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

2.1 Breeding *Brachiaria* for resistance to biotic and abiotic constraints

Highlights

- Open-pollinated progenies of 95 selected BR05 hybrids were assessed to infer reproductive mode of the parent hybrids. Of the 95 progenies, 23 were found to be fully apomictic as well as having desirable agronomic characteristics (including seed set).

- Presence/absence of SCAR marker N-14 was in good agreement with visual assessment of reproductive mode based on uniformity of OP progeny.

2.1.1 Establishment of a hybrid population as spaced plants in two field trials (Quilichao & Matazul)

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

Rationale

In each breeding cycle, several thousand new hybrids are evaluated in field trials to discard plants with obviously irrelevant phenotypes (low vigor, stemmy, diseased, deficiency symptoms). These field trials are established in two contrasting environments in Colombia. Following an initial cull on periodic visual assessments of clones at the two field sites, "pre-selected" hybrids are vegetatively propagated to CIAT-Palmira to produce propagules for further evaluations (entomology; pathology; plant nutrition; quality). Open pollinated seed is harvested (by enclosing inflorescences in mesh bags) from the ''pre-selected" plants in the CIAT-Quilichao trial to produce progenies for assessment of reproductive mode. "Pre-selects" are initially culled on seed fill (percent full spikelets, by weight) of the single-plant seed harvested.

Materials and Methods

Testcross seed was produced during 2005 at CIAT-Popayán, by exposing 565 sexual clones (series SX05NO) to pollen of *B. decumbens* cv. Basilisk in the field at CIAT-Popayán. Testcross seed was hand scarified and sown in sand in planting flats. Only 233 of the 565 sexual clones produced sufficient testcross seedlings for inclusion in the 2006 field trials.

Seedlings were individually transplanted to 10.16-cm diameter (4-in) plastic pots. Each seedling was then vegetatively propagated to produce two sets of 2250 individual genotypes in 233 testcross families. Seedlings were established in 5-plant family plots with from 1 to 4 replications depending on final family size. Four check genotypes (cvs. Basilisk, Marandu, Mulato, and Mulato II) were included in the trial, with three replications (of 5-plant plots) each. Entries (families and checks) were completely randomized among 462 5-plant plots.

Superior families and individuals within families were identified on periodic visual inspection of the two field trials. Open-pollinated seed was harvested on these "pre-selected" plants by enclosing inflorescences in mesh bags. Full caryopses were separated from empty spikelets by passing through a seed blower. Percent full seeds (weight: weight) was calculated for each individual "pre-selected" individual plant.

Results and Discussion

Initially, 353 individual testcross hybrids were identified as promising "pre-selections". Based on poor seed fill, 189 of these (53.5%) were culled. The remaining 164 selections (from 77 testcross families) will be progeny tested (to identify apomicts) and evaluated for reaction to spittlebugs, rhizoctonia foliar blight, aluminum, and forage quality.

2.1.2 Reconfirming apomictic reproduction of selected hybrids and multiplication of seed for distribution

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

Rationale

Reproductive mode (apomixis vs. sexuality) of promising hybrids is assessed by progeny test. The progeny test serves the additional function of generating a first seed increase. Where any doubt remains regarding reproductive mode, or seed multiplied is insufficient for continued testing, a further seed increase/progeny test is conducted.

Materials and Methods

Seedlings of nine BR04 series hybrids were produced and a 45-plant plot of each hybrid established at CIAT-Popayán on 18 July 2006. Seed harvest and processing is still underway at the time of preparation of this report (09-Feb-07 14:05). Seedlings of 11 MX02 series hybrids were produced and these progenies were included in a progeny trial established at CIAT-Palmira on 14 July 2006. Four 5-plant plots were established for each hybrid.

Inflorescences were enclosed in mesh bags to recover mature seed. Total weight of crude seed was recorded. Full spikelets were recovered by passing crude seed through a seed blower. Seed fill was expressed as weight: weight of full: crude seed.

Results and Discussion

Progenies of all nine BX04 and 11 MX02 hybrids being assessed were uniform, confirming apomictic reproduction. Six of the 11 MX02 hybrids were culled on poor performance. The 14 remaining hybrids will be included in further evaluations leading eventually to possible cultivar release.

2.1.3 Reproductive mode of pre-selected Brachiaria hybrids at Quilichao and Matazul

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

Rationale

Only apomictic hybrids are of interest as possible commercial cultivars. In any hybrid population, approx. half of the individuals will turn out to be sexually reproducing. A progeny test is conducted to identify fully apomictic hybrids (based on uniformity among sibs). The progeny test serves the additional functions of reconfirming (or not) desirable agronomic characteristics of the promising hybrids and of generating the initial seed increase for further distribution and testing.

Materials and Methods

OP seed of 127 BR05 series "pre-selections" was hand scarified and germinated. Thirty-two

progenies with fewer than five seedlings were culled, leaving 95 progenies. Seedlings were transplanted to 5-plant plots (one to four replications) on 14 July 2006. Entries (families) were completely randomized among available plots. Reproductive mode of each hybrid was inferred from the uniformity or variability of the siblings within families.

Seed of uniform families was recovered by enclosing inflorescences in mesh bags. Full spikelets were recovered from crude seed by passing through a seed blower. Percent full seeds were estimated on a weight: weight basis.

