM each oligonucleotide primer; 1 units Taq DNA polymerase (Invitrogen) 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, 53°C 40 seconds; step 4, 72°C for 45 seconds; step 5, go to step 2 for 35 cycles; then 72°C 4 min. The amplification products were separated by electrophoresis in a 1.2% agarose gel (Invitrogen), stained with ethidium bromide and photographed under UV lighting.

Results and Discussion

The presence of *A. implicatum* in *Brachiaria* tissues was determined by the presence of a diagnostic 500-bp amplification product (Figure 14).

In the Annual Report 2004, we reported that at 18 months after establishment, i.e., at the end of rainy season, the endophyte-infected plants (E+) showed greater values of stem biomass in both

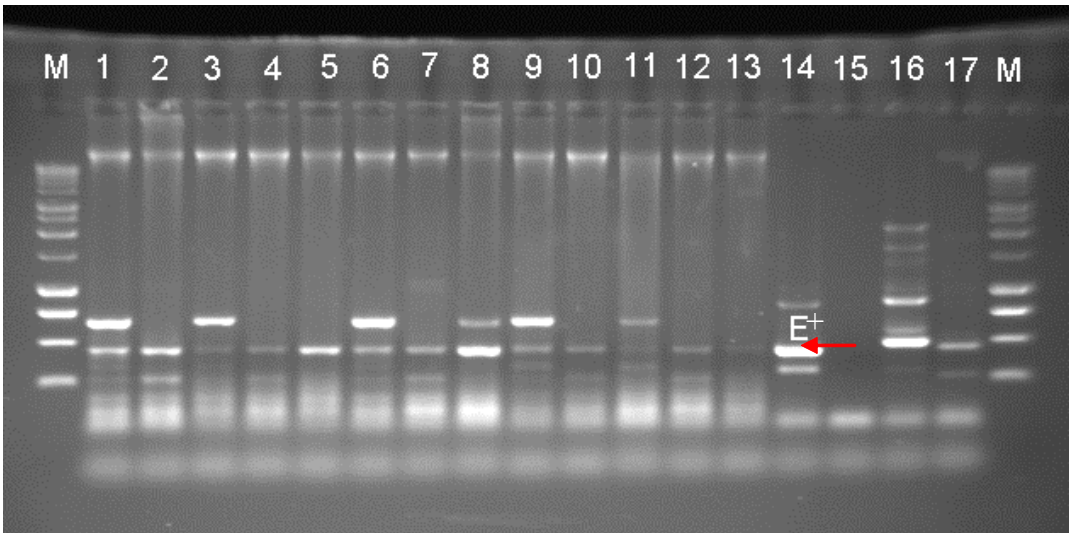

Figure 14. Specific detection of *Acremonium implicatum* in gentypes of *Brachiaria*.
Genomic DNA isolated from tissues of *Brachiaria* plants established in a field trial consisting of 2 accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110) at Matazul farm in Colombia, May, 2002. Lanes 1, 6, 11, *Brachiaria brizantha* CIAT 6780 initially endophyte-free through the use of a systemic fungicide; lanes 3,8,9, *Brachiaria brizantha* CIAT 6780 with endophyte; lanes 4,7,10, *Brachiaria brizantha* CIAT 26110 initially endophyte-free through the use of a systemic fungicide; lanes 2,5, 12, *Brachiaria brizantha* CIAT 26110 with endophyte; lane 13, cv. Mulato; lanes 14, 15, 16, 17, positive control *A. implicatum* DNA, negative control water, positive control from endopyte-positive plant from greenhouse, positive control from the field, respectively. Lane M = size marker.

accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110). In contrast to the results at the end of rainy season, at 22 months after establishment, i.e., at the end of dry season, the endophyte infected plants showed no significant increase in either green leaf or stem biomass in both accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110).

This observation is not consistent with the results from the greenhouse study where the benefits of endophyte infection were noted 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 not significantly different between the endophyte-infected plants and the endophyte free plants. This on-going field study indicated beneficial effects of endophyte infection on drought tolerance in the first year, but almost no effect in the second year. We speculated that this might be due to the re-growth of the endophyte in the endophyte free plants. There is evidence for this possibility in greenhouse grown plants.

The results of the PCR test indeed demonstrate that the initially endophyte-free plants tested positive for the diagnostic 500-bp amplified product indicating the re-growth of *A. implicatum* after fungicide treatment intended to eliminate the endophyte. This indicates that although the endophyte was successfully removed to the level that we were able to see differences demonstrating the effect of the endophyte on disease and drought resistance.

However, it is evident now that, with time, the endophyte level in the fungicide-treated plants was high enough to eliminate those differences. Work is in progress to use a biocidal product PPM (plant preservative mixture) trying to completely eliminate the endophyte from selected *Brachiaria* genotypes in order to study the long term effect of endophytes on plant development and important agronomic traits.

2.5.2 Endophyte transformation and use as gene delivery system

Contributors: J. Abello and S. Kelemu (CIAT)

Rationale

Acremonium implicatum is an endophytic fungus that forms symbiotic association with species of *Brachiaria*. The green fluorescent protein (GFP) gene, isolated from the jellyfish *Aequorea Victoria*, or its derivatives have been expressed in a wide array of organisms including plants and microbes. The practical implication of seed transmission of endophytes in *Brachiaria* is significant. Once associated with the plant, the fungus can perpetuate itself through seed, especially in apomictic genotypes of *Brachiaria*, for as long as seed storage conditions do not diminish the survival of the fungus.

