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

2.1 *Brachiaria* genotypes resistant to biotic and abiotic stresses

Highlights

- Sexual clones with resistance to spittlebug and Rhizoctonia foliar blight and high levels of Aluminum were selected.
- Modified breeding scheme: Assessment of merit of sexual clones will be assessed not only on their phenotypes but also of their hybrid progeny.

2.1.1 Selection of sexual clones

Contributors: C. Cardona, S. Kelemu, I.M. Rao, C.E. Lascano and J.W. Miles (CIAT)

Rationale

A broad-based, synthetic tetraploid sexual *Brachiaria* population is the core of the breeding program. This population has been cyclically selected over eight years (four cycles), mainly on spittlebug resistance and general agronomic performance. Stunning progress in spittlebug resistance has been achieved (see Section 2.2).

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. As the sexual population is upgraded, genetic gain is captured in apomictic genotypes by crossing selected sexual clones with elite apomicts.

Materials and Methods

Nearly 3,000 clones of the most recent cycle of the sexual population were established, as individual spaced plants, in two unreplicated field trials (CIAT-Quilichao and Matazul Farm, Puerto López) in May, 2003. Each clone was included in each of the two locations. Between May and October, the initial population was culled to 746 "pre-selections" on the basis of periodic visual inspection at the two field sites.

Between January and July 2004, These "pre-selections" were screened for spittlebug resistance in a two-stage process, including an unreplicated evaluation in which each clone was separately infested with each of the three species followed by a replicated evaluation of the best (apparently most resistant) 120 clones with the same three species.

Information on reaction to Al and Rhizoctonia were obtained simultaneously.

Results and Discussion

In July 2004, an isolated crossing block was set up by vegetative propagation of 42 selected clones established in 25-cm pots in three randomized complete blocks.

Given the large number of highly spittlebug-resistant sexual clones identified, in addition to establishing the sexual population crossing block, it was decided to cross the same 42 clones to *B. decumbens* cv. Basilisk.

2.1.2 Crossing elite sexual selections to *B. decumbens* CIAT 606

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

Rationale

The overall objective of the *Brachiaria* breeding project is to produce useful new, apomictic cultivars. *B. decumbens* CIAT 606 possesses many highly desirable characters, including edaphic adaptation and strongly stoloniferous growth habit. It is susceptible to spittlebug. Given a planned change in the breeding scheme no large field planting was scheduled for 2005. This 1-yr lull in field trials plus the high levels of multiple resistance found in many sexual clones motivated a decision to attempt to generate a large hybrid population to evaluate next year.

Given the large number of highly spittlebug-resistant sexual clones identified, in addition to establishing the sexual population crossing block, it was decided opportunistically to cross the same 42 clones to *B. decumbens* CIAT 606 to generate a large hybrid population segregating for apomixis.

Materials and Methods

An area of *B. decumbens* of approx. 1.25 ha was in the process of being prepared (mown, replanted, fenced) to serve as a "top-cross nursery" for activities in 2005. We simply accelerated this preparation and, in July 2004, were able to space plant (very approx. at 5 x 5m) four vegetative replicates of each of 120 pre-selected sexual plants in this plot of *B. decumbens*. In mid-August, when final results of the replicated, reconfirmation spittlebug screening were available, the plants of 78 clones were physically culled and the four replicates of each of the 42 most resistant clones left to cross with the surrounding *B. decumbens*.

Results and Discussion

Four hundred eighty (120 clones * 4 replicates) SX03 transplants were successfully established. Poor rainfall following planting has retarded growth of both the sexual plants and the surrounding *B. decumbens* until recent rains in September. We anticipate harvesting several thousand viable hybrid seeds from the 168 (42 clones * 4 replicates) sexual plants that remain following culling. The resulting hybrid population will be planted in 2005 in space-planted field trials in two locations.

2.1.3 Establishment and selection in the field of new hybrid clones (3000 SX x AP— Series BR03/04) clones

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 to selected apomictic accessions to generate novel apomictic hybrids.

Materials and Methods

In the sexual population planted in 2001, two clones with exceptional spittlebug resistance were identified. These two clones were each crossed during 2003, to four apomictic accessions to produce eight hybrid families. A small population of these hybrids (~420) was produced in 2003 (series BR03) and a very preliminary spittlebug screening (3 spp., 2 reps) was done

late that year. A much larger seedling population was established in 2004 (series BR04). A total of thirty-six BR03 seedlings with promising initial reaction to spittlebugs and 2,131 BR04 seedlings (and appropriate checks) were propagated to establish two unreplicated, space-planted field trials trials at CIAT Quilichao and at the Matazul Farm (Puerto López). Hybrids are from eight families, formed by crossing two spittlebug-resistant sexual selections (series SX01) by four apomictic *Brachiaria*.

Results and Discussion

The hybrid population has been culled down to 492 clones based on several visual assessments. Seed will be harvested (for progeny tests) and the "pre-selected" plants propagated from the field at CIAT-Quilichao to CIAT-Palmira during October for detailed screenings for spittlebug, aluminum, Rhizoctonia, and quality.

2.1.4 Recombination of sexual hybrids (7) with CIAT 16320 (Series BR02): Reconfirmation of reproductive mode and screening for reaction to Rhizoctonia

Contributors: J.W. Miles; S. Kelemu; J. Muñoz (CIAT)

Rationale

Our mainstream tetraploid sexual breeding population is generally very susceptible to Rhizoctonia foliar blight. The *B. brizantha* accession 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. Selected hybrids were progeny tested. Seven apparently sexual hybrids with an intermediate level of resistance were identified. By recombining these few genotypes to create a large segregating population, we expect 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 is being hand harvested as it matures.

Results and Discussion

Open-pollinated seed is being harvested currently (Sept. 2004), to establish a large population in 2005 with the expectation of being able to isolate tetraploid sexual clones with high levels of resistance.

2.1.5 Seed multiplication from apparently apomictic hybrids (36- Series BR02) for evaluation in Colombia, Mexico, and Thailand

Contributors: J.W. Miles, A. Betancourt, J. Muñoz, P. Horne, and I.M. Rao (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.

Materials and Methods

A new cohort of pre-selected, experimental apomictic hybrids is generated each selection cycle (generally every two years). From several thousand new sexual-by-apomictic hybrids first planted in Colombia in 2002 (series BR02), 36 hybrids were selected on vigor and apomictic reproduction. After assessing seed fill, 21 of these selections were culled, so that 15 lines still are under consideration. The main selection criteria have been vigor and seed fill, as well as spittlebug reaction. Given the absence of pest spittlebug species in tropical Asia, even susceptible genotypes merit testing there.

Seed of 15 selections, harvested from progeny tests conducted at CIAT in 2003 was mostly used in an unsuccessful attempt to produce sufficient plants for an agronomic trial to be planted in the Colombian Llanos. Small amounts of remnant seed of 9 of the 15 selections was sent to Peter Horne (CIAT-Laos) for initial observation trials in Laos and Thailand. All 15 selections were vegetatively propagated during 2004 and seed multiplication plots established at CIAT-Popayán at mid-year. We anticipate having sufficient seed of these lines for wider distribution (Asia, Mexico) during 2005.

Results and Discussion

Small seed multiplication plots (50 spaced plants) of 15 BR02 selections have been established at Popayán.

2.1.6 Establishment of *B. decumbens* CIAT 606 at CIAT-Popayán for formation of testcrosses in 2005

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

Rationale

The *Brachiaria* breeding program has relied to date on cyclic intra-population selection in a broad-based, synthetic, tetraploid sexual population. Since the objective of the program is to develop superior hybrids, and given the improvements achieved to date in the sexual breeding population (e.g., greatly improved spittlebug resistance), a decision has been taken to modify the population improvement scheme such that assessment of the merit of sexual clones will be based not only on their own phenotype, but also on that of their testcross (hybrid) progeny. A single apomictic ("male") tester will be crossed to candidate sexual ("female") clones in the field to generate the testcross progenies.

Materials and Methods

An area of approx. 1.25 ha of *B. decumbens* was identified at CIAT-Popayán (where *B. decumbens* flowers continuously throughout the year). The area was fenced and mown. We are already using this area in an (opportunistic) attempt to produce a large hybrid population with the 42 SX03 clones selected for resistance to three spittlebug species.

These sexual plants will be eliminated after seed harvest (early 2005), and 500 SX05 plants (resulting from recombination of SX03 selections) spaced planted (at 5 x 5 m) to be pollinated by *B. decumbens*, thus forming 500 testcross families for testing during 2006 and 2007.

Results and Discussion

The "testcross crossing block" is established and functioning. There should be no obstacle to producing the desired testcross families next year, particularly with opportune establishment of the spaced sexual plants with first semester rainy season.

2.1.7 Agronomic evaluation in Mexico apomictic hybrids from material evaluated in 2002 and 2003: Selection of candidate cultivars

Contributors: E. Guzmán (Papalotla), and J.W. Miles (CIAT)

Rationale

Approx. 125 "pre-selections" from nearly 4,000 hybrids (series MX02) established at Semilla Papalotla's Santa Elena Farm were progeny-tested during 2003 (in Mexico). Approx 60 of these appear to be highly apomictic. A number of selections were culled on poor seed fill, and 34 selected for further evaluation. Seed of these 34 apomictic selections was received at CIAT in January this year.

Evaluations of their reaction to spittlebugs, both Mexican species (in Mexico) and Colombian species (at CIAT), as well as initial seed increase have begun.

Materials and Methods

Seed was harvested from apparently apomictic progeny rows at Sta. Elena Farm (Mexico) during 2003. From seed samples of 34 progenies received at CIAT, seedlings were established and propagated for assessment of spittlebug reaction. Following quarantine clearance by Colombian authorities, 20-plant initial seed multiplication field plots were established at CIAT in September.

Results and Discussion

We anticipate having sufficient seed of these promising experimental lines for further field assessment in Colombia and SE Asia during 2005.

2.2 Screening *Brachiaria* genotypes for spittlebug resistance

Highlights

- Mass rearing of *Zulia carbonaria* and *Prosapia simulans* were successfully established.
- New sexual hybrids with high levels of antibiosis resistance to *Aeneolamia varia*, *A. reducta* and *Zulia carbonaria* were identified.
- 11 apomictic hybrids "pre-selected" for Mexican conditions showed high levels of antibiosis resistance to *Prosapia simulans*, one of the most important spittlebug species affecting *Brachiaria* in Mexico and Central America.
- The resistance of 22 sexual hybrids to *A. varia*, *Z. carbonaria*, *Z. pubescens* and *Mahanarva trifissa* was confirmed in replicated field trials conducted in Caquetá.

2.2.1 Continuous mass rearing of spittlebug species in Palmira and Macagual

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.

2.2.2 Selection of *Brachiaria* genotypes resistant to spittlebug

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

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

Rationale

Assessment of resistance to spittlebugs is an essential step in the process of breeding superior *Brachiaria* cultivars at CIAT. In 2004, intensive screening of selected hybrids was conducted under greenhouse and field conditions.

Materials and Methods

Screenings for resistance were conducted with *Aeneolamia varia, A. reducta, Zulia carbonaria,* 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

A set of 731 pre-selected sexual (SX03) hybrids was simultaneously screened for resistance to *A. varia*, *A. reducta*, and *Z. carbonaria*. We used one rep per hybrid per insect species. For comparison, we used 16 well-known checks replicated 10 times per insect species. In terms of damage scores, 78.3%, 84.3%, and 74.9% of the hybrids were rated as resistant to *A. varia*, *A. reducta*, and *Z. carbonaria*, respectively (Table 5). After percentage survival was recorded, 120 hybrids combining low damage levels and high levels of antibiosis resistance were selected for reconfirmation tests. These were conducted using five replications per genotype per insect species. Results (Table 6) clearly indicated 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 5. Frequency distribution (percentages) of resistance reactions in a set of 731sexual *Brachiaria* hybrids screened for resistance to three spittlebug species.

Category	*Aeneolamia varia*	*Aeneolamia reducta*	*Zulia carbonaria*	All three species
Resistant	64.2	75.2	59.1	39.5
Intermediate	14.1	9.1	15.8	33.9
Susceptible	21.7	15.7	25.1	26.6

The rapid progress made in incorporating resistance to spittlebug is also illustrated in Figure 3. There has been a steady increase in the frequency of resistant genotypes as a result of recurrent selection through cycles.

As part of on-going studies on mechanisms of resistance to spittlebug species of economic importance in Mexico, we screened 34 hybrids for resistance to *Prosapia simulans*. These hybrids had been pre-selected in Mexico for good adaptation and desirable agronomic characteristics. Using a level of infestation of six nymphs per plant and 10 replications, the hybrids were compared with four accessions, and two susceptible and two resistant checks.