Results and Discussion

Approximately half of the BR05 hybrids were judged to be apomictic. Percentage full seed ranged from zero to 35%. Based on seed fill, the apomicts were culled to 23, where sufficient seed for further evaluation is available.

These 23 promising BR05 hybrids along with nine BR04 hybrids and seven MX02 hybrids will be submitted to further agronomic testing leading to possible commercial release of one or more of the 39 hybrids. Our partner, Semillas Papalotla, will assume major responsibility for these advanced evaluations.

2.1.4 Genetic control and molecular markers for spittlebug and reproductive mode in *Brachiaria*: Assess presence/absence of the "apomixes locus" marker (SCAR N-14) in hybrids

Contributors: J. W. Miles, J. Tohme and C. Quintero (CIAT)

Rationale

A reliable molecular marker of the "apomixis locus" would improve the efficiency of determination of reproductive mode by eliminating the necessity of field progeny testing of approx. half of candidate hybrids. These could be culled as sexuals at a very early stage in the evaluation process – perhaps as early as prior to initial agronomic evaluation – based on marker phenotype.

Materials and Methods

Leaf tissue samples of the 95 BR05 series hybrids with cv. Basilisk included in the 2006 progeny trial were taken and standard PCR procedures applied to assess the presence or absence of SCAR marker N-14.

Results and Discussion

Marker results were in essential agreement with progeny test results, within the inevitable uncertainty of the progeny test assessment of reproductive mode. I.e., hybrids classified as facultative apomicts might, in fact, be sexuals, and hybrids classed as sexual might be facultative apomict, and many hybrids simply cannot unequivocally be classified on the field progeny test.

Thirty-five of the 95 hybrids were classified as "apomictic". Of these, all were positive for presence of the marker. Twenty-six hybrids were classified as "sexual". Twenty-five of the 26 were negative for the marker, and one positive. Of 12 hybrids classified as "sexual or facultative apomict", all 12 lacked the marker, and, hence are probably real sexuals. A number of progenies could not be classified unequivocally, and N-14 data are uninformative (Table 8).

Table 8. Visual classification of open-pollinated progenies as to reproductive mode and corresponding presence/absence of SCAR marker, N-14 of 95 BR05 progenies assessed at CIAT-Palmira.

CLASSIFICATION	TOTAL	N-14 +	N-14 -
APOMICTIC, UNEQUIVOCAL	35	35	0
SEXUAL, UNEQUIVOCAL	26	1	25
SEXUAL OR FACULTATIVE APOMICT	12	0	12
APOMICTIC OR FACULTATIVE	3	2	1
QUESTIONABLE APOMICTIC	4	1	3
FACULTATIVE APOMICTIC	4	2	2
FACULTATIVE APOMICTIC OR APOMICTIC	1	1	0
FACULTATIVE APOMICTIC OR SEXUAL	2	1	1
QUESTIONABLE FACULTATIVE APOMICTIC	5	3	2
QUESTIONABLE SEXUAL	1	1	0
UNCLASSIFIABLE	2	1	1
TOTALS	95	48	47

2.2 Potential for improving *Brachiaria humidicola* through breeding

Highlights

- Fourteen *B. humidicola* hybrid seedlings produced
- A diagnostic microsatellite marker to identify putative *B. humidicola* hybrids was found
- Nineteen *B. humidicola* accessions reported to be tetraploids are available in the *Brachiaria* germplasm collection of CIAT's Genetic Resources Unit.
- Two field crossing blocks and a small-plot, 2-replicate observation trial were established to broaden genetic base of a proposed synthetic sexual breeding population in *B. humidicola.*

2.2.1 Evaluation of open-pollinated seed harvested from putatively sexual tetraploid accession *Brachiaria humidicola* exposed to pollen of putatively apomictic tetraploid accession

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

Rationale

B. humidicola, as a species, has a number of highly desirable attributes, notably its strongly stoloniferous growth, good resilience under grazing mismanagement, and tolerance to poorly drained soil conditions. Currently available cultivars of the species have a number of defects, notably poor nutritional quality, poor seed yield, and strong physiological seed dormancy.

Materials and Methods

Two tetraploid accessions of *B. humidicola* were selected. Their reproductive modes (CIAT 26146, sexual; CIAT 26149, apomictic) were

obtained from cytological work done at Embrapa's Beef Cattle Center in Campo Grande, Mato Grosso do Sur.

Vegetative propagules of CIAT 26146 were established in 2005, as a series of single, spaced plants in a seed multiplication plot of CIAT 26149 at CIAT-Popayán and allowed to open pollinate. Seed was hand harvested on the plants of CIAT 26146. After several months to overcome dormancy, seeds were hand scarified and germinated.

A molecular marker allele present in the male parent (CIAT 26149) and absent in the female (26146), if detected in putative hybrids, would provide convincing evidence of their hybrid origin. Thus a number of microsatellite markers were assessed on the two parental genotypes. Informative markers – those present in the male (apomictic) parent and absent in the female (sexual) parent – were identified. Each of the 14 putative hybrid seedlings was assessed with one such informative microsatellite marker.