Several *Brachiaria* hybrids obtained from CIAT's forage breeding program were shown to harbor *A. implicatum*. Therefore, we may be able to exploit this association and its high seed transmission [Dongyi, H. and Kelemu, S. 2004. *Acremonium implicatum*, a seed-transmitted endophytic fungus in *Brachiaria* grasses. *Plant Disease* 88:1252-1254) by using a transgenic *A. implicatum* as a vehicle for production and delivery of gene products of agronomic interest into the host plant to enhance protective benefits and other traits, and thus improve livestock production. In addition, we want to exploit the qualities of GFP as a reporter and study the interactions between *A. implicatum* and its host *Brachiaria*.

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

Materials and Methods

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

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

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

PCR amplifications: DNA isolated (Kelemu et al. 2003. Molecular Plant Pathology 4:115-118) from putative transformant bacteria and fungus as well as control ones was analyzed using the polymerase chain reaction (PCR) using primers with sequences of *egfp* and/or *hph*. [primers glGFP3 (5‘-GCCGAGCTCAGATCTC ACTTGTACAGCT CGT-3‘) and glGFP5 (5‘-CC

GGAATTCATGAACAAGGG CGAGGAACTG-3‘)] (Fitzgerald *et al.* 2003) and hph122U (5‘-TC GATGTAGGAGGGCGTGGAT-3‘) and hph725L (5‘-CGCGTCTGCTGCTCCATACAAG-3‘) (Irie et al. 2001)]. Amplifications were carried out in a Programable Thermal Controller (MJ Research, Inc) programmed to 35 cycles comprised of 45 seconds denaturation step at 94°C (4 minutes for the first cycle), followed by 1 min at 60°C, and primer extension for 1.5 minute (10 minutes in the final cycle) at 72°C. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad Laboratories), stained with ethidium bromide, and photographed under UV lighting.

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

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

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

Results and Discussion

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

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

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

The protocol that we developed for the transformation of this endophytic fungus is based on the protocol described by Mullins *et al.* (2001, Phytopathology 91:173-180)) for the pathogenic fungus *Fusarium oxysporum*. However, modifications were needed for a successful transformation of *A. implicatum*. For example, *A. implicatum* is a slow growing fungus, and thus the recommended concentration of cefotaxime (200 µM) to inhibit the growth of *A. tumefaciens*, was not sufficient enough to prevent bacterial growth from impeding the growth of *A. implicatum*.

Results from the experiments we conducted indicated that cefotaxime concentrations at 500 µM was sufficient to inhibit the growth of *A. tumefaciens* while allowing *A. implicatum* putative transformants to grow on selection media. Introducing TYNG medium instead of MM [for 1L medium: 2.05g K_2HPO_4, 1.45g KH_2PO_4, 0.15g NaCl, 0.5g $MgSO_4$ $7H_2O$, 0.07g $CaCl_2 \bullet 2H_2O$, 0.0025g FeSO4 $\bullet$ $7H_20$, 0.5g $(NH_4)_2SO_4$] has

reduced the time needed to reach the required bacterial concentration (OD_{600} = 0.75) from 48 hours to only 16. In addition, the TYNG medium eliminated the cell aggregation problem we encountered with the growth of *A. tumefaciens* (particularly with strain LBA4404) in MM and that interfered with the transformation process.

A better transformation efficiency was obtained with *A. tumefaciens* strain AGL-1 (Table 21; Figure 15). Although *A. implicatum* transformants containing either pSK1019 (*trpC* promoter) or pCAMBIA 1300 (CaMV35S promoter) were obtained, a significantly higher number of transformants were obtained with pSK1019 (Table 21). However, this suggests that the CaMV35S promoter can function in *A. implicatum* although at a much lower efficiency. The colony size of transformants in both cases is similar with an average size of 19-mm after 12 days of incubation at 28^{O}C on the selection medium.

The results indicate that the transformation efficiency is directly influenced by the length of the co-cultivation (*A. tumefaciens* and *A. implicatum*) period (Table 21, Figure 15). As the co-cultivation period increased from 48 h to 72 h, the efficiency increased from 542 transformant colonies to 1084 in the case of mycelial transformation protocol; and from 271 to 542 for conidial transformation (Table 21, Figure 15). Similar results have been reported for transformation of *Magnaporte grisea* (Rho *et al.* 2001. Mol. Cells 3:407-411) and *F. oxysporum* (Mullins *et al.* 2001). The efficiency of transformation also differed depending on whether we used mycelia or conidia for transformation (Figure 15). The best and optimum transformation results were obtained with *A. tumefaciens* strain AGL-1, plasmid pSK1019 under the control of *trp*C promoter either with mycelial or conidial transformation. However, mycelial transformation consistently generated significantly higher number of transformants than when conidia were used to transform (Figure 15).

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

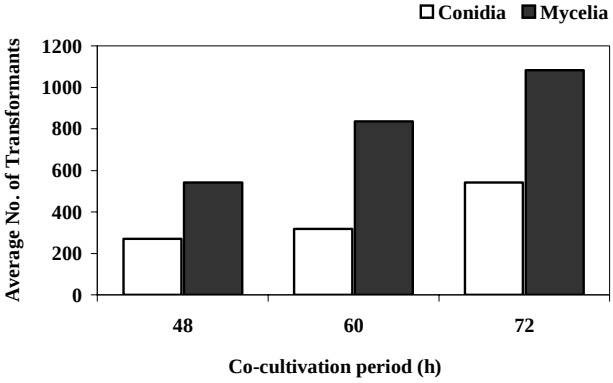

Figure 15. The effect of *Agrobacterium tumefaciens* strain AGL-1 and *Acremonium implicatum* co-cultivation period on transformation efficiency. The *A. tumefaciens* strain contains plasmid pSK1019 that has enhanced green fluorescent protein *(egfp)* gene under the promoter of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from the fungus *Cochliobolus heterostrophus*. It also contains a hygromycin B resistance gene hph, controlled by the *Aspergillus nidulans trp*C promoter. The data presented are the average of three plates per treatment.