Results shown in Table 7 indicate that 11 hybrids have antibiosis resistance to *P. simulans*. This

Table 6. Levels of resistance to three spittlebug species in selected sexual *Brachiaria* hybrids

Genotype	Damage scores			Percentage nymph survival		
	Aeneolamia varia	*Aeneolamia reducta*	*Zulia carbonaria*	*Aeneolamia varia*	*Aeneolamia reducta*	*Zulia carbonaria*
			Elite hybrids			
SX03/2483	1.0	1.0	2.4	8.0	0.0	26.7
SX03/2226	1.0	1.0	1.7	3.3	6.7	16.7
SX03/2061	1.3	1.0	1.3	16.7	0.0	16.7
SX03/4043	1.3	1.0	1.2	10.0	10.0	6.7
SX03/3744	1.0	1.4	1.6	13.3	6.7	3.3
SX03/4351	1.1	1.4	1.4	20.0	13.3	10.0
SX03/3882	1.0	1.3	1.2	13.3	20.0	10.0
SX03/2053	1.0	1.0	2.4	20.0	20.0	33.3
SX03/1100	1.0	1.5	2.7	25.0	21.7	13.3
SX03/4224	1.4	1.2	1.2	20.0	23.2	4.2
SX03/0282	1.0	1.0	1.5	30.0	6.7	6.7
SX03/0770	1.3	1.3	1.2	30.0	10.0	3.3
SX03/1090	1.3	1.7	1.7	30.0	17.3	13.3
SX03/1408	1.0	1.2	1.2	26.7	23.3	13.3
SX03/2784	1.5	1.1	2.0	26.7	16.7	0.0
			Checks			
CIAT 36062[a]	1.0	1.4	2.2	25.0	21.7	60.0
SX01NO/0102 [a]	1.6	1.0	2.2	26.7	10.0	20.0
CIAT 0606 [b]	4.6	3.8	4.0	91.7	75.0	53.3
BRX-44-02 [b]	4.8	4.6	3.8	83.3	80.0	68.3

[a] Resistant check; [b] Susceptible check.

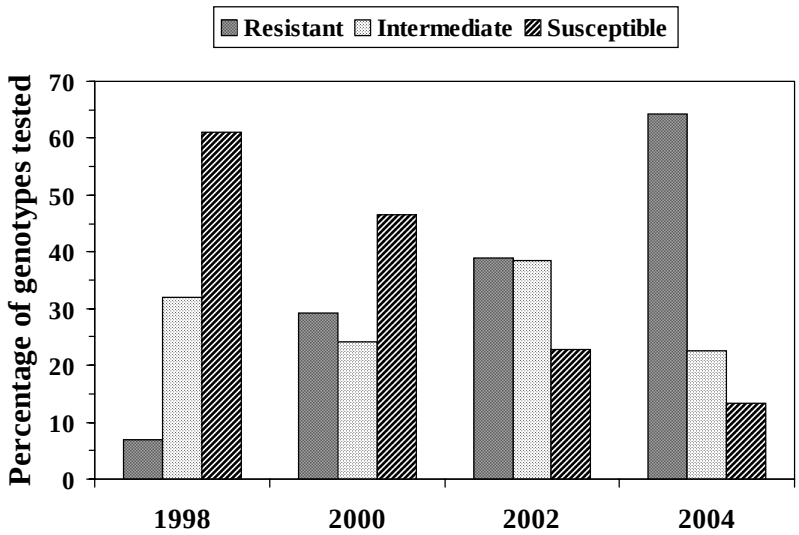

Figure 3. Progress in the incorporation of resistance to *Aeneolamia varia* in *Brachiaria*; note the steady increase in the frequency distribution of resistance genotypes and the decline in the frequency of susceptible genotypes as a result of continuous cycles of selection.

information will be crossed with that obtained in Mexico with the species *Aeneolamia albofasciata* and *A. postica* (part of Ulises Castro's M. Sc. thesis on mechanisms of resistance to Mexican species). In progress is the evaluation for resistance to *A. varia*, *A. reducta* and *Z. carbonaria* of 422 apomictic hybrids derived from crosses between the highly resistant sexual hybrid SX01NO/0102 and *B. decumbens* 'Basilisk' and other susceptible genotypes. The main purpose of this study is to identify patterns of segregation of resistance for each of the spittlebug species involved. Results will be reported in 2005.

Table 7. Levels of resistance to *Prosapia simulans* in *Brachiaria* hybrids pre-selected for Mexican conditions.

Genotype	Damage scores	Percentage nymph survival	Rating	Genotype	Damage scores	Percentage nymph survival	Rating
		Hybrids				Hybrids	
MX 1905	1.1	3.3	Resistant	MX 1660	2.9	47.9	Susceptible
MX 1561	1.3	5.6	Resistant	MX 1769	3.0	3.3	Resistant
MX 3056	1.6	1.7	Resistant	MX 1548	3.1	50.0	Susceptible
MX 1423	1.8	1.7	Resistant	MX 1565	3.1	31.7	Susceptible
MX 1880	1.8	29.6	Intermediate	MX 2775	3.1	40.7	Susceptible
MX 3641	2.0	25.0	Intermediate	MX 1638	3.2	56.7	Susceptible
MX 2295	2.2	10.0	Resistant	MX 3861	3.2	66.7	Susceptible
MX 1809	2.2	16.7	Resistant	MX 2273	3.2	6.2	Resistant
MX 1388	2.2	16.7	Resistant	MX 2090	3.2	23.3	Susceptible
MX 3567	2.2	26.7	Intermediate	MX 1614	3.3	46.7	Susceptible
MX 2552	2.2	33.3	Intermediate	MX 3626	3.4	71.7	Susceptible
MX 1788	2.3	31.5	Susceptible	MX 3582	3.7	75.9	Susceptible
MX 3426	2.3	26.7	Intermediate			Checks	
MX 1263	2.4	42.6	Susceptible	CIAT 16827	1.2	5.0	Resistant
MX 3731	2.4	18.5	Resistant	CIAT 26110	1.8	21.7	Resistant
MX 2135	2.5	48.3	Susceptible	CIAT 36087	1.8	1.7	Resistant
MX 2531	2.6	50.0	Susceptible	CIAT 36061	3.1	27.1	Intermediate
MX 1942	2.6	41.7	Susceptible	CIAT 36062	1.8	0.0	Resistant check
MX 3850	2.7	50.0	Susceptible	CIAT 06294	1.6	6.7	Resistant check
MX 2783	2.7	60.0	Susceptible	CIAT 0606	3.6	50.0	Susceptible check
MX 3213	2.8	9.2	Resistant	BRX-44-02	4.0	68.3	Susceptible check
				LSD 5%	1.97	12.5	

2.2.2.2 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 an artificial infestation technique that allows us to properly identify resistance under field conditions. The purpose of field evaluations is 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, 12 major screening trials (four with *A. varia*, four with *Zulia carbonaria*, two with *Z. pubescens*, and one with *Mahanarva trifissa*) were conducted in Caquetá in 2004.

The main purpose of these trials was to reconfirm resistance in 22 sexual hybrids (SX01) previously selected in Palmira under greenhouse conditions.

Results and Discussion

As shown in Table 8, virtually all of the sexual hybrids showed adequate levels of field resistance to all four species tested. Consistently, average damage scores were significantly lower than those obtained with the susceptible checks, CIAT 0606 and BRX-44-02. Tiller ratios for the sexual hybrids were significantly higher than those of susceptible checks, suggesting that antibiosis resistance present in the hybrids protects the plants from intense insect damage, allowing the plant to grow and produce new tillers. On the contrary, susceptible plants lose tillers As in previous occasions there were significant ($P < 0.01$) negative correlations between damage scores and tiller ratios (r = -0.844 for *A. varia*, -0.887 for *Z. carbonaria*, -0.785 for *Z. pubescens*, and -0.697 for *M. trifissa*). This means that damage scores are useful in predicting tiller losses resulting from intense insect damage.

One of the commercial checks (CIAT 36087, 'Mulato 2') was resistant. Surprisingly, the commercial check CIAT 36061 ('Mulato'), which

Table 8. Damage scores and tiller ratios obtained with 22 selected sexual *Brachiaria* hybrids and checks tested for resistance to *Aeneolamia varia* (Av), *Zulia carbonaria* (Zc), *Z. pubescens* (Zp), and *Mahanarva trifissa* (Mt) under field conditions.

Genotype	Damage scores				Tiller ratios[a]			
	Av	Zc	Zp	Mt	Av	Zc	Zp	Mt
SX01/NO/0067	1.8	2.1	1.6	1.3	1.09	1.21	1.29	1.62
SX01/NO/0102	2.0	1.7	1.6	1.4	1.29	1.76	1.92	1.60
SX01/NO/0159	1.6	1.8	1.5	1.3	1.37	1.66	1.53	1.82
SX01/NO/0233	2.7	1.9	1.8	1.9	0.94	1.29	1.43	1.33
SX01NO/0263	2.0	1.9	1.6	1.2	1.34	1.48	1.55	1.76
SX01/NO/0446	1.8	1.8	1.6	1.1	1.09	1.26	1.38	1.47
SX01/NO/0878	1.9	1.8	1.8	1.7	1.38	1.44	1.65	1.71
SX01/NO/1090	1.9	1.9	1.8	1.2	1.06	1.61	1.26	1.24
SX01/NO/1175	1.8	2.0	1.8	1.3	1.12	1.26	1.35	1.43
SX01NO/1186	2.1	2.1	1.9	1.4	1.22	1.46	1.34	1.18
SX01NO/1710	1.7	2.0	1.6	1.5	1.33	1.52	1.46	2.72
SX01/NO/2017	1.9	2.0	1.6	1.3	1.48	1.39	1.72	1.83
SX01/NO/2420	1.9	1.8	1.6	1.7	1.35	1.57	1.46	1.18
SX01/NO/2619	1.7	1.7	1.7	1.2	1.11	1.46	1.69	1.49
SX01/NO/3168	1.8	1.8	1.8	1.4	1.10	1.33	1.52	1.38
SX01/NO/3178	1.9	1.8	1.8	1.3	1.12	1.41	1.34	1.47
SX01/NO/3390	2.1	2.3	1.8	1.9	0.92	1.14	1.30	1.14
SX01/NO/3439	1.9	1.9	1.6	1.0	1.25	1.61	1.58	2.22
SX01/NO/3615	1.7	1.8	1.7	1.4	1.22	1.54	1.32	1.47
SX01/NO/4506	2.1	2.1	1.7	1.6	0.92	1.17	1.29	1.44
SX01/NO/4785	1.9	2.0	1.6	1.1	1.22	1.27	1.57	1.83
SX01/NO/4861	1.7	1.7	1.7	1.6	1.28	1.64	1.62	1.74
Mean 22 hybrids	1.9b	1.9b	1.7b	1.4b	1.19b	1.43b	1.48a	1.59a
CIAT 36087	2.0	1.6	1.6	1.4	1.31	1.60	1.50	1.50
CIAT 36061	1.7	1.5	1.3	1.3	1.44	2.00	1.59	1.71
Mean commercial checks	1.8b	1.6bc	1.4c	1.3b	1.37a	1.80a	1.54	1.60a
CIAT 36062	1.6	1.3	1.4	1.1	1.64	1.92	1.69a	1.89
CIAT 6294	1.1	1.4	1.2	1.1	1.22	1.58	1.33	1.46
Mean resistant checks	1.3c	1.3c	1.3c	1.1b	1.43a	1.75a	1.51a	1.67a
CIAT 0606	4.0	3.1	2.9	3.7	0.37	0.62	0.59	0.46
BRX-44-02	4.5	3.5	3.4	4.0	0.30	0.64	0.70	0.54
Mean susceptible checks	4.2a	3.3a	3.1a	3.8a	0.33c	0.63c	0.64b	0.50b

a. Tillers per plant at the end of the infestation process/Tillers per plant at the beginning of the infestation process
Means of 10 reps per genotype per species, 4 trials in the case of *A. varia* and *Z. carbonaria*, two trials with *Z. pubescens* and one trail 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.

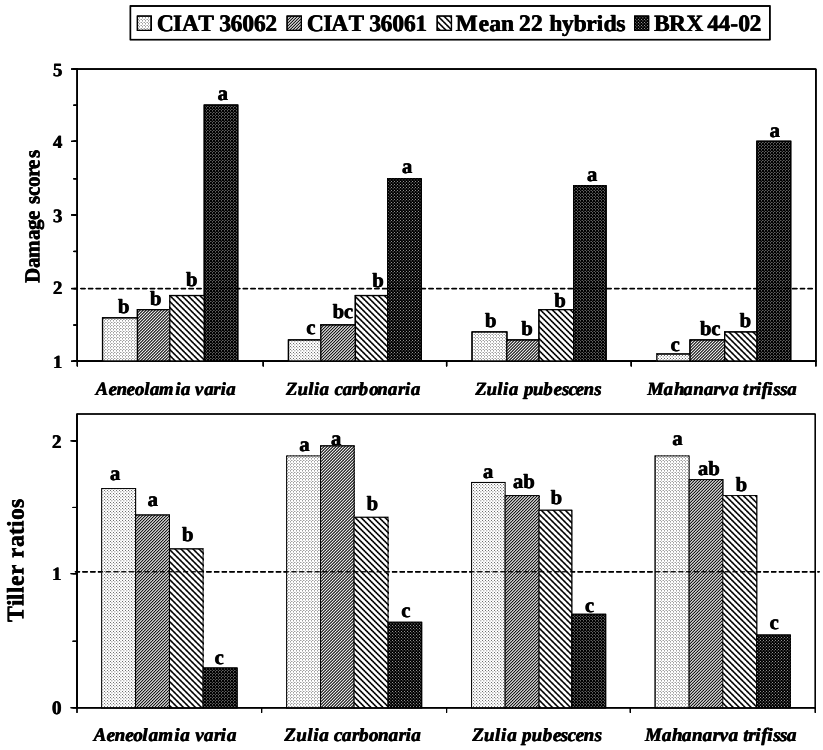

Figure 4. Resistance to four spittlebug species in selected *Brachiaria* genotypes tested under field conditions. Dotted lines represent cut-off points for resistance rating and selection. Within a given spittlebug species, bars with the same letter are not significantly different at the 5% by LSD. Each species analyzed separately.

is not antibiotic to any spittlebug species, showed a very interesting level of field tolerance both in terms of damage scores and tiller ratios (Figure 4). Damage scores obtained with the 22 sexual hybrids and assorted *Brachiaria* accessions in the greenhouse correlated very well (r = 0.76; $P < 0.01$) with damage scores recorded in the field (Figure 5). This is further proof that the technique we are using to screen for resistance in the field is a reliable one to reconfirm resistance detected under greenhouse conditions.