Results and Discussion

Fourteen putative hybrid seedlings were obtained. Detection of the band present in the male parent and absent in the female in all 14 of putative hybrids confirmed that all putative hybrids are in fact true hybrids. These results open the possibility of directed genetic improvement in *B. humidicola*, particularly if inheritance of reproductive mode (sexuality vs. apomixis) is found to be simply inherited.

2.2.2 Screen tetraploid *B. humidicola* germplasm accessions for reproductive mode

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

Rationale

Having demonstrated the feasibility of achieving genetic recombination between two reproductively characterized tetraploid *B. humidicola* accessions, it should be feasible to access all of the genetic diversity in CIAT's collection of *B. humidicola* germplasm at the tetraploid level. A first step to accessing this genetic diversity in a plant breeding program is to characterize reproductive mode in each of the tetraploid *B. humidicola* germplasm accessions. Existing information on reproductive mode of these accessions is incomplete and may be erroneous.

Materials and Methods

CIAT's collection of *B. humidicola* contains 19 accessions identified as tetraploid, based on work done at Embrapa's Beef Cattle Research Center and published in Penteado et al., 2000. Vegetative material of these 19 accessions was collected from the *Brachiaria* collection maintained at CIAT-Popayán by the Genetic Resources Unit. Three plantings have been established with these accessions (and 14 tetraploid hybrids): a space-planted crossing block established with vegetative transplants at CIAT-Popayán; a crossing block of pot-grown plants at CIAT-Palmira, and a small-plot (4-plant plots) 2-replicate observation trial at CIAT-Quilichao. OP seed will be harvested and individual genotypes – accessions and hybrids – will be progeny tested to assess their reproductive behavior.

Results and Discussion

Only one of the accessions (CIAT 16517) is flowering profusely at the time this report was prepared (13 February 2007), but this accession is mis-identified since it is not *B. humidicola*. Flowering is beginning on plants of other accessions at CIAT-Popayán, but not at CIAT-Palmira or at CIAT-Quilichao. No seed has been harvested to date (12-Feb-2007) from any of the three field plantings.

2.2.3 Recombination of tetraploid *B. humidicola* germplasm accessions to broaden genetic base of a proposed synthetic sexual breeding population

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

Rationale

To allow effective *B. humidicola* plant breeding, a broad-based, sexual breeding population needs to be synthesized from available tetraploid germplasm.

Materials and Methods

All available germplasm accessions reported as tetraploid, as well as 14 tetraploid hybrids are being allowed to open pollinate in field trials in order to: 1) assess reproductive mode by progeny testing all tetraploid genotypes, and 2) identify fully sexual genotypes in hybrid populations to recombine into a broad-based, synthetic sexual *B. humidicola* breeding population.

Results and Discussion

Several "crossing blocks" have been established. Seed produced in these crossing blocks, resulting from uncontrolled open pollination, will allow progeny testing of hybrids and accessions.

Superior individuals in the (segregating) progenies of sexual accessions will then have to be progeny tested to identify sexual hybrids. These hybrids will then form the "founder" parental set for the proposed tetraploid sexual *B. humidicola* breeding population.

2.2.4 Assessing reproductive mode of *Brachiaria humidicola* hybrids

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

Rationale

We know nothing of the inheritance of reproductive mode in *B. humidicola*. Progeny testing the 14 hybrids available to date will provide preliminary (but probably not conclusive) information on inheritance of apomixis in this species.

Materials and Methods

Open-pollinated seed from each of the 14 *B. humidicola* hybrids will be used to establish progenies.

The reproductive mode of each hybrid will be inferred from the uniformity or variability of its progeny.

Results and Discussion

None of the *B. humidicola* hybrids is flowering yet in any of the field plantings.

2.3 Screening *Brachiaria* genotypes for spittlebug resistance

Highlights

- A high level of combined (multi-species) antibiotic resistance to spittlebug was identified in a number of hybrids, including nine apomictic hybrids, in spite of all BR05 hybrids being from crosses with the highly susceptible cv. Basilisk
- Very high levels of antibiotic resistance to *P. simulans*, *M. trifissa* and *Z. pubescens* were detected in elite apomictic hybrids (series BR04) previously selected for high resistance to *A. varia*, *A. reducta*, and *Z. carbonaria*.
- High levels of antibiosis resistance to six major spittlebug species were found in 6 apomictic hybrids of the series BR05. Three of these hybrids combine resistance to spittlebug with adaptation to acid soils
- Three apomictic hybrids of the MX02 series (selected in previous evaluations for resistance to *Prosapia simulans*, *A. varia*, *A. reducta, Z. carbonaria*, and *M. trifissa*) also showed resistance to *Z. pubescens*
- High levels of antibiotic resistance to three major Brazilian spittlebug species were detected in the commercial cv. Mulato II.

2.3.1 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 both nymphs and adults of all major spittlebug species present in Colombia. Insects produced in our mass rearing facilities are used for greenhouse evaluations in Palmira and field evaluations in Caquetá. Our mass rearing and mass screening techniques have proved to be successful in Brazil and Mexico.

2.3.2 Identification of *Brachiaria* genotypes resistant to spittlebug

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

2.3.2.1 Greenhouse screening of *Brachiaria* accessions and hybrids for resistance to multiple spittlebug species

Rationale

Assessment of resistance to spittlebugs is an essential step in the process of breeding superior *Brachiaria* cultivars at CIAT. In 2006, intensive screening of selected hybrids was conducted under greenhouse and field conditions. A grand total of 1,150 available genotypes were evaluated. All six major spittlebug species were used.