Table 21. Putative *Acremonium implicatum* transformant colonies per Petri dish of selection medium.

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

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

The putative *A. implicatum* transformants selected on hygromycin B containing agar media were further examined using fluorescence microscope, PCR and Southern blot analysis. The PCR method allowed us to quickly examine and further confirm putative transformants that have been selected on antibiotic selection media (Figures 16 and 17). To determine the copy number of the transferred T-DNA, genomic DNA from19 randomly picked transformants from each experimental condition was digested with *Hind*III and anlalyzed with Southern blot. The results exhibited genomes with inserts ranging from a single insert to 5 inserts (data not shown), while the negative control, untransformed *A. implicatum*, showed no hybridization. No correlation existed between the average copy number of T-DNA per genome and the co-cultivation period, the mycelial or conidial transformation or other variables introduced in the experiments.

Microscopic examinations of selected transformants demonstrated strong expression of *egfp* as evidenced by the intense fluorescence emission. All parts of the fungal structure including conidia, mycelia, germinating conidia showed emission. These results demonstrate that the fungal promoter glyceraldehyde-3-phosphate dehydrogenase (GPD) isolated from *Cochliobolus heterostrophus* functions well for expression of genes in the endophytic fungus *A. implicatum* (Photo 2a).

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

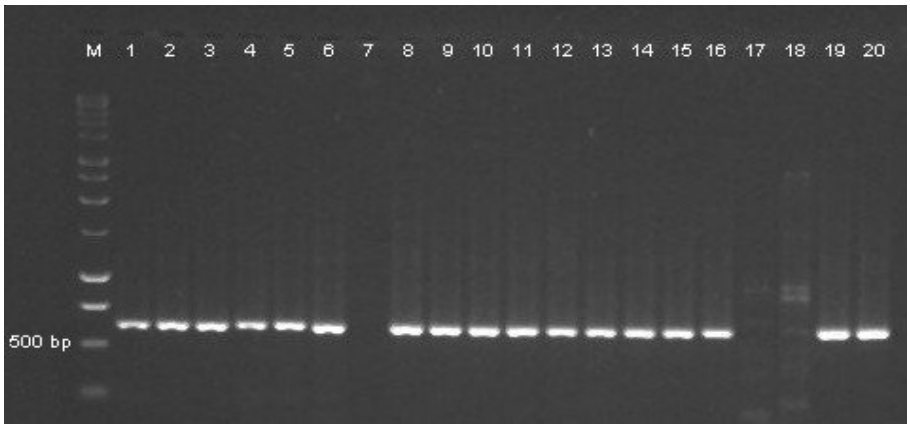

Figure 16. Polymerase chain reaction (PCR) amplifications, with primer specific for sequences of hygromycin B resistance gene (hph), of template DNA isolated from *Acremonium implicatum* transformants. Lanes M = molecular marker; template DNA from: 1 = conidia transformed with pSK1019 in *A. tumefaciens*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

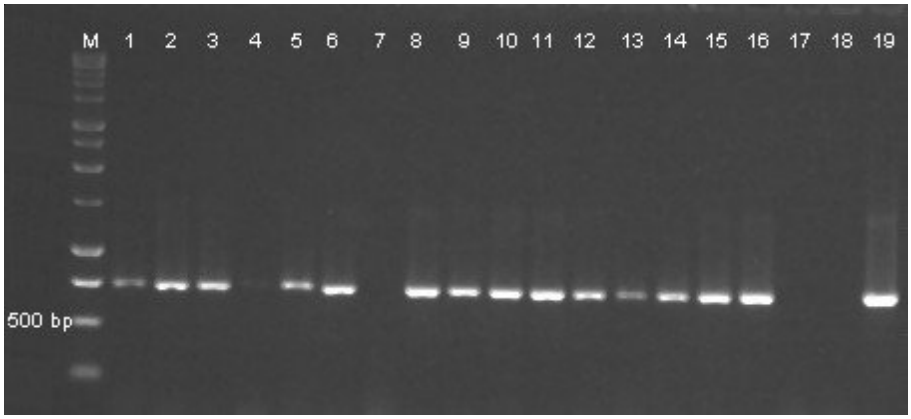

Figure 17. Polymerase chain reaction (PCR) amplifications, with primer specific for sequences of enhanced green fluorescent protein (*egfp*) gene, of template DNA isolated from *Acremonium implicatum* transformants. Lanes M = molecular marker; 1 = conidia transformed with pSK1019 in *A. tumefaciens*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