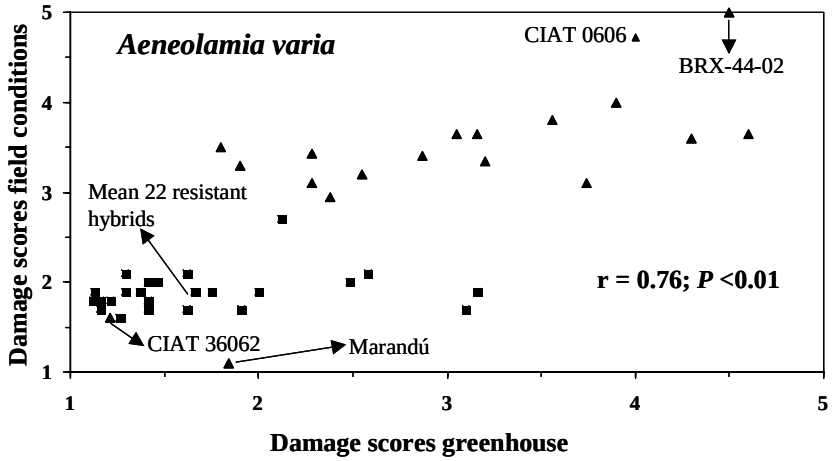

Figure 5. Damage scores obtained with selected sexual *Brachiaria* hybrids (%) and accessions (%) tested for resistance to *Aeneolamia varia* under greenhouse and field conditions.

2.3 Identify host mechanisms for spittlebug resistance in *Brachiaria*

Highlights

- The antibiosis to nymphs in the resistant *Brachiaria* hybrid CIAT 36062 causes significant sub-lethal effects on the reproductive biology of resulting adults.

- The high immature mortality and sub-lethal effects of antibiosis on resulting adults caused by the resistant *Brachiaria* hybrid CIAT 36062 have a major impact on the demography of *A. varia*.

2.3.1 Effect of host plant resistance on the demography of *Aeneolamia varia*

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

Rationale

Varying levels of antibiosis resistance to nymphs of several spittlebug species have been well characterized in a number of resistant *Brachiaria* genotypes. The effects of antibiosis on the biology of nymphs have also been studied. Not much 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. 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*. 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 *A. varia* were conducted. For this, 18 life tables (nine fecundity, nine complete) were constructed. Treatment combinations are shown in Table 9.

Table 9. Treatment combinations to study possible sub-lethal effects of intermediate and high levels of nymphal antibiosis on adults of *Aeneolamia varia*.

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

[a] BRX44-02 is a highly susceptible accession; CIAT 6294 (an accession) and CIAT 36062 (a resistant hybrid) possess intermediate and high levels of antibiosis resistance to nymphs of *A. varia*, respectively.

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.

Results and Discussion

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

Both resistant genotypes caused significant effects on the demography of *A. varia*. For simplicity, we will limit the discussion to the results obtained with the most resistant genotype, CIAT 36062. In general, rearing of nymphs of *A. varia* 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 10). Females feeding on the susceptible genotype BRX-44-02 weighted significantly more than those feeding on the resistant genotype. Age-specific survival and age-specific fecundity curves for *A. varia* adults are presented in Figure 6. 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 CIAT 36062 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 *A. varia* adults were significantly affected by the antibiotic effect of CIAT 36062 on the nymphs (Table 11).

Females originating from nymphs reared on 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 reared on the susceptible genotype. We conclude that antibiosis to nymphs in the resistant *Brachiaria* hybrid CIAT 36062 causes significant sub-lethal effects on the reproductive biology of resulting adults.

Table 10. Life history parameters of *Aeneolamia varia* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (BRX 44-02) or resistant (CIAT 36062) *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		
BRX 44-02 (S)	BRX 44-02 (S)	5.73a	3.79a	130.4ab	93.0a
BRX 44-02 (S)	CIAT 36062 (R)	4.90b	3.66ab	147.8a	92.6a
CIAT 36062 (R)	BRX 44-02 (S)	5.46ab	3.28b	108.0bc	80.6b
CIAT 36062 (R)	CIAT 36062 (R)	4.37c	3.10c	86.1c	80.4b

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

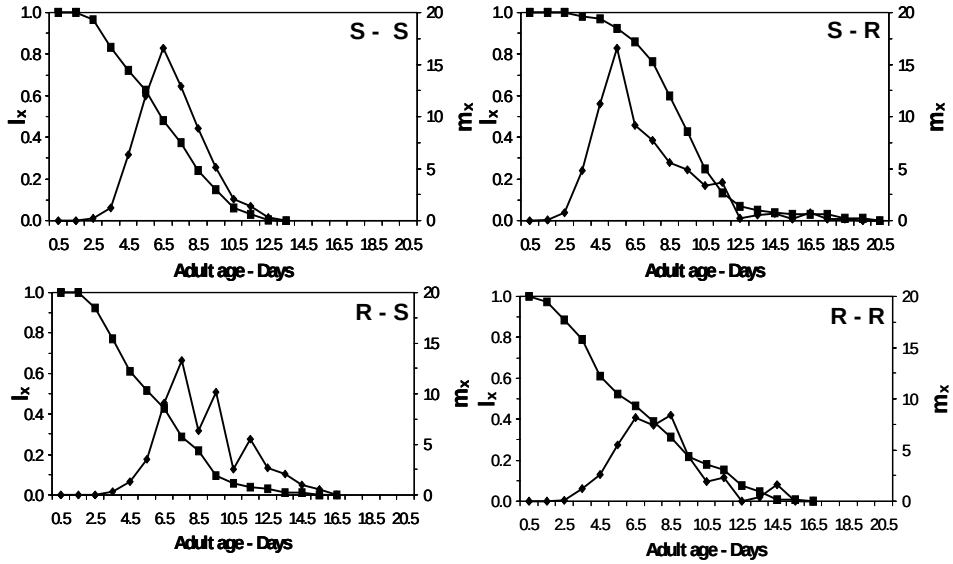

Figure 6. Age-specific survival (l_x) (%) and age-specific fecundity (m_x) (f&) curves for adults of *Aeneolamia varia* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (BRX 44-02); R, resistant genotype (CIAT 36062).

Table 11. Fecundity life-table statistics for *Aeneolamia varia* adults as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (BRX 44-02) or resistant (CIAT 36062) *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)	Mean generation time (T)	Finite rate of increase (λ)
BRX 44-02 (S)	BRX 44-02 (S)	65.8a	0.724a	5.8b	2.06a
BRX 44-02 (S)	CIAT 36062 (R)	69.5a	0.747a	5.7b	2.11a
CIAT 36062 (R)	BRX 44-02 (S)	52.5b	0.576b	6.9a	1.77b
CIAT 36062 (R)	CIAT 36062 (R)	42.2b	0.574b	6.3a	1.80b

[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 *Aeneolamia varia*

To measure the total impact of antibiosis resistance on the demography of *A. varia*, 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 *A. varia* adults are presented in Figure 7. The antibiosis to nymphs present in the resistant genotype CIAT 36062 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 of about 15 days 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.

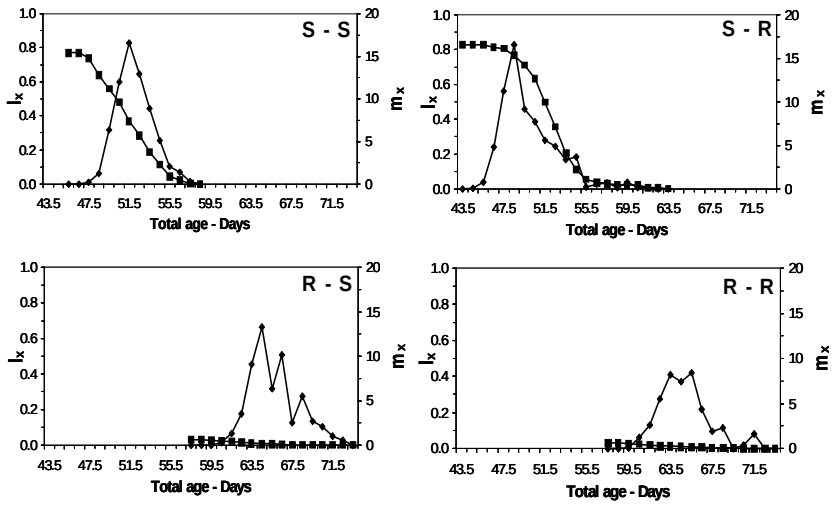

Figure 7. Age-specific survival (l_x) (%) and age-specific fecundity (m_x) (f&) curves for *Aeneolamia varia* as affected by all possible combinations of food substrate for adults and nymphs. First initial in letter combinations indicates the food substrate for nymphs followed by the initial for the food substrate for resulting adults. S, susceptible genotype (BRX 44-02); R, resistant genotype (CIAT 36062).

As a result of high immature mortality and sub-lethal effects on resulting adults, all demographic statistics of the *A. varia* population tested were significantly affected by the antibiosis present in CIAT 36062 (Table 12).

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 9.5 to 10.3% whereas those on the resistant genotype would grow by 0.4-0.8% (Table 12). We conclude that high immature mortality and sub-lethal effects of antibiosis on resulting adults caused by the resistant *Brachiaria* hybrid CIAT 36062 have a major impact on the demography of *A. varia*.

Table 12. Life-table statistics for *Aeneolamia varia* as affected by all possible combinations of rearing immature stages and feeding resulting adults on susceptible (BRX 44-02) or resistant (CIAT 36062) *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)	Mean generation time (T)	Finite rate of increase (λ)
BRX 44-02 (S)	BRX 44-02 (S)	50.7a	0.090b	43.3b	1.095b
BRX 44-02 (S)	CIAT 36062 (R)	57.7a	0.098a	41.5c	1.103a
CIAT 36062 (R)	BRX 44-02 (S)	1.6b	0.008c	54.4a	1.008c
CIAT 36062 (R)	CIAT 36062 (R)	1.3b	0.004c	53.8a	1.004c

[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).

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

Highlights

- Developed a visual rating system for screening for resistance to Rhizoctonia foliar blight disease.
- Identified *Brachiaria* genotypes with resistance to *Rhizoctonia solani*.

2.4.1 Developing a rating scale for rhizoctonia foliar blight disease of *Brachiaria*

Contributors: Carolina Zuleta, Gustavo Segura, John Miles and Segenet Kelemu (CIAT)

Rationale

Disease management through the use of host resistance, when available, remains to be the most preferred, practical and environmentally friendly method. Differences in reaction to *Rhizoctonia solani* exist in genotypes of *Brachiaria.* The assessment of such differences requires not only a reproducible inoculation method but also a reliable rating scale. The ability to uniformly induce disease and measure resistance accurately is essential in a breeding program for developing resistant cultivars. To meet this need, we developed a simple 0-5 disease rating scale.

Materials and Methods

Brachiaria materials*:* Eighty-seven *Brachiaria* genotypes that showed varying degrees of disease reaction were used. Fully developed leaves of same maturity were detached from each of the 87 genotypes of the BR-02 series (codes given by the IP-5 forage breeding program). In addition, control resistant (CIAT 16320) and susceptible (CIAT 36061) controls were included in all the tests.

Fungal inoculum and inoculation: Isolate *R. solani* originally collected from CIAT 36061 was used as inoculum. Sclerotia of R. solani isolates originally isolated from species of Brachiaria were germinated on potato dextrose agar (PDA) at 28 °C. Mycelial discs (6 mm in diameter) were cut out of the actively growing 2 days old PDA culture for inoculation. Fully developed young leaves collected from each of the 87 *Brachiaria* genotypes were trimmed to about 12 centimeters in length. Two leaves were placed in a Petri dish of 15 cm in diameter containing Whatman #3 filter paper soaked with 5.5 ml sterile water. A mycelial disc was placed on the center of each leaf and the Petri dish was sealed with parafilm and incubated at room temperature (approximately 25 °C) on a large table in the lab. Tests were conducted in a completely randomized design with three replications.

Evaluation: The leaf samples were photographed 96 hours after inoculations. The disease areas were defined, as shown in Photo 2, using Photo Editor and Paint Brush of Microsoft. Once the images of disease areas were defined (white areas diseased, black areas healthy; see Photo 2), they were analyzed using WinRHIZO software (Regent Instruments Inc.) to determine percentage of leaf area infected.