Materials and Methods

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

Results and Discussion

In 2005 we reported on the levels of resistance to *A. varia*, *A. reducta,* and *Z. carbonaria* in 141 apomictic BR04 hybrids. Elite materials were screened in 2006 for resistance to *Z. pubescens, Mahanarva trifissa*, and *Prosapia simulans*. As usual, correlations between damage scores and percentage nymph survival were high and significant ($P < 0.01$): 0.889 for *P. simulans*, 0.939 for *M. trifissa*, and 0.917 for *Z. pubescens*. Very high levels of antibiotic resistance to all three spittlebug species were detected in these selected genotypes (Table 9).

Table 9. Percentage nymph survival in selected *Brachiaria* genotypes screened for resistance to three major spittlebug species. Means ± SEM of five replications per genotype.

Genotype	Spittlebug species		
	Prosapia simulans	*Mahanarva trifissa*	*Zulia pubescens*
BR04NO/1751	0	0	0
BR04NO/1819	0	0	0
BR04NO/1889	0	3.3 ± 3.3	0
BR04NO/1900	0	10.0 ± 6.7	6.7 ± 6.7
BR04NO/2007	6.7 ± 6.7	0	0
BR04NO/2109	0	0	0
BR04NO/2405	0	0	0
BR04NO/2455	0	0	0
BR04NO/2515	6.7 ± 6.7	10.0 ± 6.7	0
BR04NO/2557	0	0	0
BR04NO/2793	0	0	0
BR04NO/3119	33.4 ± 10.5	3.3 ± 3.3	3.3 ± 3.3
CIAT 6294[1]	10.0 ± 3.7	1. 7 ± 1.7	11.7 ± 7.9
CIAT 36062[1]	5.0 ± 2.5	0	3.3 ± 3.3
CIAT 36087[2]	0	0	0
SX01NO/0102[1]	0	0	0
BRX-44-02[3]	92.8 ± 4.9	80.0 ± 4.8	61.7 ± 7.9
CIAT 0606[3]	81.7 ± 6.8	85.0 ± 3.9	75.9 ± 4.0

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

Ten hybrids — BR05NO/0048, BR05NO/0267, BR05NO/0293, BR05NO/0537, BR05NO/0563, BR05NO/0760, BR05NO/0913, BR05NO/1402, BR05NO/1447, BR05NO/1520 — are highly resistant to six different spittlebug species (Table 10). Further, 6 of these 10 multiple species resistant hybrids — BR05NO/0334, BR05NO/0537, BR05NO/0563, BR05NO/0760, BR05NO/0913, and BR05NO/1520 — are apomicts, and hence candidates for cultivar release.

Three of the hybrids — BR05NO/0334, BR05NO/0537, and BR05NO/0563 — combine resistance to four spittlebug species and tolerance to aluminum with apomictic reproduction.

Table 10. Percentage nymphal survival in selected *Brachiaria* genotypes screened for resistance to six species of spittlebug.

Genotype	*Aeneolamia varia*	*Aeneolamia reducta*	*Zulia carbonaria*	*Zulia pubescens*	*Prosapia simulans*	*Mahanarva trifissa*
BR05NO/0048	0	3.3	3.3	0	13.3	0
BR05NO/0267	16.7	0	0	0	0	0
BR05NO/0293	0	3.3	0	0	11.7	0
BR05NO/0537	23.3	20.0	16.7	16.7	33.3	16.7
BR05NO/0563	13.3	10.0	6.7	0	6.7	6.7
BR05NO/0760	10.0	30.0	20.0	3.3	16.7	6.7
BR05NO/0913	3.3	3.3	13.3	0	0	0
BR05NO/1402	20.0	6.7	10.0	3.3	30.0	13.3
BR05NO/1447	0	13.3	3.3	0	23.3	6.7
BR05NO/1520	4.2	0	0	0	3.3	0
CIAT 6294[1]	36.6	76.7	30.0	26.7	46.7	0
CIAT 36062[2]	3.3	23.3	23.3	6.7	20.0	0
SX01NO/0102 01NO/0102[2]	6.7	0	0	0	23.3	0
CIAT 36087[3]	13.3	40.0	6.7	0	6.7	0
CIAT 0606[4]	93.3	93.3	93.3	90.0	76.7	66.7
BRX 44-02[5]	90.0	100.0	76.7	66.7	80.0	80.0

[1] Resistant commercial check (cv. Marandu).
[2] Resistant checks.
[3] Resistant commercial check (cv. Mulato II).
[4] Susceptible commercial check (cv. Basilisk).
[5] Susceptible check (Tetraploid *B. ruziziensis*).

In 2004 we reported on varying levels of resistance to *Prosapia simulans* (one of the most important species affecting *Brachiaria* in Mexico) in 34 apomictic hybrids (coded MX). These hybrids had been pre-selected in Mexico for good adaptation and desirable agronomic characteristics.

In 2005 we conducted a series of replicated tests to evaluate the resistance of these genotypes to four major species present in Colombia. In 2006 the genotypes were tested for resistance to *Z. pubescens*. Those with high levels of antibiotic resistance are shown in Table 11.