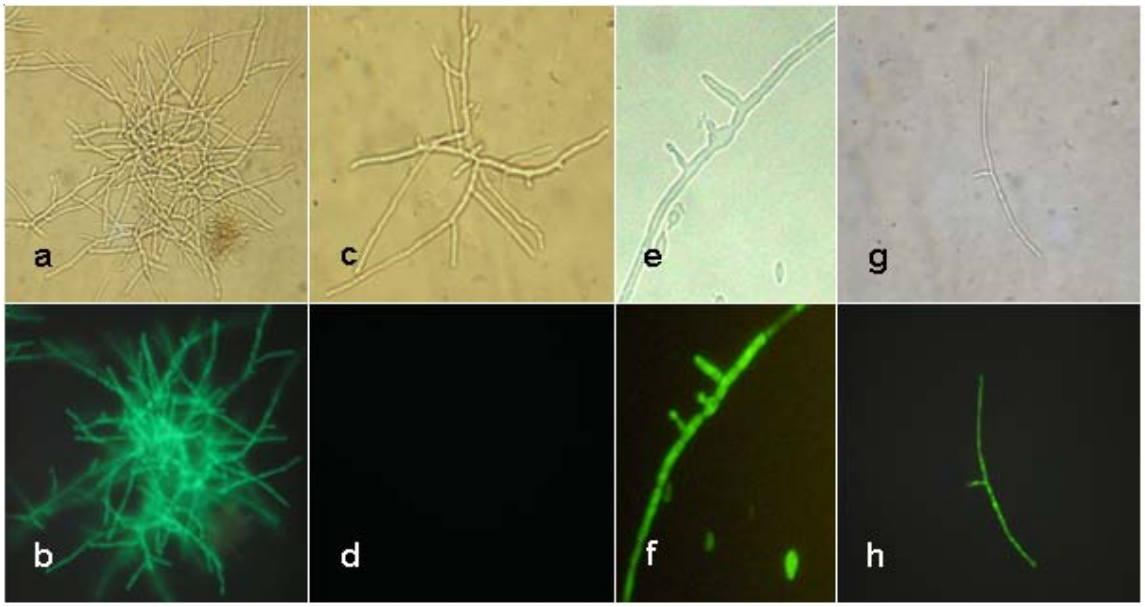

Photo 2. Structures of *Acremonium implicatum* strain 6780 201v transformed with green fluorescent protein gene (*egfp)* and observed microscopically with UV light. Photos a, c, e and g under normal light; b = fluorescence emission from transformed mycelia under Leica D filter (355 and 425 nm); c and d = control untransformed *A. implicatum* strain 6780 201v without and with UV light, respectively; f and h = transformed structures emitting green fluorescence under UV light with H3 filter (420 and 490 nm).

Preliminary data showed that *Brachiaria* tissues taken from plants inoculated with GFP-transformed *A. implicatum* expressed fluorescence emission (Photo 4). Photo 3a shows the gfp-expressing transgenic *A. implicatum* used to inoculate *Brachiaria* plants. This will allow us to study the endophyte-*Brachiaria* interaction, endophyte distribution within the plant tissue, and stability in the seed. This will in turn allow us to examine the potential use of this endophyte as a gene delivery and expression system in plants.

Although various transformation systems have been developed and reported for many fungi, successful application of the technology is still not routine in many species. Furthermore, developing an efficient transformation system for a previously untransformed fungus can be a technical obstacle. This work describes the transformation and expression of the GFP-encoding gene in an isolate of *A. implicatum*, an endophyte in species of *Brachiaria*. We have demonstrated that both the mycelia and conidia of *A. implicatum* can efficiently be transformed using *A. tumefaciens*. To the best of our knowledge, this is the first report on transformation of this endophytic fungus.

The stable integration and expression of the introduced gene into the genome of the recipient fungus indicate that the endophyte may be an excellent tool for delivering and expressing genes of agronomic importance such as disease and insect resistance to host plants. For this to be successful, the practical implication of high seed transmission of *A. implicatum* in *Brachiaria* is significant: once associated with the plant, the fungus can perpetuate itself through seed,

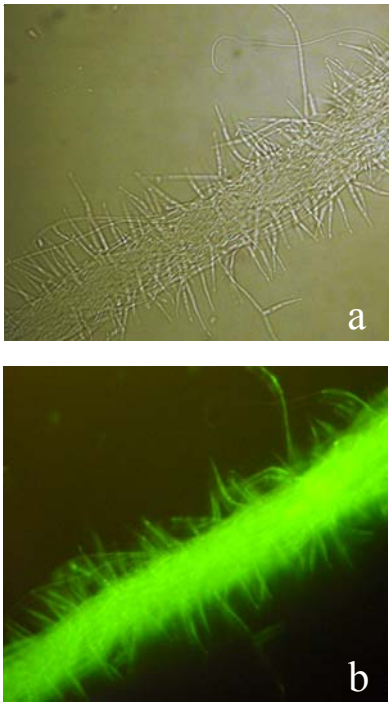

Photo 3. Mycelium of *Acremonium implicatum* transformed with enhanced green fluorescent protein (*egfp)* encoding gene: a) mycelium observed microscopically (40x) under normal lighting, b) the same mycelium observed under UV lighting, and demonstrating fluorescence emission.

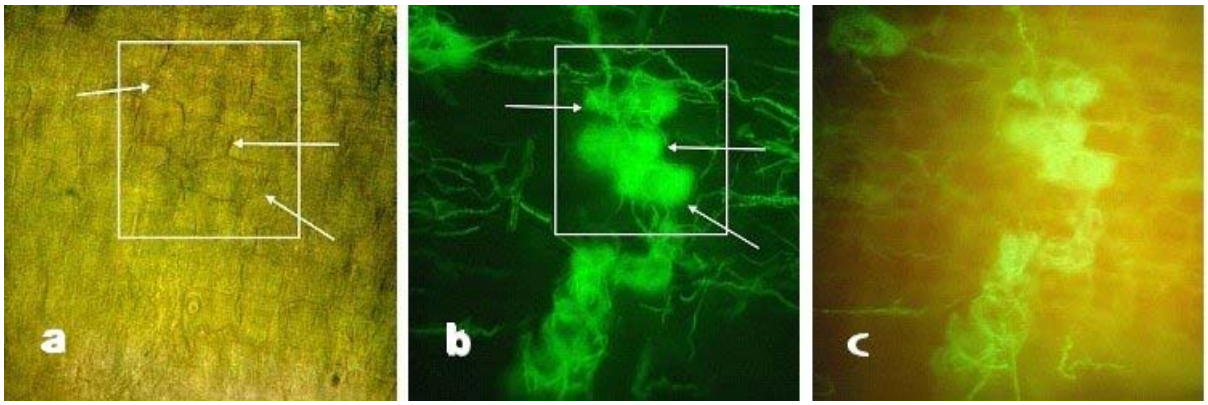

Photo 4. *Brachiaria* tissues from plants inoculated with *Acremonium implicatum* strain 6780 201v transformed with green fluorescent protein gene (*egfp*) [transformed strain shown in Figure 2. (a) under normal lighting, (b) fluorescence emission under UV light with Leica D filter, (c) fluorescence emission under UV light with Leica H3 filter.