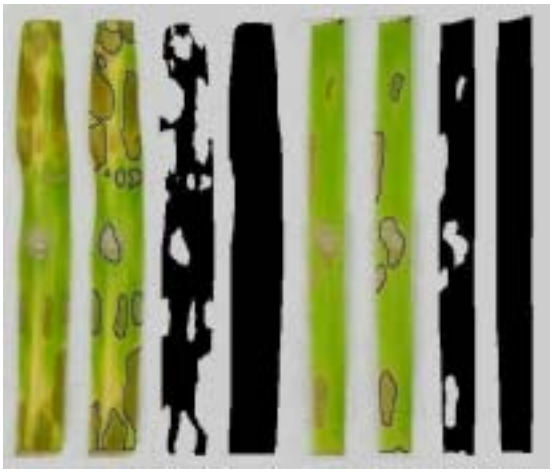

Photo 2. Defining precise percentage of rhizoctonia foliar blight diseased leaf area in species of *Brachiaria* (shown also in AR2003).

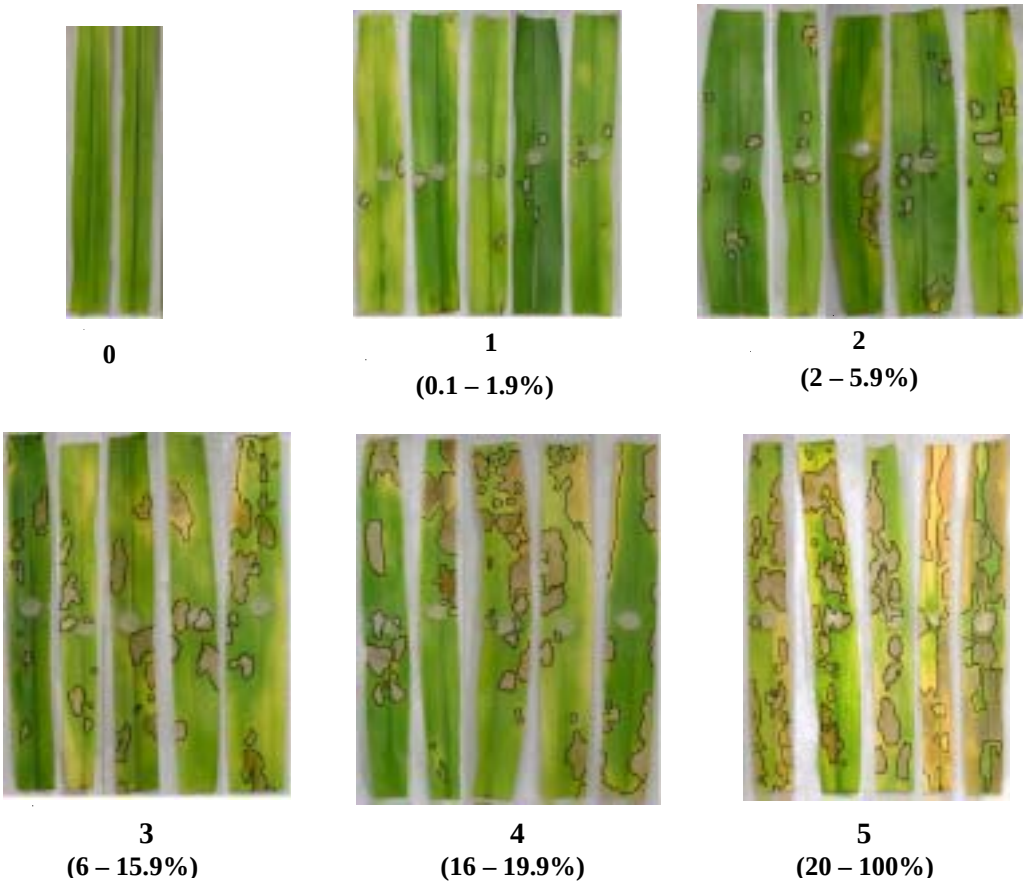

Photo 3. A visual rating system for rhizoctonia foliar blight disease in species of Brachiaria, corresponding to ranges of lesion areas, where 0 = 0%, 1 = 0.1-1.9%, 2 = 2-5.9%, 3 = 6-15.9%, 4 = 16-19.9%, and 5 = 20-100%.

Results and Discussion

After 96 hours of incubation and disease symptom development, pictures of leaves demonstrating various levels of disease reaction were taken and lesion areas calculated using the WinRHIZO software (Regent Instruments Inc.) as described under *materials and methods* section. A visual rating system corresponding to ranges of lesion areas was developed, where 0 = 0%, 1 = 0.1-1.9%, 2 = 2-5.9%, 3 = 6-15.9%, 4 = 16-19.9%, and 5 = 20-100% (Photo 3).

Because results of visual disease rating systems can vary from person to person (what seems a rating of 2 by one person may be rated as 1 by another), we further verified the accuracy of the rating scale. Two people (one who intimately worked to develop the scale, but not the second one) used the scale to evaluate the same materials independently. The two evaluators came up with similar results (with a correlation of 84%). In addition, the data independently generated by the two evaluators had correlations of 83% and 87%, respectively, with the actual precise disease lesion areas.

Using this newly developed rating scale of 0-5, 87 *Brachiaria* genotypes of the codes BR02- series and resistant control CIAT 16320 and susceptible control CIAT 36061 were evaluated. Ten of these (BR02-1995, -1811, -1919, -1667, -1917, -1717, -1805, -1973, - 465, -1968) and CIAT 16320 scored less than 3 (with BR02-1995 with the lowest score i.e. high level of resistance). The new rating scale coupled with the detached leaf inoculation method have enhanced our screening efficiency enabling us to evaluate hundred of plant materials in a relatively short period of time.

2.4.2 Screening *Brachiaria* genotypes for resistance to *Rhizoctonia* foliar blight disease

Contributors: Carolina Zuleta, Gustavo Segura, Ximena Bonilla, John Miles and Segenet Kelemu

Rationale

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

Rhizoctonia solani complex consists of an economically important group of soil-borne pathogens that infect various plant species worldwide. The fungus is a basidiomycete and does not produce any asexual spores (conidia). Occasionally the fungus produces sexual basidiospores. The pathogen survives in soil in the form of thick-walled mycelia commonly called sclerotia, associated with organic debris. These sclerotia can germinate and produce hyphae that can infect host plants.

In *Brachiaria*, initial symptoms 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 resulting in entire leaf or plant death. As the plant cells die due to infection, the fungal hyphae continue to grow and colonize dead tissue, eventually forming sclerotia for another cycle of infection. The fungus infecting *Brachiaria* belongs to anastomosis group AG-1 (Kelemu et al. 1995. Tropical Grasslands 29:257-262). (There are about 12 anastomosis groups described in *R. solani*). The emergence of *R. solani* as an important *Brachiaria* pathogen is perhaps attributed to the development of high quality, high tillering Brachiarias and an increase in fertilization.

Host resistance, when available, is the most practical and cheapest means of disease control. The ability to uniformly induce disease and measure resistance accurately is crucial in a breeding program for developing resistant cultivars. The objective of this work is to identify resistant materials among *Brachiaria* genotypes

Materials and Methods

Storage of isolates: Fungal sclerotia produced either in PSY broth (20 g peptone, 20 g sucrose, 5 g yeast extract , 1 L deionized water) or on potato dextrose agar (PDA) were air-dried on sterile Whatman filter paper in a laminar flow hood. Dry sclerotia were placed in sterile glass tubes and stored at 4 °C.

Inoculum production and inoculation on detached leaves in Petri dishes*:* Sclerotia of *R. solani* isolates originally isolated from species of *Brachiaria* were germinated on potato dextrose agar (PDA) at 28 °C. Mycelial discs (6 mm in diameter) were cut out of the actively growing 2 days old PDA culture for inoculation. Fully developed young leaves were collected from each of the 745 *Brachiaria* genotypes evaluated, and trimmed to about 12 centimeters in length. Two leaves were placed in a Petri dish of 15 cm in diameter containing Whatman #3 filter paper soaked with 5.5 ml sterile water. A mycelial disc was placed on the center of each leaf and the Petri dish was sealed with parafilm and incubated at room temperature (approximately 25 °C) on a large table in the lab (Photo 4).

Inoculation on plants in greenhouse tests: Mycelial discs were cut out of actively grown PDA cultures as described above. The 73 selected materials from the detached leaf tests plus resistant and susceptible controls were used. A disc was placed in contact with each plant stem at the base and wrapped with parafilm (Photo 5). Inoculated plants were kept in the greenhouse at about 28 ^{o}C, high humidity (≥ 90 %), using a randomized complete block design and 4 replications over time. Plants were evaluated 15 days after inoculation.

Photo 4. Large -scale artificial inoculation of detached *Brachiaria* leaves placed in Petri dishes of 15 cm in diameter containing wet Whatman #3 filter paper. A mycelial disc was placed on the center of each leaf and the Petri dish was sealed with parafilm and incubated at room temperature (approximately 25 °C) on a large table in the laboratory.

Evaluation of resistance: Detached leaves were collected from 745 *Brachiaria* genotypes of SX03-series. CIAT 16320 and CIAT 36061 were included as resistant and susceptible controls, respectively, in all the experiments. The samples were randomized and experiments repeated three times over time. The plant materials were evaluated for disease reaction 96 hours after inoculation, using a 0-5 rating scale we developed, where 0 = 0%, 1 = 0.1-1.9%, 2 = 2-5.9%, 3 = 6-15.9%, 4 = 16-19.9%, 5 = 20-100% (Photo 3).

Results and Discussion

The 745 genotypes evaluated differed in their reactions to *R. solani*. Ninety percent of these (672 genotypes) exhibited severe foliar blight symptoms. Ten percent of the plant materials (73) showed average disease ratings below 3.0. Of the materials with average ratings above 3.0, 323 had average rating values between 3.0 and 3.7. The remaining 349 hybrids scored with ratings above 3.7.

To further verify the results, the 73 genotypes with ratings of less than 3.0 along with the susceptible check (CIAT 36061) and resistant control (CIAT 16320) were re-evaluated with 10 repetitions in a completely randomized design. The repetitions were increased to 10 in order to reduce the variation further. All the 73 selected materials (SX03-8, -11, -130, -203, -227, -252, -257, -258, -261, -282, -290, -293, -325, -520, -846, -864, -869, -872, -881, -946, -979, -1085, -1100, -1195, -1225, -1367, -1384, -1444, -1450, -1479, -1489, -1500, -1504, -1536, -1873, -1922, -1982, -1997, -2011, -2053, -2068, -2145, -2166, -2226, -2391, -2424, -2425, -2431, -2483, -2494, -2545, -2617, -2626, -2640, -2716, -2763, -2873, -2892, -2909, -3017, -3282, -3551, -3566, -3604, -3709, -3736, -3744, -3753, -3799, -3884, -4194, -4235, -4312), [Note: These codes of the SX03-series were assigned by the *Brachiaria* breeder (J. Miles)] consistently showed disease ratings below 3.0, indicating that the method of inoculation and evaluation as well as the plant tissue reactions to the pathogen are reproducible and consistent.

Photo 5. Reaction of *Brachiaria* plants (left, susceptible; right, resistant) to *Rhizoctonia solani*. A mycelial disc was placed in contact with each plant stem at the base and wrapped with parafilm Inoculated plants were kept in the greenhouse at about 28 °C, high relative humidity (≥ 90 %).

Inoculations conducted on complete plants under high humidity conditions in the greenhouse generally resulted in higher levels of disease reactions along with more variability than those observed on detached leaves. Among the 73 selected *Brachiaria* genotypes that were tested in the greenhouse, 19 genotypes (SX03- 2166, -1479, -252, -1982, -214, -3551, -3753, -4194, -3884, -3799, -3604, -2425, -2494, -257, -3017, -2716, -3282, -2431, -4312) had high level of resistance. Some of the genotypes that were evaluated as intermediate reaction to the disease using the detached leaf inoculation method developed high levels of disease reaction in glasshouse tests.

In conclusion, the detached leaf inoculation and evaluation method is very useful in screening large numbers of genotypes very quickly at a lower labor and material cost. The technique enabled us to quickly discard large number of susceptible materials and select a few materials for greenhouse tests using complete plants. Therefore, a combination of the two methods allowed us to screen large number of materials for resistance to the disease rapidly and reliably.

2.5 Elucidate the role of endophytes in tropical grasses

Highlights

- The presence of the fungal endophyte *Acremonium implicatum* in some hybrids of *Brachiaria* confirmed.
- *Acremonium implicatum* transformed with green fluorescent protein gene for the first time.
- A methanol extract compound from endophyte-infected *Brachiaria* plants showed strong fungal inhibition activity.
- Found that endophyte infection had no significant effect on dry season performance after 2 years of establishment of two accessions of *Brachiaria brizantha*.

2.5.1 PCR analysis and screening of *Brachiaria* genotypes for endophytes

Contributors: Tomoko Sakai (JICA), Martin Rodriguez and Segenet Kelemu (CIAT)

Rationale

The fungus *Acremonium implicatum* can develop an endophytic association with *Brachiaria* species that is asymptomatic. Endophyte-plant associations are widespread in nature. Grasses harboring nonpathogenic and intercellular endophytes benefit in various ways such as having enhanced drought tolerance and vigor, and increased resistance to attacks from insect pests and pathogens. Systemic infections of grasses in the *Festuca* and *Lolium* genera with *Neotyphodium* species and the corresponding teleomorph *Epichlöe* species have been studied extensively. These fungi are often transmitted by seed to the next host generation.