Work done in Brazil by Alejandro Pabón in cooperation with the Empresa de Pesquisa Agropecuaria de Minas Gerais (EPAMIG) revealed high levels of resistance to three major Brazilian spittlebug species in cv. Mulato II (Table 12). Mulato II was recently released in Brazil and elsewhere.

Table 11. Damage scores and percentage nymph survival in selected *Brachiaria* apomictic hybrids tested for resistance to *Zulia pubescens*. Means ± SEM of ten replications per genotype.

Genotype	Damage scores[1]	Percentage nymph survival
MX02NO/1905	1.0 ± 0	0
MX02NO/2273	1.3 ± 0.10	0
MX02NO/3056	1.3 ± 0.14	0
MX02NO/1388	1.4 ± 0.19	3.3 ± 2.22
MX02NO/3213	1.6 ± 0.18	3.3 ± 3.32
MX02NO/1769	1.7 ± 0.15	5.0 ± 2.55
MX02NO/1423	1.3 ± 0.16	5.6 ± 3.73
MX02NO/1561	1.3 ± 0.13	6.7 ± 4.43
MX02NO/3861	1.3 ± 0.13	8.3 ± 5.12
CIAT 6294[2]	1.6 ± 0.12	31.7 ± 6.77
CIAT 36062[2]	1.2 ± 0.11	0
SX01NO/0102[2]	1.0 ± 0.05	1.7 ± 1.66
CIAT 36087[3] (Mulato II)	1.3 ± 0.07	0
BRX-44-02[4]	4.8 ± 0.11	90.7 ± 2.78
CIAT 0606[4]	3.9 ± 0.16	81.6 ± 4.61

[1] On a 1 – 5 visual score scale (1, no visible damage; 5, plant dead).
[2] Resistant check.
[3] Resistant commercial check.
[4] Susceptible check.

Table 12. Levels of resistance to three Brazilian spittlebug species in CIAT 36087 (cv. Mulato II).

Genotype	*Notozulia entreriana*		*Deois flavopicta*		*Deois schah*	
	Damage scores[a]	Percentage survival	Damage scores[a]	Percentage survival	Damage scores	Percentage survival
CIAT 0606[b]	3.7a	76.0a	4.3a	84.7a	4.3a	80.9a
CIAT 6294[c]	1.9b	25.5b	2.4b	25.3b	2.0b	28.2b
CIAT 36087	1.6c	12.2c	1.8c	14.8c	1.7b	16.6b

[a] On a 1 – 5 visual score scale (1, no visible damage; 5, plant dead).
[b] Susceptible commercial check (cv. Basilisk).
[c] Resistant commercial check (cv. Marandu).
Means within a column followed by the same letter are not significantly different at the 5% level by LSD. Means o three trials, 10 repetitions per trial with *N. entreriana* and *D. flavopicta*; 20 repetitions with *D. schach*.

We continued with our studies on possible genotype x by insect species interactions. Pending data with two species from Mexico and final statistical analysis, our results suggest the occurrence of interesting genotype x spittlebug species interactions (Table 13), which may have important implications for breeding for resistance. This work (including field data from Caquetá, four spittlebug species) will be reported in full in 2007.

Table 13. Mechanisms of resistance to eight spittlebug species in selected *Brachiaria* genotypes. R, high antibiosis; I, intermediate antibiotic resistance; T, tolerance; S, susceptibility.

Genotype	*Aeneolamia varia*	*Aeneolamia reducta*	*Zulia carbonaria*	*Zulia pubescens*	*Prosapia simulans*	*Mahanarva trifissa*	*Notozulia entreriana*	*Deois schach*
CIAT 06294	I	T	T	R	R	R	R	R
CIAT 16106	S	T	R	S	T	S	R	R
CIAT 16307	R	R	I	R	R	R	-	-
CIAT 16310	-	-	-	R	R	R	-	-
CIAT 16493	I	I	S	T	T	R	S	S
CIAT 16827	I	T	T	I	R	R	R	R
CIAT 16829	R	T	T	I	I	R	R	R
CIAT 16830	R	I	I	T	I	R	R	R
CIAT 16835	I	T	T	I	I	R	R	R
CIAT 16843	I	S	S	S	S	I	R	R
CIAT 16844	I	S	I	S	S	S	R	R
CIAT 16867	S	S	T	S	S	S	T	T
CIAT 16886	S	S	T	T	S	S	T	T
CIAT 26110	T	T	T	R	R	R	T	R
CIAT 26288	S	S	S	S	S	I	S	S
CIAT 36061	S	S	S	R	I	R	T	T
CIAT 36062	R	R	R	R	R	R	R	R
CIAT 36087[a]	T	T	R	R	R	R	R	R
SXNO/0102[b]	R	R	R	R	R	R	-	-
CIAT 0606[c]	S	S	S	S	S	S	S	S
CIAT 0654[c]	S	S	S	S	S	S	-	-

[a] co. Mulato II.
[b] Resistant check.
[c] Susceptible check.