especially in apomictic genotypes of *Brachiaria*, for as long as seed storage conditions do not diminish the survival of the fungus (Dongyi and Kelemu, 2004, Plant Disease 88:1252-1254) Several *Brachiaria* hybrids obtained from CIAT's forage breeding program were shown to harbor *A. implicatum*. In addition, we want to exploit the qualities of GFP as a reporter and study the interactions between *A. implicatum* and its host *Brachiaria*.

2.6 Effect of an antifungal protein isolated from seeds of the tropical forage legume *Clitoria ternatea* on disease control

Highlight

- Demonstrated that spraying crude preparation of the antifungal protein "Finotin" was effective in reducing diseases and increasing yield of tomato plants in the field.

2.6.1 Disease control under greenhouse and field conditions of Finotin (antifungal proteins)

Rationale

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

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

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

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

Materials and Methods

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

Plant inoculation and extract applications: A highly virulent isolate of the pathogen *Phaeoisariopsis griseola*, causal agent of angular leaf spot, was grown on V8 agar at 24°C

for 12 days. Conidia were collected and suspended in sterile distilled water at a concentration of 2 x 10^4 conidia per mL. This inoculum was used on *Phaseolus vulgaris* variety Sprite (a susceptible one) bean plants.

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

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

Results and Discussion

Effect of antifungal protein Finotin on bean angular leaf spot: The crude protein extract from seeds of *C. ternatea* CIAT 20692 showed antifungal activity *in vitro* on the pathogen *P. griseola* (data not shown). Conidia treated with the crude protein failed to germinate 32 or 96 hours after treatment (Photo 5).

Plants treated with the crude antifungal protein preparation consistently developed fewer angular leaf spot disease lesions than the control plants that were treated with sterile distilled water (Figure 18). Had a purified protein been used to control the disease on bean plants, the level of disease control would perhaps have been even higher.

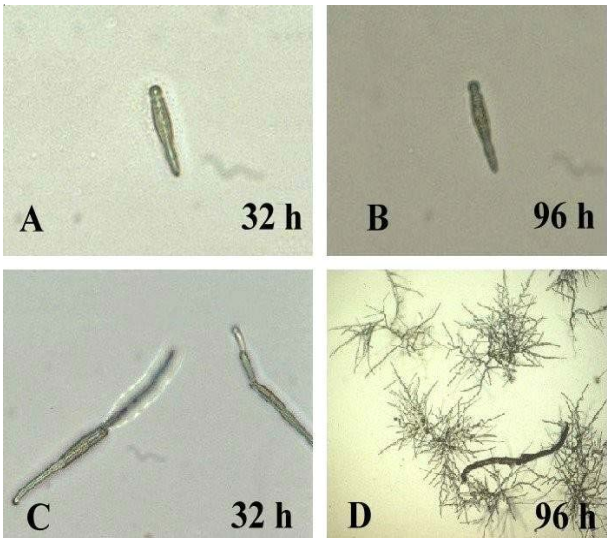

Photo 5. Treatment of *Phaeoisariopsis griseola* conidia with the antifungal protein finotin. Conidia failed to germinate in the presence of the antifungal protein finotin, 32 and 96 hours (A and B) after treatment, whereas those treated with sterile water germinated (C and D). [Annual Report 2004].

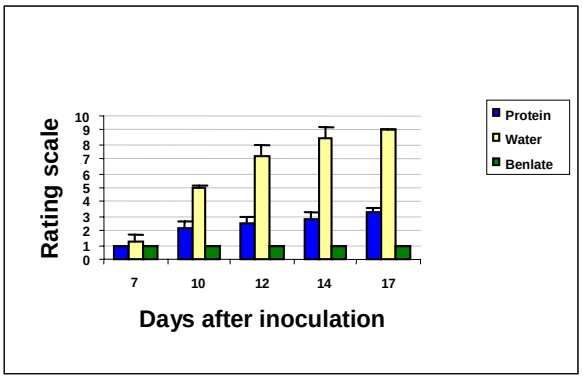

Figure 18. Angular leaf spot disease development in artificially inoculated bean plants following treatment with crude antifungal protein preparations isolated from *C. ternatea* CIAT 20692, the fungicide benlate, or water control (from AR-2004).

Effect of antifungal protein finotin on tomato diseases: Tomatoes are generally susceptible to a number of diseases under natural conditions. The purpose of these experiments is to develop a simple disease control strategy for small producers using this antifungal protein. Plants sprayed with the crude protein preparation once or twice a week developed better, had fewer disease symptoms, had more plant biomass, and produced more tomatoes than control plants (Photo 6 and Figure 19).

Photo 6. Tomato plants sprayed with crude antifungal protein preparations: control-water (1), once (2) and twice (3).