Many *Brachiaria* species are apomictic and reproduce asexually through seed. Apomictic reproduction permits plant genotypes to breed true through seed. This type of reproduction also offers advantages to research on endophyte-host associations and use. If the specific endophyte in question were seed-transmitted, almost all seeds of an endophyte-infected apomictic plant would contain the endophyte, as well as being

genetically identical to each other. We had demonstrated for the first time that *A. implicatum* is transmitted through seeds of *Brachiaria* grasses (Dongyi and Kelemu, 2004, Plant Disease, *in press*). A previously developed polymerase chain reaction (PCR)-based method used a pair of endophyte-specific primers to amplify a diagnostic, 500-bp, DNA fragment for a rapid and reliable detection of *A. implicatum* in tissues of *Brachiaria* grasses (Kelemu et al., 2003, Mol. Plant Pathol. 4:115-118). We used this method to examine tissues of *Brachiaria* hybrids for the presence or absence of *A. implicatum*.

Materials and Methods

Plant materials: Forty-one genotypes of *Brachiaria* (CIAT 16320, SX99/0711, SX99/0574, SX99/0275, BR99NO/4015, FM9503/S046/024, BR99NO/4132, SX99/3564, SX99/2514, SX99/2857, SX99/1370, SX99/2606, SX99/1260, CIAT 606, SX99/2173, SX99/3690, SX99/2621, SX99/2280, SX99/0835, SX99/0823, SX99/0246, SX99/2663, SX99/3770, SX99/1630, SX99/2030, SX99/1513, FM9201/1873, BRUZ4X/4402, SX99/0731, SX99/0029, CIAT 6294, SX99/1616, SX99/3488, SX99/2115, SX99/1145, SX99/2162, SX99/0236, SX99/2341, SX99/2927, SX99/1833, SX99/1622) provided by the forage-breeding program of CIAT were tested. These were maintained either in the greenhouse or planted in field plots.

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 ^{o}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)).

PCR analysis: PCR was carried out using the 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); 1.25ìM each olygonucleotide 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* leaf tissues was determined by the presence of a diagnostic 500-bp amplification product (eg. Photo 6). Plants belonging to the genotypes SX99/1833, SX99/0236, SX99/2162, SX99/2663, SX99/0823, SX99/1260 tested negative for the presence of the 500-bp amplified DNA fragment and thus, for the endophyte *A. implicatum*, whereas all remaining genotypes examined tested positive. Genotypes CIAT 16320, CIAT 606, SX99/0275, SX99/2857, SX99/2173, SX99/2621, and SX99/2280 showed strong associations with the endophyte under field and greenhouse conditions. These results indicate that the *A. implicatum-Brachiaria* associations are widespread naturally. Many of the genotypes were maintained both in the greenhouse and in the field. Some genotypes tested either positive or

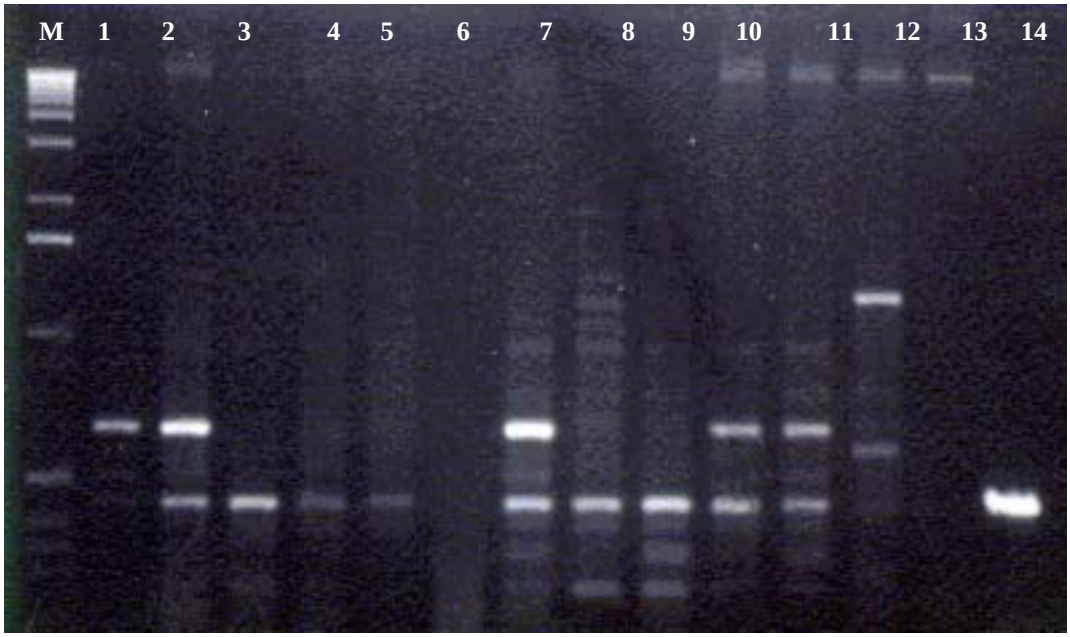

Photo 6. Specific detection of *Acremonium implicatum* in hybrids of *Brachiaria*.
Genomic DNA isolated from leaves of *Brachiaria* plants maintained in the greenhouse. Lanes 1-12, CIAT 06294 (05), BR99NO/4132 (06), SX99/0574(09), SX99/2030 (20), SX99/1513 (23), SX99/0236 (27), SX99/3564 (28), CIAT 16320 (32), BR99NO/0415 (37), FM9503/S046/024 (45), SX99/3770 (51) and SX99/2162 (17), respectively. Lane 13 is template DNA from *Rhizoctonia solani* as negative control. Lane 14 is positive control, genomic DNA from endophytic fungus *Acremonium implicatum*. Lane M = 1Kb-ladder.

negative depending on whether they were maintained in the field or in the greenhouse. Although we don't know the exact reasons for some of these inconsistencies, we suspect that environmental conditions may play a role in the amount and distribution of endophyte mycelia within the plant tissues.

2.5.2 Analysis of alkaloid profile results (collaboration with University of Kentucky, USA)

Contributors: Christopher Schardl (Department of Plant Pathology, University of Kentucky), Gustavo Segura, Ximena Bonilla and S. Kelemu (CIAT)

Rationale

Endophytic fungi associated with temperate grasses are known to produce a variety of alkaloids. The endophytes *Neotyphodium coenophialum* (previously known as *Acremonium coenophialum*), from tall fescue (*Festuca arundinacea* Schreb.), and *N. lolii* (*A. lolii*), from perennial ryegrass (*Lolium perenne* L.), have been a major focus of research associated with toxicity in livestock grazing on endophyte-containing pastures. In most cases, the toxic syndromes have been attributed to alkaloids in endophyte-containing tall fescue and perennial ryegrass.

Although several livestock disorders have been associated with cattle grazing some *Brachiaria* pastures, the exact cause or causes of these syndromes are unknown. These syndromes include conditions called "fallen cow" which affects cows in late gestation or early lactation and grazing *B. decumbens* cv. Basilisk; and "swollen face", which occurs in horses grazing

B. humidicola pastures in the Brazilian *Cerrados*. Deaths have been reported in Brazil, Colombia and Venezuela. In Brazil, livestock grazing *B. decumbens* suffer a hepatic disorder that affects young animals, causing weight lose and even death. In the State of Mato Grosso, Brazil, sheep grazing *B. decumbens* have suffered poisoning with symptoms such as swelling and dermatitis of the face, ears and eyelids, and blindness (de Lemos et al. 1996. Cienc. Rur. Santa Maria 24:109-113.). Although the causes of these disorders are still unknown, endophytic fungi may possibly play a role.

A collaboration has been set up with Dr. Christopher Schardl, Department of Plant Pathology, University of Kentucky, to determine the alkaloid profile in *Acremonium*/*Brachiaria* associations, with funds made available by USAID-University linkage programs. Some preliminary results are presented here below.

Materials and Methods

Plant samples: Freeze-dried plant samples were prepared from endophyte-infected and endophyte-free *Brachiaria* grasses in our laboratory (CIAT) and were sent to Kentucky. The endophyte-containing grasses were infected with the fungal endophyte *Acremonium implicatum*.

Fungal isolates: In addition eleven isolates of the endophyte *A. implicatum* were sent to Kentucky in pure cultures. Test fungi to be used and described in a paper by Abou-Jawdaw et al. (2002, Journal of Agricultural and Food Chemistry, 50, 3208-3213) are *Botrytis cinerea, Alternaria solani, Penicillium* sp., *Cladosporium* sp., *Fusarium oxysporum* f. sp. *melonis*, *Verticillium dahlia*, *Phytoptora infestans, Colletotrichum* , and *Rhizoctonia solani*. Since the *Dreschleria* complex was shown to be affected by the endophyte *A. implicatum* (Kelemu et al.. 2001. An endophyte of the tropical forage grass *Brachiaria brizantha*: isolating, identifying, and characterizing the fungus, and determining its antimycotic properties. Canadian Journal of Microbiology 47:55-62), this would need to be included in our analysis as well as some of the listed ones from above.

Sample analysis: Samples of freeze-dried plant materials were extracted by two different methods; soxhlet, which is basically a warm methanol reflux, and by shaking in methanol at room temperature. In pilot experiments, other solvents were also used on the soxhlet. Solvents are of analytical grade from Aldrich.

Assay for fungicidal activity: Extracts from freeze-dried plant samples were applied on to Petri dishes containing potato dextrose agar (PDA) (extract from endophyte-free (E-) plant samples to half of a plate and extract from endophyte-containing (E+) sample to the other half). The plates were then sprayed with spores of *Colletotrichum graminicola* and incubated for fungal growth. The dark color indicates abundant growth of *C. graminicola* (Photo 7).

Chemical compound analysis: Extracts exhibiting fungal growth inhibition will further be looked at with thin layer chromatography (TLC) autobiography tests. For the TLC autobiography (Hamburger and Cordell, 1987, Journal of Natural Products, 1, 19-22), aliquots of these extracts will

Photo 7. Growth inhibition of *Colletotrichum graminicola* by methanol extract compounds from freeze-dried leaf tissues of *Brachiaria* containing the endophyte *Acremonium implicatum*. The dark color indicates abundant growth of *C. graminicola*, whereas the clear transparent zone shows growth inhibition.

be spotted onto a silica gel plate in duplicate and run using different solvent systems, initially chloroform-methanol (9:1) as reported by Abou-Jawdaw et al. (2002, Journal of Agricultural and Food Chemistry, 50, 3208-3213). The duplicate plate is for later isolation of compounds from the corresponding areas on test plates that exhibited inhibition of growth.

These plates will be dried at 37°C for one hour to remove solvent residue before a suspension of spores of the different test organisms in potato dextrose broth is sprayed onto the plates and control plates (fresh silica gel plates and silica gel plates run in solvent). These plates are grown in a humidity chamber for several days to observe zones of inhibition. The corresponding areas on the unsprayed plates are then scraped and analyzed via GC-MS or LC-MC to identify compounds of interest.

Results and Discussion

In pilot experiments, other solvents were used on the soxhlet, but only the methanol extraction method gave compounds that resulted in growth inhibition of test fungi. The difference in growth on the plates between inhibited and uninhibited is quite dramatic (Photo 7).

The main problem is that some of the samples that were sent as endophyte-free also showed inhibition. One explanation is that there was a possible error in labels being switched (particularly E- and E+) either in the labs or at the APHIS offices that received the samples for examinations before forwarding them to the University of Kentucky. Alternatively, perhaps we are just seeing differences in plant genotypes, with the endophyte playing no role.

However, the results from similarly numbered plants are not always consistent either when we disregard the presence or absence of endophytes, but only consider the plant genotype. Thus, the most plausible explanation is that some sample labels had been switched somewhere along the line.

We plan to resend samples and repeat the tests.

2.5.3 Endophytes as gene delivery system

Contributors: Javier Abello and Segenet Kelemu (CIAT)

Rationale

Ever since D. C. Prasher cloned a cDNA for the green fluorescent protein (GFP) gene from the jellyfish *Aequorea victoria* in 1992 (Gene 111:229-233), this gene or its derivatives have been expressed in a wide array of organisms including plants and microbes. The protein (27 kDa) absorbs light at maxima of 395 and 475 nm and emits at a maximum of 508 nm. The 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. GFP-expressing transgenic fungal isolates have been used for analysis of *in planta* fungal development and interaction.

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 subcellular organelles. They are useful for monitoring gene expression and protein localization.

This work describes the transformation and expression of the GFP gene in an isolate of *Acremonium implicatum*, and endophyte in species of *Brachiaria*, in order to study endophyte-*Brachiaria* interactions as well as to examine the potential use of endophytes as gene delivery systems. 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*. 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* (in press)] 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*.

In this study, we used two GFP expression vectors, pWGFP20 and pC74, to transform *A. implicatum*. In this initial phase of the work our immediate objective is to develop an efficient transformation protocol for *A. implicatum*.

Materials and Methods

GFP expression vectors: Vectors pWGFP20 and pCT74 were kindly provided by Dr. Jin-Rong Xu of Purdue University, USA, and Dr. Lynda Ciuffetti of Oregon State University, respectively. Vector pWGFP-20 has successfully been used to transform *Magnaporthe grisea*. Vector pCT74 is a GFP expression vector for filamentous fungi that expresses the protein from a *Tox*A promoter of *P. tritici-repentis*. It has successfully been used in *Fusarium sambucinum*, *Botrytis cinerea*, *Pyrenophora tritici-repentis*, *Alternaria alternata*, *Cochliobolus sativus*, *Sclerotinia sclerotiorum*, *Colletotrichum magna*, and *Verticillium dahliae*.