2.3.2.2 Field screening of *Brachiaria* accessions and hybrids for resistance to several spittlebug species

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

Rationale

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

Materials and Methods

Using the experimental unit described in our 1998 Annual Report, the genotypes (usually 10 replicates) are initially infested in the greenhouse with an average of 10 eggs per stem. Once the infestation is well established, with all nymphs feeding on the roots, the units are transferred to the field and transplanted 10-15 days after infestation. The infestation is then allowed to proceed without interference until all nymphs have developed and adults emerge some 30-35 days thereafter.

The plants are then scored for damage by means of the 1-5 visual scale utilized in greenhouse screenings. The number of stems per clump is counted before and after infestation and a tiller ratio (tillers per plant at the end of the infestation process/tillers per plant at the beginning of the infestation process) is then calculated. Using this

methodology, 12 major screening trials (three with *A. varia*, four with *Zulia carbonaria,* three with *Z. pubescens*, and two with *Mahanarva trifissa*) were conducted in Caquetá in 2005.

The main purpose of these trials was to reconfirm resistance in 36 apomictic hybrids (BR04) and 18 CIAT accessions that had been previously evaluated in Palmira under greenhouse conditions.

Results and Discussion

Using tiller ratios (the ratio between tillers per plant at the end of the infestation process and tillers per plant at the beginning of the infestation process) as the main selection criterion, we found that most of the BR04 hybrids tested were susceptible to spittlebug. Those combining resistances to two or more species are listed in Table 14.

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

Genotype	Spittlebug species			
	Aeneolamia varia	*Zulia carbonaria*	*Zulia pubescens*	*Mahanarva trifissa*
	Selected hybrids			
BR04NO/1197	1.18	0.97	0.90	1.08
BR04NO/1950	1.06	0.81	1.15	0.99
BR04NO/2007	1.07	0.87	1.01	1.02
BR04NO/2069	1.27	0.89	1.10	0.82
BR04NO/2093	1.02	0.93	0.88	1.01
BR04NO/2405	1.00	0.96	1.07	0.92
BR04NO/2557	1.19	0.93	1.09	0.98
BR04NO/2833	1.28	1.13	1.03	0.88
BR04NO/2940	1.02	1.04	0.91	0.84
BR04NO/2983	1.09	1.04	0.94	0.97
BR04NO/3056	1.27	0.88	0.95	0.88
BR04NO/3077	1.02	0.79	1.07	0.84
BR04NO/3119	1.20	0.96	1.12	0.89
Mean selected hybrids	1.12a	0.94a	1.02a	0.93b
	Resistant checks			
CIAT 6294	1.06	1.00	1.06	1.09
CIAT 36062	1.09	1.00	1.00	1.10
Mean resistant checks	1.07a	1.00a	1.03a	1.09a
	Commercial check			
CIAT 36087 (Mulato II)	1.13a	1.00a	1.02a	1.00ab
	Susceptible checks			
CIAT 0606	0.49	0.36	0.58	0.48
BRX44-02	0.43	0.43	0.53	0.40
Mean susceptible checks	0.46b	0.39b	0.56b	0.44c

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

2.4 Identify host mechanisms for spittlebug resistance in *Brachiaria*

Highlights

- A reliable mass screening technique to screen for resistance to adult feeding damage was developed.
- Adult survival was not affected by *Brachiaria* genotypes used in the experiments at the level of infestation used, which suggests that antibiosis does not play a role in resistance to adult feeding damage.

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

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

Rationale

Our studies have clearly identified nymphal antibiosis as the main mechanism of resistance to several different species of spittlebug in many different *Brachiaria* genotypes. In fact, we have also been able to document rapid progress in the incorporation of antibiosis resistance to nymphs in sexual and apomictic hybrids developed through a recurrent selection-breeding scheme. Given that adults can be as damaging as the nymphs, it is widely accepted that antibiosis to nymphs should be combined with an acceptable level of tolerance to adult feeding damage. However, nothing is known about mechanisms of resistance to adult feeding damage in *Brachiaria*. For this reason, and for the first time, in 2005 we initiated a series of studies aimed at characterizing tolerance to adult damage as a possible component of resistance to spittlebug. In 2006 we successfully developed a reliable screening technique to evaluate *Brachiaria* genotypes for resistance to adult feeding damage.

Materials and Methods

Three spittlebug species were utilized: *A. varia, A. reducta*, and *Z. carbonaria*. Based on results obtained in 2005, twenty-day old plants were infested with four neonate adults per plant and the infestation was allowed to proceed until all adults died (usually, 8-10 days after infestation). Percentage adult survival was recorded on a daily basis. Damage scores in a 1-5 visual damage score scale were taken 10 days after infestation.

To measure chlorophyll loss as a result of adult feeding, we used a SPAD-502 chlorophyll meter 10 days after infestation. Four representative readings per plant were taken and their averages were recorded. SPAD index values were then calculated with respect to the uninfested checks. At the end of the trial, when all insects had died, plants were cut at soil level and dried in an oven at 40^{o} C. Percentage biomass losses were calculated with respect to the uninfested checks. Damage scores and percentage biomass losses were used to calculate functional plan loss indices. These methodologies were used to test for resistance six *Brachiaria* genotypes of well-known reaction to nymphal attack. Two evaluation units were compared: Muslin cages and plastic bottles.