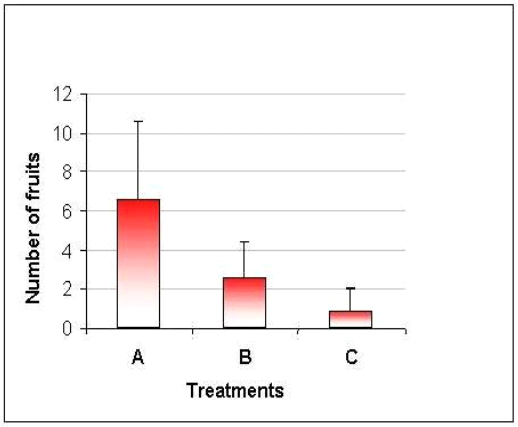

Figure 19. Average tomato fruit yield per tomato plant in plants treated with crude protein preparations twice a week (A), once a week (B) and water only (C).

The protein finotin, is shown to be inhibitory to the growth of a range of important plant pathogenic fungi and at least one important bacterium pathogenic to common bean, as well as two important species of bruchids, *Z.subfasciatus* and *A. obtectus* (Kelemu et al., 2004. Plant Physiology and Biochemistry 42: 867-873). These findings raise the possibility that finotin may contribute to the high level of disease and insect resistance observed in *C. ternatea* in the field. Finotin is released from seeds when the seed coat is mechanically damaged creating a zone of fungal growth inhibition *in vitro*. The antifungal activity of finotin is not affected by high temperatures, which made attractive for the direct use of this protein in disease management under field and greenhouse conditions. The results presented here demonstrate that a disease control strategy can be developed for small producers using this antifungal protein.

2.7 Isolating the gene encoding a biocidal protein named "Finotin"

Highlight

- Progress was made in defining the amino acid sequence of the antifungal protein "Finotin". Data obtained suggest that the cDNA clone generated could be classified as a member of the nsLTP which has been shown to inhibit growth of a number of pathogens.

Contributors: Martin Rodriguez and S. Kelemu (CIAT)

Rationale

Diseases and pests are major biological production constraints in a wide-range of crops. Plants, when attacked by harmful agents, can trigger an array of defense mechanisms. Pathogens and pests, in turn, have an array of matching mechanisms and evolve to overcome and compromise plant defense systems. One type of plant defense mechanism is the synthesis of proteins/peptides or low-molecular weight compounds following mechanical wounding or attack by biological agents. Biocidal or antimicrobial proteins are widely distributed in nature and are synthesized by various organisms. A number of plant-derived proteins that have antimicrobial or insecticidal properties have been isolated and characterized from various plants.

One such example is the isolation, purification and characterization of a highly basic small protein, designated 'finotin', from seeds of *Clitoria ternatea* (Kelemu et al. 2004. Plant Physiology and Biochemistry 42: 867-873). This protein has broad and potent antifungal, antibacterial and insecticidal properties, indicating that it may contribute to the high level of disease and insect resistance observed in *C. ternatea* in the field. We have subsequently demonstrated that plants sprayed with the crude protein preparation consistently developed fewer lesions of various diseases than the control plants both in greenhouse and field experiments (Kelemu et al. 2005. Phytopathology 95:S52).

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

Materials and Methods

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

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

Synthesis of cDNA: Although there are various methods for doing so, complementary DNA (cDNA) is often synthesized from mature (i.e. fully spliced) mRNA using reverse transcriptase enzyme, which operates on a single strand of mRNA and generating its complementary DNA based on the pairing of RNA base pairs (A, U, G, C) to their DNA complements (T, A, C, G). In this study, the synthesis was conducted using 200 ng mRNA, 12-µM of BD SMART II™ A oligonucleotide and 12-µM of *Primer* 5'-RACE CDS in a 10-µl volume. This was incubated at 70°C for 2 min, then placed on ice. Two-µl of 5x buffer, 1-µl of DTT (20 mM), 1-µl of 10 mM dNTP and 1-µl of BD PowerScript Reverse Transcriptase were subsequently added to the mixture and incubated at 42°C for 2 h (the reverse transcriptase scans the mature mRNA and synthesizes a sequence of DNA that complements the mRNA template). Fifty-µl of Buffer Tricina-EDTA was added and further incubated at 70 °C for 7 minutes to deactivate the reaction. For synthesis of cDNA for the 3´ end, 500-ng mRNA and 1-µl (100-ng/µl) of primer oligo-$(dT)_{25}$ were mixed and incubated at 70°C for 8 min followed by cooling the mixture on ice. Subsequently, 10-µl of 5X buffer, 1-µl of 25mM dNTP, 2-µl of 100 mM DTT and 1-µl of SuperScript™ III RT (200 U/µl) were added to the mixture and incubated for 1 h at 50°C. At the end the mixture was deactivated by heat treatment at 70°C for 10 min. Information on oligonucleotides used in this study is given in Table 22.

Polymerase chain reaction of cDNA: We contracted Cornell University's biotechnology unit to sequence finotin. However, the protein (finotin) sequence data obtained from Cornell Biotech was not satisfactory, and as a result we used sequences of an antifungal protein (from Clitoria ternatea) reported by Osborn et al., 1995 (FEBS Lett. 368: 257-262) to generate degenerate primers (Table 22). A 25-µl PCR mixture contained 1.5-mM of $MgCl_2$, 200-ìMof dNTPs, 0.5-ìM of each olgonucleotide (UPM and FINOR5), 200-ng cDNA, 1 unit of Taq DNA polymerase, and 1x PCR buffer. Amplifications were programmed with 35 cycles of a 30 second (3 min for the first cycle) denaturation step at 94 °C, annealing for 45 seconds at 50 °C, and prime extension for 45 seconds at 72 °C.

Table 22. Sequences of primers and adaptors used in this study.