Bacterial transformation and plasmid extraction: Vector DNA was sent either on Whatman 3 MM paper (pCT74) or in a tube (pWGFP20). To recover the plasmid from the filter paper we cut out a marked circle (where the plasmid DNA was placed), placed it in an eppendorf tube, added 50 µl of 10 mM Tris, pH 7.6, vortexed and let it rehydrate for about 5 minutes. After brief centrifugation, the supernatant liquid was used to transform competent bacterial cells. Plasmid DNA was isolated using standard alkaline lysis procedure (Sambrook et al., 1989).

Electroporation- mediated transformation of Agrobacterium tumefaciens LBA 4404: Bacterial cells were cultured in Luria agar supplemented with 0.1% glucose and incubated for 2 days at 28 °C. Cells from a single colony were transferred to Luria broth medium (100 ml) supplemented with 0.1 % glucose and grown at 28 °C in a shaker at 250 rpm till it reached an optical density of OD_{600}=1.2. Once it reached this growth density, the culture was kept in ice. Bacterial cells were collected by centrifugation at 4000 rpm for 10 minutes. The cells were resuspended in 750 µl of 10 % glycerol to generate a concentration of about 7 x 10^9 bacterial colony forming units/ ml. Aliquots of this suspension were distributed in eppendorf tubes containing 45 µl and stored at -80 °C. For electroporation, a mixture of bacterial cells (40 µl) and 1 µl of plasmid DNA (concentration 200-500 ng/µl) were placed in 0-2 cm electrode gap Gene Pulser® cuvettes (Bio-Rad), and pulsed at various voltages. Electroporated cells were added to SOC medium [Tryptone (Difco) 20 g, Yeast-extract (Difco) 5 g, NaCl 0.5 g. This was autoclaved and when the medium cooled, filter sterilized solution of 1 M $MgCl_2$ (10 ml), 1 M Mg SO_4 (10 ml), 1 M glucose 20 ml was added] and incubated for 1.5 hr at 28 °C in a shaker at 250 rpm. The cells were then plated on agar medium containing ampicillin (50 µg/ml).

Agrobacterium tumefaciens–mediated transformation of Acremonium implicatum: Monoconidial cultures of *A. implicatum* were cultured on fresh potato dextrose agar at 28 °C for conidial production. Conidia were collected by adding sterile water containing 0.15 M NaCl to the plates and gently rubbing with a sterile glass rod. The conidial suspension was filtered through

sterile Whatman # 2 to remove large particles (mycelia).

A. tumefaciens LBA4404 containing either pWGFP20 or pCT74 was cultured in minimal medium [2.05g K_2HPO_4, 1.45 g KH_2PO_4, 0.15 g NaCl, 0.50 g $MgSO_4.7H_2O$, 0.1g $CaCl_2$. $6H_2O$, 0.0025g $FeSO_4.7H_2O$, 0.5g $(NH_4)_2SO_4$, 2.0 g glucose, 1 L water] supplemented with ampicillin (50 µg/ml) at 28°C for 2 days. The *A. tumefaciens* were diluted to optical density (OD_{600}) of 0.45, 0.54 and 0.5 in induction medium [40 mM 2-(N-Morpholino) ethanesulfonic acid (MES) pH 5.3, 10 mM glucose, 0.5 % (v/v) glycerol] with or without 200µM of acetosyringone. The cells were grown for 6 hours. The various concentrations of cells were mixed with an equal volume of each of the three conidial concentrations ($3.25x10^6$, $12.5x10^6$ y $18.1x10^6$). A sample of each mix (200 µl per plate) was placed on nitrocellulose filter (0.45-µm pore size) and plated on cocultivation medium (which is the same as the induction medium, but it contains half of the glucose) with or without 200 µM of acetosyringone. After incubation for 2 days at 25°C, the filter was transferred to minimal medium containing hygromycin B (50 µg/ml) as a selection agent for transformants and cefotaxime (200µM) to eliminate *A. tumefaciens* cells. Individual transformants were transferred to small centrifuge tubes of 2 ml volume containing 1.5 ml of YMG agar medium (4 g glucose, 10 g malt extract, 4 g yeast extract, 10 g agar, 1L) supplemented with hygromycin B (50 µg/ml) and incubated until conidia formation. Conidia of individual transformants were suspended in sterile distilled water and plated on potato dextrose agar containing hygromycin B (50 µg/ml). Monoconidial cultures of each transformant were created. Conidia from these cultures were stored in 20% glycerol at –80 °C for further analysis.

Results and Discussion

The transformation of *A .tumefaciens* through electroporation generated few successful transformants on the selection medium. The vectors were isolated from the transformants to further confirm their presence. *A. implicatum* is a slow growing fungus. Although a recommended concentration of cefotaxime (200 µM) was used to inhibit the growth of *A. tumefaciens*, there was still enough bacterial growth to further impede the growth of *A. implicatum*. However, we managed to have fungal transformants free of *Agrobacterium* although the green fluorescence emitting appears to be weak with those containing the vector pWGFP20 (Photo 8). Work is in progress to get a more pronounced emission with those containing pCT74.

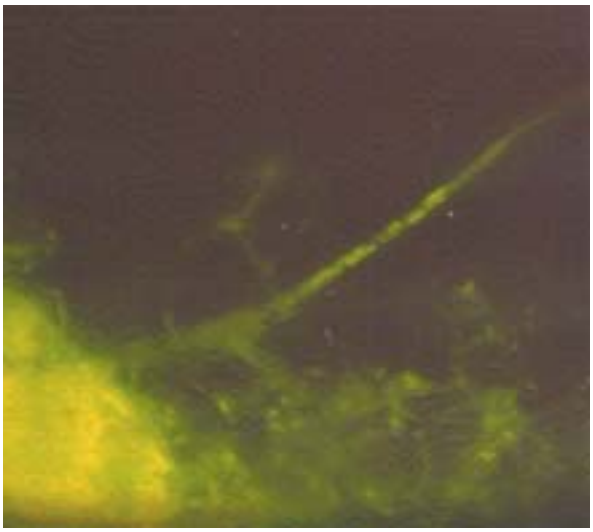

Photo 8. *Acremonium implicatum* transformed with vector pWGFP20 containing green fluorescent protein.

2.5.4 Drought tolerance in endophyte-infected plants under field conditions

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

Rationale

We showed before with soil-grown plants in the greenhouse 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 2002, to validate the findings from the greenhouse study, we initiated a field study in the Llanos of Colombia to quantify the impact of endophytes in improving drought tolerance and persistence in *Brachiaria*. Last year, 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*. This year, we conducted further field evaluation to confirm the role of endophytes in improving dry season tolerance of *Brachiaria* grasses.

Materials and Methods

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

Results and Discussion

At 18 months after establishment, i.e., at the end of rainy season, the endophyte-infected plants (E+) showed greater values of stem biomass (Figure 8a) in both accessions of *Brachiaria brizantha* (CIAT 6780 and CIAT 26110). Between these two accessions, CIAT 6780 was more productive. Endophyte infection had no significant effects on leaf and stem nutrient composition at the end of rainy season (Table 13). Leaf TNC content was not affected by endophyte infection but stem TNC content was slightly higher in CIAT 26110 than in CIAT 6780 but endophyte infection had no effect. Leaf and stem ash contents were also not affected by endophyte infection.

Results on shoot nutrient uptake showed that uptake of N, P, K, Ca and Mg was greater with endophyte-infected (E+) plants than that of uninfected (E-) plants (Figure 8b).

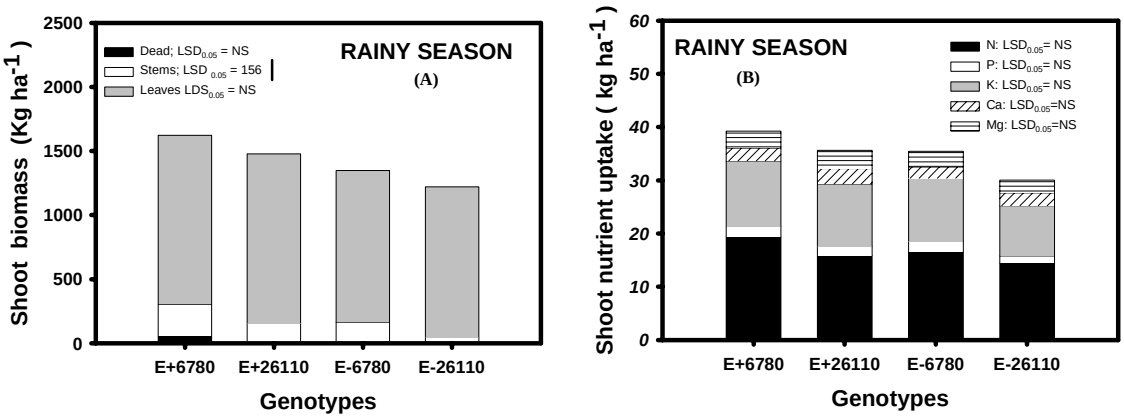

Figure 8. Influence of endophyte infection on (a) shoot (green leaves + stem) biomass production and (b) nutrient uptake of two accessions of *Brachiaria brizantha* CIAT 6780 and CIAT 26110 at 18 months after establishment (at the end of rainy season). E+ are endophyte-infected plants while E- are endophyte-free plants.

Table 13. Influence of endophyte infection on stem biomass and leaf and stem nutrient content of two accessions of *Brachiaria brizantha* CIAT 6780 and CIAT 26110 at 18 months after establishment (at the end of rainy season). E+ are endophyte-infected plants while E- are endophyte-free plants.

Genotype	CIAT 6780		CIAT 26110		
Endophyte infection	E +	E -	E +	E -	$LSD_{0.05}$
Stem biomass (t/ha)	248	164	156	48	156
Leaf N content (%)	1.33	1.32	1.14	1.18	NS
Leaf P content (%)	0.13	0.15	0.13	0.11	NS
Leaf K content (%)	0.74	0.86	0.79	0.77	NS
Leaf Ca content (%)	0.16	0.16	0.21	0.20	NS
Leaf Mg content (%)	0.20	0.21	0.25	0.20	NS
Leaf TNC content (mg g^{-1})	164	160	141	164	NS
Leaf ash content (%)	5.77	5.61	6.58	5.45	1.05
Stem N content (%)	0.84	0.81	0.71	0.69	NS
Stem P content (%)	0.11	0.12	0.10	0.09	NS
Stem K content (%)	0.99	1.23	1.23	0.99	NS
Stem Ca content (%)	0.11	0.12	0.11	0.12	NS
Stem Mg content (%)	0.18	0.22	0.21	0.21	NS
Stem TNC content (mg g^{-1})	117	125	123.1	136	16.8
Stem ash content (%)	5.42	6.03	6.36	5.47	0.52

Table 14. Influence of endophyte infection on (a) shoot biomass production and (b) nutrient uptake of two accessions of *Brachiaria brizantha* CIAT 6780 and CIAT 26110 at 22 months after establishment (at the end of dry season). E+ are endophyte-infected plants while E- are endophyte-free plants.

Genotype	CIAT 6780		CIAT 26110		
Endophyte infection	E +	E -	E +	E -	$LSD_{0.05}$
Leaf N content (%)	1.60	1.55	1.29	1.39	NS
Leaf P content (%)	0.15	0.14	0.11	0.10	0.05
Leaf K content (%)	1.18	1.02	0.95	0.85	0.14
Leaf Ca content (%)	0.31	0.25	0.19	0.26	0.04
Leaf Mg content (%)	0.27	0.30	0.16	0.21	NS
Leaf TNC content (mg g^{-1})	121	123	134	108	NS
Leaf ash content (%)	7.47	7.07	5.49	5.73	NS
Stem N content (%)	1.15	1.24	0.97	0.93	NS
Stem P content (%)	0.13	0.11	0.09	0.08	NS
Stem K content (%)	1.08	1.00	1.06	1.02	NS
Stem Ca content (%)	0.21	0.18	0.11	0.14	NS
Stem Mg content (%)	0.28	0.29	0.18	0.22	NS
Stem TNC content (mg g^{-1})	109	115	112	88.3	NS
Stem ash content (%)	7	6.78	6.12	6.2	0.86

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 (Figure 9a) 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. But endophyte free plants showed the tendency of greater dead biomass. Leaf nutrient composition indicated that leaf K was significantly greater in endophyte infected CIAT 6780 than the endophyte free plants (Table 14). Leaf and stem TNC content were significantly greater in the endophyte infected plants of CIAT 26110 than the endophyte free plants (Table 14). Results on nutrient uptake at the end of dry season also

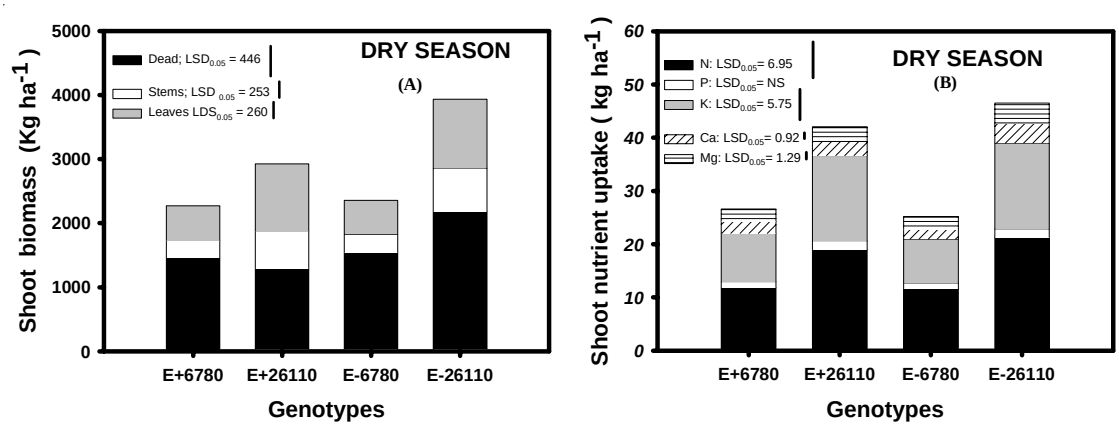

Figure 9. Influence of endophyte infection on (a) shoot biomass production and (b) nutrient uptake of two accessions of *Brachiaria brizantha* CIAT 6780 and CIAT 26110 at 22 months after establishment (at the end of dry season). E+ are endophyte-infected plants while E- are endophyte-free plants.

showed that the uptake of N, P and K was not significantly different between the endophyte-infected plants and the endophyte free plants (Figure 9b). This on-going field study indicated beneficial effects of endophyte infection on drought tolerance in the first year and almost no effect in the second year. This might be due to the regrowth of the endophyte in the endophyte free plants. There is evidence for this possibility in greenhouse grown plants. We are testing the persistence of the endophytes in these plants in order to see if the lack of effects is due to the disappearance of the endophytes in the infected plants.