Results and Discussion

At the level of infestation used in these experiments, adult survival was not affected by the genotypes. This means that antibiosis does not seem to play a role in resistance to adult feeding damage. Susceptible and tolerant reactions of genotypes were very similar using the muslin cage technique or the plastic bottle technique (Table 15). The latter has been adopted to conduct large-scale screenings of *Brachiaria* genotypes for resistance to adult feeding damage.

Table 15. Levels of resistance to adults of three spittlebug species in *Brachiaria* genotypes detected by means of two screening methodologies.

Genotype	Damage scores[a]		Percentage chlorophyll loss		FPLI (%) [b]	
	Muslin cages	Plastic bottles	Muslin cages	Plastic bottles	Muslin cages	Plastic bottles
			Aeneolamia varia			
CIAT 0654	4.6a	4.3a	42.3a	41.5a	93.4	93.5
CIAT 0606	3.4b	3.6b	27.7b	26.1b	85.5	67.4
CIAT 6294	1.8d	1.9e	6.8d	15.7c	26.7	17.3
CIAT 36062	2.9c	2.7cd	12.2cd	22.8c	70.6	48.1
CIAT 36087	2.2d	2.2de	6.8d	20.9bc	42.1	47.3
SX01NO/0102	3.2bc	3.0c	17.1bc	25.1b	75.5	64.5
Zulia carbonaria						
CIAT 0654	5.0a	4.8a	65.7a	77.6a	100.0	96.4
CIAT 0606	3.9c	3.7b	37.9b	49.0b	78.0	72.3
CIAT 6294	2.6d	2.0d	12.0c	26.0d	49.5	50.5
CIAT 36062	4.3bc	2.9c	39.2b	39.4c	87.8	61.5
CIAT 36087	2.6d	2.3d	26.1b	30.8d	44.2	48.0
SX01NO/0102	4.7ab	3.1c	66.9a	40.3bc	96.1	75.4
Aenolamia reducta						
CIAT 0654	-	4.0a	-	35.3a	-	83.7
CIAT 0606	-	3.1b	-	26.0a	-	71.2
CIAT 6294	-	2.0c	-	6.5c	-	53.6
CIAT 36062	-	2.9b	-	27.3a	-	61.6
CIAT 36087	-	1.8c	-	12.7b	-	52.4
SX01NO/0102	-	2.9b	-	34.4a	-	67.1

[a] On a 1 – 5 visual scale (1, no visible damage; 5, severe damage, plant killed).
[b] Functional Plant Loss Index = $1 - \{[\frac{\text{Weight of infested plant}}{\text{Weight of uninfested plant}}] \times [1 - \frac{\text{(damage score)}}{5}]\} \times 100$

2.5 Define interactions between host and pathogen in *Brachiaria*

Highlight

- A total of 10 Brachiaria hybrids (9 from the series RZ 05 and 1 from the series BR05) exhibited similar resistance (2.0-2.5) to *Rhizoctonia* foliar blight than the check (CIAT 16320)

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

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

Rationale:

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

R. solani is a basidiomycete fungus that does not produce any asexual spores (called conidia). In nature, the fungus reproduces mainly asexually and exists as vegetative mycelia and/or dense sclerotia. In the absence of a susceptible host, these sclerotia, that are irregular-shaped, brown to black structures, can survive in soil and on plant debris for several years.

The fungus can also survive as mycelia by colonizing soil organic matter as a saprophyte. When a susceptible host is available, sclerotia can germinate and produce hyphae that can infect host plants. The fungus is a very common soil-borne pathogen that primarily infects below ground plant parts in a great diversity of plant species, but can also infect above ground plant parts such as pods, fruits, and leaves and stems as is the case with *Brachiaria*. In *Brachiaria*, infected leaves first appear water-soaked, then darken, and finally turn to a light brown color. As symptoms progress, lesions may coalesce quickly during periods of prolonged leaf wetness and temperatures between 21 and 32 °C.

Disease management through the use of host resistance, when available, remains to be the most practical and environmentally friendly strategy. A number of constitutive factors including cell wall calcium content, and cuticle thickness may contribute to resistance.

Other factors expressed after infection also play a role in resistance. These components of resistance may also be influenced by factors such as age and maturity of the plant as well as other external factors such as plant nutrition and environmental conditions (e.g. field vs controlled environmental growth conditions).

Differences in reaction to *R. solani* exist in genotypes of *Brachiaria.* The ability to uniformly induce disease and measure resistance accurately is crucial in a breeding program for developing resistant cultivars. Measurement of resistance is based on quantification of disease symptoms or the growth and expansion of the pathogen on its host.

The objectives of this study are to: 1) artificially inoculate and induce uniform disease development in selected *Brachiaria* genotypes generated by CIAT's tropical forages project, 2) accurately measure resistance and identify resistant materials among these *Brachiaria* genotypes.

Materials and Methods

Plant materials: Two-hundred nine *Brachiaria* genotypes (127 with BR05 series and 82 with RZ 05 series) provided by the breeding program were planted in the field at Macagual ICA/ CORPOICA Research Station in Florencia, Caquetá. CIAT 16320, CIAT 36061 and CIAT 36087 were included as controls. The field location is highly conducive to the development of the disease, with mean annual relative humidity of 84 %, an average temperature of 25°C and an annual rainfall of 3793 mm.