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

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

Results and Discussion

Amplified DNA fragments (using cDNA as a template) ranging from 120 to 650 bp were isolated and cloned. Ten combinations of primers, 7 sense and anti-sense orientations and a universal primer, were used on 4 different preparations of cDNAs. A total of 37 clones were generated and of these, 17 have been sequenced so far. The sequence of one such clone is shown in Figure 20. The sequence data demonstrated homology to genes encoding nonspecific lipid transfer proteins (nsLTPs) from plants. These findings may be significant because nsLTPs have been reported to play a role in plant defense systems. A number of peptides (small proteins with sizes ranging 2-10 kDa) including nsLTPs have been reported to be involved in plant defense mechanisms. It is generally believed that seed proteins with antimicrobial activity may play a role in the protection of seeds against harmful microbes. Nonspecific lipid transfer proteins are basic, 9-kDa proteins with conserved cysteines and present in high amounts in plants. One promising clone with homology to nsLTPs is tentatively designated CtLTP. The clone is not complete, but based on sequences from nsLTP of other plants, only a small portion from the 3' end is missing (Figure 21).

The biological function of nsTLPs is still not well understood. However, a number of studies have demonstrated growth inhibition of a range of pathogens by nsTLPs. For example Cammue et al. (1995, Plant Physiol. 109:445-455) isolated a protein from seeds of onions (*Allium cepa* L.) that had sequence homology to nsLTPs and that was a potent groth inhibitory effect against 14 different pathogens.

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

Figure 20. Sequence analysis of a cDNA clone synthesized from mRNA of seeds of *Clitoria ternatea*. The figure shows nucleotide sequences and the corresponding deduced amino acid sequences The sequence highlighted in green represents a possible TATA box sequence where as the yellow highlighted region represents a sequence commonly present in various LTP genes. The cysteine amino acid sequences are circled. The sequence of primer FINOR5 (anti-sense) is underlined.

Figure 21. Amino acid sequence comparisons among various plant nsLTPs that are associated with plant defense systems. CtLTP: sequences deduced from this study; AtLTP: nsLTP of *Arabidopsis thaliana*; OsLTP: nsLTP of *Oryza sativa*; TaLTP: nsLTP of *Triticum aestivum*; HvLTP2: nsLTP of *Hordeum vulgare*; VuLTP2: nsLTP of *Vigna unguiculata*.

2.8 Plant growth promoting and nitrogen fixing bacteria associated with *Brachiaria*

Highlights

- Demonstrated that through tissue culture and spraying antibiotics (cefotaxime and vancomycin) we could eliminate endophytic bacteria in *Brachiaria*, which is necessary step to determine their nitrogen fixing properties.

- Showed that through introduction of bacteria isolated from a Brachiaria hybrid (CIAT 36062) to the *Brachiaria* hybrid cv. Mulato (CIAT 36061), exhibited improved growth (more litters and root development) relative to the control (indigenous bacteria only).

- Developed a specific primer useful to detect endophytic bacteria associated with *Brachiaria* using the step PCR instead of nested PCR.

2.8.1 Elimination of bacteria from *Brachiaria* plants using tissue culture methods

Contributors: P. Fory, X. Bonilla and S. Kelemu (CIAT)

Rationale

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

Endophytic bacteria that reside in plant tissues without causing any visible harm to the plant have been isolated from surface-sterilized *Brachiaria* tissues. Three bacterial isolates 01-36062-R2, 02-36062-H4, and 03-36062-V2 were isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively, that tested positive for sequences of the *nif*H gene (the gene that encodes nitrogenase reductase) [IP-5 Annual Report 2003]. 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 has been used with a number of PCR primers that amplify the gene from microbes and other samples by a number of researchers.

As stated in the 20003 Annual Report, 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. The analysis matched isolate 02-36062-H4 with *Agrobacterium rubi* at 0.845 similarity index. The name *A. rubi* is synonymous to *Rhizobium rubi*. 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). A nitrogen-fixing endophytic strain of *Klebsiella pneumoniae* (Kp342) has been isolated from a nitrogen-efficent line of maize (Chelius and Triplett, 2000. Applied and Environmental Microbiology 66:783-787). This strain has been described to have a very broad host range and is capable of colonizing the interior of many plants with fewer than 10 cells in the inoculum (Dong et al., 2003. Plant Soil 257:49-59). More recently, endophytic colonization and nitrogen fixation in wheat were demonstrated upon inoculation with *Klebsiella pneumoniae* strain Kp342 (Iniguez et al., 2004. Molecular Plant Microbe Interaction 17:1078-1085).

Nitrogen fixation is conducted by phylogenetically diverse groups of prokaryotes. Tropical forage grasses could be ideal for investigating associations with nitrogen fixing bacteria because of their perennial nature and low chemical inputs including fertilizers. *Brachiaria* CIAT 36062 was chosen for these studies because it remains green even under conditions of low nitrogen input. Subsequently, we have determined that other genotypes of *Brachiaria* such as CIAT 36061 (cv. Mulato) also contained endophytic bacteria that tested positive for sequences of the *nif*H gene.

The main objectives of this initiative are to: 1) isolate and characterize indigenous endophytic and rhizospheric bacteria responsible for nitrogen fixation in association with species of *Brachiaria*, and 2) determine their phenotypic properties. In order to achieve the second objective, we have conducted various experiments to create genetically identical plants with or without endophytic bacteria. A key to achieving this is a successful method to eliminate indigenous bacteria. In 2004, we reported that none of the antibiotic or heat treatment procedures successfully eliminated the bacteria that tested positive for *nif*H gene sequences. We also reported that we initiated studies on the possibility of eliminating endophytic bacteria associated with *Brachiaria* CIAT 36062 through tissue culture and regeneration procedures. We report here initial results on elimination of endophytic bacteria.