2.6 Association of bacteria with *Brachiaria* genotypes

Highlight

- A mutant strain was marked for resistance to rifampicin from the original bacterial strain isolated from *Brachiaria* CIAT 36062 and that tested positive for *nif*H gene sequences that was introduced into *Brachiaria* CIAT 36061.

2.6.1 Characterization of endophytic bacteria isolated from *Brachiaria*

Contributors: Raul Sedano, Carolina Zuleta and Segenet Kelemu (CIAT)

Rationale

Endophytic bacteria that reside in plant tissues without causing any visible harm to the plant have been isolated from surface-sterilized *Brachiaria* tissues. The primary point of entry for many of these bacteria is the root zone, although aerial plant parts like flowers and stems may also be entries. Once inside a plant, they may either be localized at the point of entry or spread throughout. Bacterial endophytes have been reported to live within cells, in the intercellular spaces or in the vascular system of various plants.

Many plant-growth-promoting bacteria (PGPB) that include a diverse group of soil bacteria are thought to stimulate plant growth by various mechanisms such as plant protection against pathogens, providing plants with fixed nitrogen, plant hormones, or solubilized iron from the soil. 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 nifH gene (the gene that encodes nitrogenase reductase) [IP-5 Annual Report 2003]. 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 ioslted 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).

The objective of this study is to isolate and characterize bacterial strains with potential plant growth promoting properties.

Materials and methods

Marking bacterial cells for antibiotic resistance: An overnight culture of bacterial cells (strain 01-36062-R2) were plated on nutrient agar medium containing rifampicin (50 µg/ml) and incubated at 28 C for 48 h. Individual colonies which appeared on the medium were transferred on to freshly prepared medium containing the same concentration of rifampicin. The growing colonies were transferred on to freshly prepared medium containing 50µg/ml rifampicin. The same process was repeated until a mutant bacterium was obtained which grew the same on rifampicin-containing medium at a concentration of 50 µg/ml as well as on medium containing no rifampicin. Dilution series of the mutant bacterium were plated on nutrient agar medium with and without rifampicin to determine that the mutant grew equally on both media. Growth curves of the mutant bacterium were also conducted in nutrient broth media with and without rifampicin. The growth of the rifampicin-resistant mutant strain was determined in comparison with that of the original isolate from which the mutant was derived. In addition, nested PCR amplifications were conducted on both the marked mutant and the original bacterial isolate to make sure that the *nifH* gene sequences can be detected in both.

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

Inoculation of Brachiaria: Rifampicin–resistant bacterial cells were used to inoculate *Brachiaria* CIAT 36061 (Mulato) plants. Plants were inoculated with the rifampicin-resistant mutant either by injection or immersing the roots in bacterial suspension for 48 hours. In the root immersion inoculation method, roots of 19 plants were washed with sterile distilled water. The plants were then transferred to a suspension of bacterial cells (rifampicin resistant mutant derived from strain 01-36062-R2 at a concentration of optical density (OD_{600} = 0.1). The plants were removed from the suspension two days later and rinsed with sterile distilled water. They were then planted in sterile soil. Mutant bacterial cell suspensions (200 µL of OD_{600} = 0.1) were injected into stems and leaves of each plant (a total of 19 plants). Control plants were treated with sterile distilled water.

Evaluation of inoculated plants: root tissues or above ground tissues were macerated in 200 µL sterile distilled water, 3, 7, 12, 21, 26 and 75 days after inoculations. A dilution series was made and plated on nutrient agar containing rifampicin at a concentration of 50 µg/mL. The colonies were counted after 48 hours incubation at 28 C. The values were used to calculate the approximate number of colony forming units per tissue sample. The isolated bacteria were also tested with nested PCR to determine whether they were positive for *nif*H gene sequences.

Results and discussion

Bacterial cells were re-isolated from inoculated plants (hybrid *Brachiaria* CIAT 36061; cv. Mulato) on nutrient agar medium containing rifampicin (50 µg/mL) as late as 75 days after inoculation. No bacterial cells that can grow on the rifampicin-containing media were isolated from control plants. These rifampicin-resistant bacterial colonies also tested positive for sequences of *nif*H (Photo 9). In summary, we took a bacterial strain isolated from what appeared to be a nitrogen-efficient *Brachiaria* CIAT 36062 and that tested positive for *nif*H sequences, marked it for resistance to the antibiotic rifampicin at 50 µg/mL, introduced it to

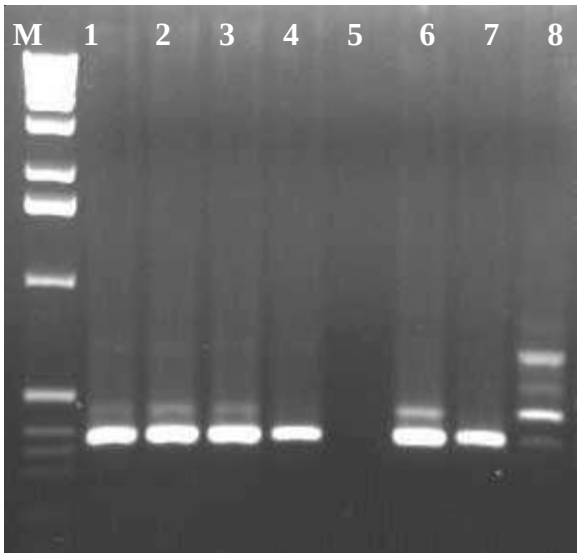

Photo 9. Nested PCR analysis of rifampicin-resistant bacterial colonies reisolated from artificially inoculated CIAT 36061 (cv. Mulato) plants, for *nif*H gene sequences. Lanes 1-4 are rifampicin-resistant independent bacterial colonies re-isolated from Mulato plants 23 days after inoculations (lanes 1 and 2 DNA of bacteria isolated from leaves; lanes 3 and 4 isolated from roots). Lane 5 is negative control. Lanes 6 and 7 are positive control and DNA from original positive bacterium from which rifampicin resistant mutants were derived, respectively. Lane 8 is a randomly picked bacterium. Lane M is size marker.

cv. Mulato and re-isolated it 75 days after inoculations, indicating that the bacterium was established in artificially inoculated plants of cv. Mulato.

The rifampicin-resistant bacterial cells were isolated both from leaves and roots of inoculated plants, although the bacterial population is not evenly distributed in all the leaves. The bacterium was consistently re-isolated from root tissues. Although both inoculation methods (plant injections or root immersions) gave successful results, more bacterial cells were recovered following root immersion inoculations and thus, root immersion method is a better inoculation method. Not surprisingly, the bacterial cell population in *Brachiaria* was much lower than that observed for a plant pathogenic bacterium such as *Xanthomonas campestris* pv. *graminis*.

By introducing this strain into cv. Mulato, we can now study the effect of the bacterial strain on the growth of Mulato plants in comparison with genetically identical plants without the bacterial strain.

2.6.2 Creation of plants with and without endophytic bacteria

Contributors: Raul Sedano and Segenet Kelemu (CIAT)

Rationale

An important component of the rhizosphere (the soil portion that forms the complex habitat of plant roots) is the actively growing microbial community that flourishes due to organic nutrients in root exudates. The microbes that colonize the rhizosphere affect plant and rot biology in relation to nutrition, development and general health. Microorganisms that colonize the rhizosphere can have various effects on plants.

Some are pathogens that have deleterious effects, whereas others have beneficial ones. Among the beneficial effects, nitrogen fixation is conducted by phylogenetically diverse groups of prokaryotes. Evidence on nitrogen fixation by rhizospheric bacteria associated with grass roots was first presented in the tropics (Döbereiner and Day, 1976. Associated symbioses in tropical grasses: characterization of microorganisms and nirogen-fixing sites. In: W. E. Newton and C. J. Nyman ed. Proc. of the 1st International Symposium on nitrogen fixation, Washington State Univ. Press, Pullman, pp. 518-538). Plant growth promoting beneficial microbes have various mechanisms of actions including production of secondary metabolites such as antibiotics, phosphate solubilization, and siderophore production.

Brachiaria CIAT 36062 stays green under both greenhouse and field conditions even under limited nitrogen conditions and it appears to be nitrogen-efficient. A bacterium was consistently isolated from *Brachiaria* CIAT 36062. The bacterial strain tested positive for *nif*H gene

(encodes nitrogenase reductase) sequences in a nested PCR test.

In this study, we attempted to create genetically identical clones of CIAT 36062 with and without the bacterium in order to study the effect of the bacterium on plant growth and development.

Materials and Methods

Treatment with antibiotics*:* In an attempt to eradicate bacteria associated with *Brachiaria* CIAT 36062, plant tillers were treated with 5 different antibiotics; ampicillin (30 µg/mL), kanamycin (15 µg/mL), streptomycin (15 µg/mL), nalidixic acid (20 µg/mL), and chloromphenico (20 µg/mL) either sequentially or in combinations by immersing the roots in antibiotic solutions in beakers.

Tillers were soaked in antibiotic solutions for a total of 3 days, i.e. either sequentially treating the tillers in individual antibiotic solutions approximately every 14 hours or for 3 days in solution mix of all three antibiotics. Tillers were then rinsed with sterile distilled water and planted in sterile sand subsequently receiving nutrient solutions.

Heat treatment: Roots of young *Brachiaria* tillers were soaked in hot water at 75 °C for 1.5, 3, 6,10, 20 minutes in an attempt to remove bacterial association from plants and create genetically identical clones with and without bacteria. The tillers were subsequently transferred to sterile sand receiving nutrient solutions.

DNA isolations: DNA extraction was done using a modified protocol based on combinations of standard methods, which includes growing bacterial cells in liquid media LB (tryptone 10g, yeast extract 5g, NaCl 10g, 10 ml of 20% glucose in 1 L of distilled water), treatment of cells with a mixture of lysozyme (10 mg.ml in 25 mM Tris-Hcl, ph 8.0) and RNase A solution, and extraction of DNA with STEP (0.5% SDS, 50 mM Tris-HCl 7.5, 40 mM EDTA, proteinase K to a final concentration of 2mg/ml added just before use. The method involves cleaning with phenol-chloroform and chloroform/isoamyl alcohol and precipitation with ethanol. Wizard Genomic DNA purification Kit (Promega, Madison, WI) was used to extract DNA from *Brachiaria* tissues. The quality of DNA was checked on 1 % agarose gel.

PCR amplifications: Three primers were used, which were originally designed by Zehr and McReynolds (1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. 55: 2522-2526) and Ueda, et al. (1995. Remarkable N2- fixing bacterial diversity detected in rice roots by molecular evolutionary análysis of *nifH* gene sequences. J. Bacteriol. 177: 1414-1417), to amplify fragments of *nifH* genes.

Amplification steps described by Widmer et al (1999. Analysis of *nifH* gene pool complexity in soil and litter at a douglas fir forest site in the Oregon cascade mountain range. Applied and Environmental Microbiology 65:374-380) were adopted.

Staining roots method: In order to sample new root tissues for presence or absence of bacteria after antibiotic treatment, roots were stained with safranine (0.5%) dissolved in water. Right after antibiotic treatments were completed, the roots were immersed in the stain for an hour, rinsed with sterile distilled water and transferred to pots containing sterile sand. Plants received nutrient solution.

Results and Discussion

None of the antibiotic treatments nor the heat treatment procedures successfully eliminated bacteria that tested positive for *nif*H gene sequences. Samples were collected 4 weeks after treatments. Template DNA isolated from root tissues grown after antibiotic treatment (white colored ones, see Photo 10) as well as from those treated with antibiotics (red colored ones, Photo 10) all produced the diagnostic 370

Photo 10. Roots of *Brachiaria* stained with 0.5% safranine solution dissolved in water. Roots stained red were treated with antibiotics; new root growth after treatment is seen as whitish.

bp amplification nested PCR product (data not shown).