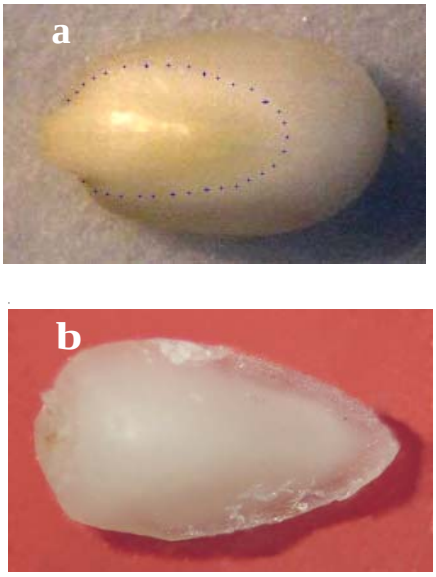

Photo 7. *Brachiaria* seed preparation for the isolation of embryos. (a) seed scarified and marked for isolation of its embryo, (b) embryo isolated free from the endosperm.

Materials and Methods

Plant material: Seeds of *Brachiaria* CIAT 36062 and CIAT 36061 (cv Mulato) were manually scarified and surface sterilized with 70% ethanol for 2 minutes, then with 2.5% NaOCl solution for 10 minutes, and rinsed four times with sterilized distilled water. The seed were then left immersed in sterilized distilled water for an hour in order to soften the endosperm. Longitudinal cuts were made in each seed to separate the embryo from the endosperm as shown in Photos 7a and b.

Plant growth media: The basic medium used was basal MS medium (Murashige and Skoog, 1962. Physiol Plant 15:473-479). This medium was subsequently modified for each sequence of propagation depending on the objective: a) induction medium (IM), b) regeneration medium (RM), and rooting medium (RM).

The isolated embryos were cultured aseptically in petri dishes (8-10 embryos per dish) containing induction medium (IM), with a composition of basal salt mixture of MS medium 4.49 g/L; casein, enzymatic hydrolysate 100 mg/L; 2,4-dichloro-phenoxyacetic acid 2 mg/L, 6-Benzylaminopurine (6-BAP) 0.2 mg/L, and supplemented with 30 g/L sucrose, 5 g/L agar, and incubated at 28°C for 12 weeks, and sub-culturing embryogenic calli every 3 weeks (Photo 8).

Photo 8. Embryogenic calli induction of *Brachiaria* seed embryos on induction medium.

Five embryogenic calli were transferred to each of a wide-mouth bottle (65mm x 45 mm of diameter) containing regeneration medium (basal salt mixture of MS medium 4.49 g/L; vitamin B5, 112.6 mg/L; sucrose 30 g/L; naphthalene-acetic acid 0.1 mg/L; kinetin 0.4 mg/L; gel rite 2.0 g/L; inositol100 mg/L; activated carbon 2.0 g/L; 5 g/L agar with or without antibiotics that inhibit the growth of bacteria (cefotaxime 100 mg/L and vancomycin 20 mg/L) and incubated first for a week under full light condition and then transferred to a growth chamber with a photoperiod of 16/8 hours (light/dark) at 24°C. As early as three weeks of incubation, green shoot formation was observed indicating successful plant development (Photo 9).

Once the plants reached a height of approximately 3 cm, they were transferred to a bigger bottle (130-mm x 75-mm of diameter) containing rooting medium (basal salt mixture of MS medium 2,245 g/L; vitamin B5, 112.6 mg/L; sucrose 30 g/L; casein, enzymatic hydrolysate 100 mg/L; gel rite 2.0 g/L5 g/L agar, with or without antibiotics that inhibit the growth of bacteria (cefotaxime 100 mg/L and vancomycin 20 mg/L) and incubated in a growth chamber under conditions described above for 2 weeks.

Photo 9. *Brachiaria* plant regeneration from embryogenic calli.

Fully developed seedlings were transferred to beakers containing sterile distilled water and maintained at room temperature for 5 days, with a daily change of water. This was done to allow plants adapt to external conditions free from rich culture media. The seedlings were subsequently transplanted onto pots containing sterilized 95% sand and 5% soil, and placed in a growth chamber with 12/12 hours (light/dark) and a temperature of 26±1°C. Each week and for a total of 3 weeks plants that were treated with antibiotics in regeneration and rooting media were also sprayed with the same concentration of antibiotic solutions.

Bacterial isolation from Brachiaria tissues: Isolations were conducted from leaf, stem and root tissues of the seedlings of CIAT 36062 and CIAT 36061 generated from embryogenic calli. Each tissue was sliced to approximately 1-cm size and surface sterilized with sterilized 1% NaOCl solution for 2 minutes, then with 70% ethanol for one minute, and rinsed three times with sterilized distilled water. Each sample was then macerated with mortar and pestle in 1 ml of sterile distilled water. One hundred-ìl of this sample was taken and a dilution series conducted, and a total of 100-ìl of each dilution was spread on plates containing nutrient agar medium (Difco, Detroit, MI). The plates were incubated at 28°C for 24 hours. Independent bacterial colonies were counted.

Results and Discussion

The methods described above were effective in plant development from embroyogenic calli for both *Brachiaria* genotypes: CIAT 36062 and CIAT 36061. No macroscopic plant development differences were observed among control and antibiotic-treated plants. Two evaluations were made for the presence or absence of endophytic bacteria at 2 and 5 weeks after the last spray applications of antiobiotics. Bacterial colonies appeared from leaf, stem and root samples of control plants whereas samples from antibiotic-treated plants largely remained bacterial-free, although a few colonies appeared (Photos 10 and 11). We do not know yet whether these few

(Continued)