New root growth did not seem to be affected by safranine staining procedure. High plant mortality resulted after the heat treatment procedure.

We are currently studying the possibility of eliminating endophytic bacteria associated with *Brachiaria* CIAT 36062 through tissue culture and regeneration procedures. If successful, we will be able to generate genetically identical clones with or without bacteria and study subsequent plant development.

2.7 Antifungal proteins in tropical forages

Highlight

- Fungal pathogens in beans and *Brachiaria* controlled in direct spray applications of an antifungal protein isolated from *Clitoria ternatea* seeds

2.7.1 An antifungal protein from *Clitoria* and its direct application in disease control

Contributors: Gustavo Segura, George Mahuku and Segenet Kelemu (CIAT)

Rationale

When wounded, or attacked by harmful microorganisms, plants can trigger an array of potent defense mechanisms, one of which is to synthesize proteins, peptides and low-molecular-weight compounds that have antimicrobial effects. Antimicrobial proteins and peptides are widely distributed in nature and are synthesized not only by plants but also by bacteria, insects, fungi and mammals.

Seeds use strategies to germinate and survive in soils that are inhabited by a wide range of microfauna and microflora. Various antifungal and/or antibacterial proteins such as chitinases, â-glucanases, thionins, ribosome-inactivating proteins and permatins have been detected in seeds. Antimicrobial proteins and peptides have been isolated and characterized 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 *in vitro*. This belief is further supported by their ability to confer resistance (to pathogens) in transgenic plants containing genes that encode them. The list of antifungal proteins from various organisms is long, with new ones continuously being discovered.

Other plant-derived proteins have insecticidal properties that can, for example, protect seeds from attack by larvae of various bruchids and inhibit the growth and development of *Helicoverpa punctigera* (Wallengren) larvae.

Of particular interest are plant-derived proteins called cyclotides (circular proteins in which the N and C termini are linked via a peptide bond), which have antimicrobial and insecticidal properties. Ocatin, a protein isolated from the Andean tuber crop oca (*Oxalis tuberosa* Mol.), is reported to have antibacterial and antifungal effects.

We reported the isolation, purification and characterization of a protein with an antifungal, antibacterial and insecticidal properties from seeds of *Clitoria ternatea* (L.) [*IP-5 AR 2003*; Kelemu, S., Cardona, C., and Segura, G. 2004. *Plant Physiology and Biochemistry* (in press); Kelemu, S., Cardona, C., and Segura, G. 2004. *Phytopathology* 94:S50). In this study, we examined the direct applications of the crude preparations of the protein in disease control on various plants.

Materials and Methods

Protein extractions: Large quantities of seeds *Clitoria ternatea* CIAT 20692 were produced on field plots at CIAT headquarters in Palmira, Colombia, for protein extractions. Seeds (100 g) of *C. ternatea* CIAT 20692 were surface-sterilized in 3.25% NaOCl solution for 10 min, then in 70% ethanol (3 min), and rinsed 6 times with sterile distilled water. The seeds were left in sterile distilled water overnight to facilitate maceration. The imbibed seeds were then macerated in 1,000 mL of sterile distilled water with a sterilized mortar and pestle. The macerated solution was filtered through several layers of cheesecloth to get rid of the seed debris. The filtrate was then centrifuged at 4 ^{o}C in tubes (50 mL) at 13 000 × g for an hour. To remove any potential microbe associated with the filtrate, the supernatant was filtered through 0.22-µm-pore-size cellulose acetate membranes. Aliquots (7 mL) of the filtrate were distributed in 15-mL tubes and lyophilized for 7 hours.
The lyophilized samples were stored at - 20 ^{o}C for further use.

This lyophilized crude protein extracts were re-suspended in sterile distilled water (10 % of the original volume) to conduct the antifungal activity bioassay on plants.

Inoculum: A highly virulent isolate (PG8 HND) 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 bean plants. This variety is one of the most susceptible varieties to *P. griseola*.

Rhizoctonia solani, causal agent of foliar blight disease of *Brachiaria* was used as inoculum. Inoculum production and inoculation methods were as described in section 2.4.2.

Plant inoculations and treatment applications: Seeds of a highly susceptible bean variety (Sprite) were planted in pots in the greenhouse at CIAT headquarters. Seventeen-day old bean plants (15 plants per treatment) were first sprayed with, either 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 at a concentration of 2 x 10^4 conidia per mL. The inoculated plants were placed in a humidity chamber for 4 days. They were then transferred to the greenhouse for development and symptom expression. Treatments with crude antifungal protein, benlate or sterile water continued every 2 days.

Disease evaluations were conducted 7, 10, 12, 14 and 17 days after inoculation.

Brachiaria CIAT 36061, which is highly susceptible to *R. solani*, was used in this test. Fully developed detached leaves were used for inoculations as described in Materials and Methods under section 2.4.2.

Treatment of *P. griseola* conidia with the protein Finotin: A conidial suspension of 2 x 10^4 conidia per mL was diluted 10^{-2} - 10^{-5} and examined under a microscope in order to determine the right concentration with evenly

distributed and separated conidia. The dilution 10^{-4} was chosen for treatment with the antifungal protein Finotin and further examination. Twenty-µl of this conidial suspension was placed on a slide and subsequently covered with a thin layer of potato dextrose agar medium. A 200-µl crude protein preparation (the same concentration that was used to spray onto bean plants) was applied on the agar. Control slides had water instead of the antifungal protein. 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, 2, 7, 24, 32 and 96 hours to observe the development of individual conidia.

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). 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 (Photo 11; Figure 10). Had a purified protein been used to control the disease on bean plants, the level of disease control would perhaps have been even higher.

It is interesting to note that even a crude protein extract sprayed directly onto plants provided protection against the disease. Experiments are currently in progress to control tomato diseases under field conditions and natural infections using crude protein preparations. Tomatoes are generally susceptible to a number of diseases.

The purpose of these experiments is to develop a simple disease control strategy for small producers using this antifungal protein.

Effect of antifungal protein on conidia of *P. griseola* in vitro: Conidia treated with crude protein or sterile water along with a layer of potato dextrose agar as described earlier reacted differently. Conidia failed to germinate in the presence of the antifungal protein Finotin 96 hours after treatment, whereas those treated with sterile water germinated and converted into mycelia (Photo 12).

Thus, one of the mechanisms of pathogen control by the protein may be by preventing fungal spore germination. However, a more detailed work on plant tissue as well as on culture is needed to fully establish the mechanism of disease control by this protein.

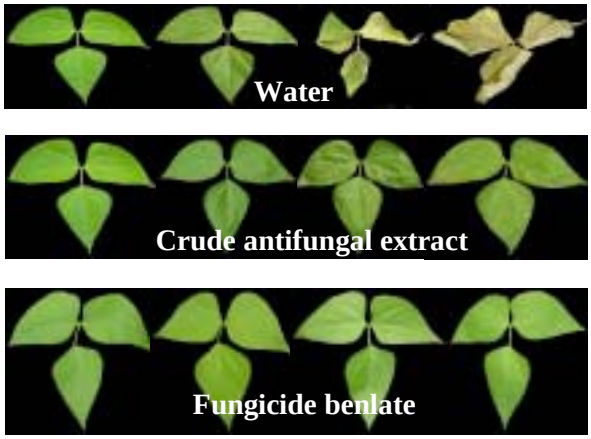

Photo 11. Treatment of bean plants with crude protein extract from seeds of *C. ternatea* CIAT 20692 against the fungal pathogen *P. griseola*, causal agent of angular leaf spot disease. Plants treated with the crude antifungal protein preparation consistently developed fewer angular leaf spot disease lesions than the control plants that were treated with sterile distilled water.

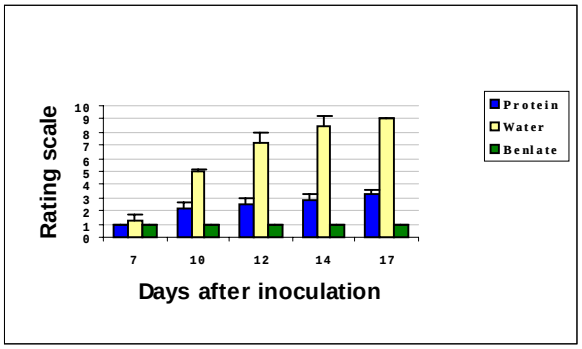

Figure 10. Angular leaf spot disease development in artificially inoculated bean plants following treatment with crude antifungal protein preparations isolated from *C. ternatea* CIAT 20692, the fungicide benlate, or water control.

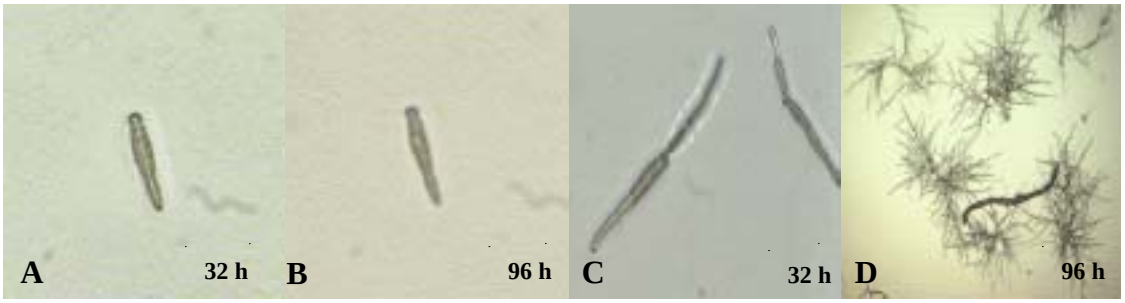

Photo 12. Treatment of *Phaeoisariopsis griseola* conidia with the antifungal protein Finotin. Conidia failed to germinate in the presence of the antifungal protein Finotin 32 and 96 hours (A and B) after treatment, whereas those treated with sterile water germinated and grew into mycelia (C and D) .

Effect of antifungal protein Finotin on Brachiaria foliar blight: Detached *Brachiaria* CIAT 36061 leaves sprayed with crude protein extract and subsequently inoculated with *R. solani* mycelial discs developed very limited or no foliar blight lesions, whereas control leaves developed severe lesions (Photo13) when evaluated 72 hours after inoculation. Although we don't intend to use the antifungal protein for direct applications in the control of foliar blight disease in *Brachiaria* (it will be impractical to do so), we are exploring the possibilities of transforming some of the endophytic microbes associated with *Brachiaria* with the gene encoding the protein.

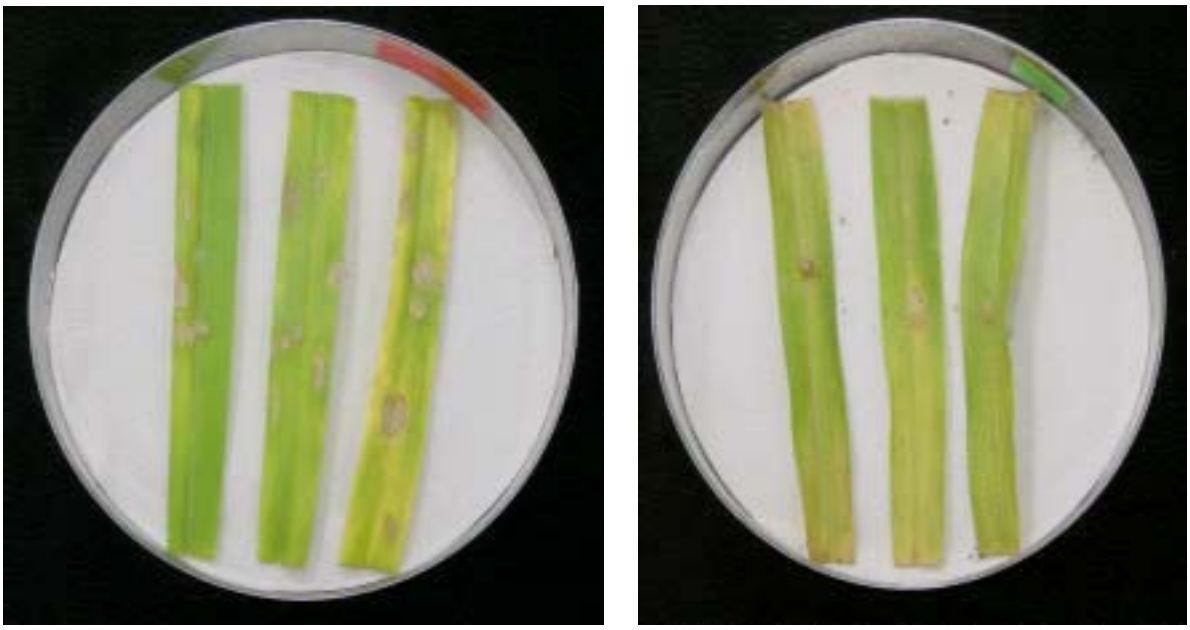

Photo 13. Detached *Brachiaria* CIAT 36061 leaves sprayed with crude antifungal protein isolated from the forage legume *Clitoria ternatea* (L.), and subsequently inoculated with *Rhizoctonia. solani* mycelial discs (right) and control leaves (left).