Field layout, artificial inoculations and disease evaluations: Twelve plants (that were generated from the same mother plant) of each of the *Brachiaria* genotypes were transplanted from a CIAT glasshouse to the field site in Caquetá. The space between plants was 80 cm, and 2 m between blocks.

The entries were replicated 4 times in a randomized complete block design. Plants were inoculated one month after transplanting by placing 0.7 g dry sclerotia of *R. solani* isolate 36061 on the soil surface at the base of each plant. Plants were evaluated for disease reaction 15, 25, 35, 45, 55 and 65 days after inoculations, using the 0 – 5 (0 = no visible infection; 5 = 20 - 100% of the aerial portion of the plant infected) scale that we developed earlier and reported in the 2004 Annual Report.

Results and Discussion

Disease symptoms developed in susceptible genotypes 10-15 days after inoculations. There was a high degree of correlation in disease evaluation data among the various evaluation

dates. The resistant control CIAT 16320 was consistently evaluated at scale less than 2. The disease evaluation data taken 65 days after inoculations represented well-developed disease symptoms that correlated well with data taken at various dates.

Based on the results we formed three groups:

Group 1 (highly resistant):
Ten genotypes, RZ05/3635, BR05/0262, RZ05/2721, RZ05/3551, RZ05/3634, RZ05/3738, RZ05/2738, RZ05/2919, RZ05/3394, and RZ05/3575 were evaluated at an average between 2.0 and 2.5.

Group 2 (moderately resistant):
Eighty-one others, R05/0048, BR05/0377, BR05/0555, BR05/0591, BR05/1482, CIAT 36087, BR05/0753, BR05/0760, BR05/0777, BR05/1359, RZ05/2699, RZ05/2816, RZ05/2842, RZ05/3021, RZ05/3362, RZ05/3397, RZ05/3405, BR05/0156, BR05/0537, RZ05/3063, BR05/0114, BR05/0115, BR05/0118, BR05/0379, BR05/0629, BR05/0714, BR05/0744, BR05/0913, BR05/0914, BR05/1455, RZ05/2682, RZ05/2764, RZ05/2942, RZ05/3173, BR05/0071, BR05/0092, BR05/0408, BR05/0549, BR05/0586, BR05/0701, BR05/1352, RZ05/3226, RZ05/3343, RZ05/3472, RZ05/3524, RZ05/3579, BR05/0731, BR05/0746, RZ05/3361, RZ05/3645, BR05/0303, RZ05/0462, RZ05/3158, RZ05/3541, RZ05/3576, RZ05/3434, BR05/0605, BR05/0707, BR05/1467, BR05/1717, RZ05/2838, BR05/0830, BR05/1433, BR05/1434, BR05/1469, BR05/1494, BR05/1865, BR05/1872, RZ05/2786, RZ05/3106, RZ05/3262, RZ05/3335, RZ05/3452, RZ05/3483, RZ05/3539, BR05/0150, BR05/1149, RZ05/2937, RZ05/3630, BR05/0475, BR05/0561 scored with an average rating scale of 2.6-2.9.

Group 3 (susceptible):
All remaining 118 materials, BR05/1830, BR05/0637, BR05/0642, BR05/0933, BR05/1440, BR05/1609, BR05/0406, BR05/0733, BR05/1449, BR05/0265, BR05/1401, RZ05/2938, RZ05/2985, BR05/1853, RZ05/3101, RZ05/3332, BR05/1879, RZ05/3371, RZ05/3528, RZ05/3608, RZ05/3333, RZ05/3590, BR05/1426, BR05/1462, BR05/1611, RZ05/2932, BR05/0508, BR05/0545, BR05/0671, BR05/0708, BR05/1460, RZ05/2801, RZ05/2847, RZ05/3107, RZ05/3355, RZ05/3574, BR05/0120, BR05/0284, BR05/0351, BR05/1574, BR05/1706, RZ05/3128, RZ05/3312, RZ05/3495, BR05/0117, BR05/0334, BR05/0609, BR05/0931, BR05/1464, RZ05/3244, RZ05/3466, BR05/0702, BR05/1302, RZ05/2802, RZ05/3485, RZ05/2992, BR05/0990, BR05/1520, BR05/1857, RZ05/3365, BR05/0354, BR05/0743, BR05/1173, BR05/1308, BR05/1402, BR05/1447, BR05/1623, RZ05/3589, BR05/1249, BR05/1586, BR05/0577, BR05/0627, BR05/0995, BR05/1344, BR05/1435, BR05/1444, BR05/1475, BR05/1738, RZ05/2831, RZ05/3311, BR05/0563, BR05/1361, BR05/1420, BR05/1479, BR05/1835, BR05/0244, BR05/0267, BR05/0891, BR05/1019, BR05/1429, BR05/1826, BR05/1493, BR05/1702, RZ05/3377, BR05/1376, BR05/0020, BR05/0159, BR05/0293, BR05/1040, BR05/1480, RZ05/3359, BR05/0209, BR05/1490, RZ05/2668, RZ05/2873, RZ05/3253, BR05/1331, BR05/1610, BR05/1883, RZ05/3398, CIAT 36061, RZ05/2641, RZ05/3378, RZ05/3391, RZ05/3367, RZ05/3629, BR05/1059, BR05/1647, RZ05/3585, RZ05/3616 scored between 3.0-5.0.

In Figure 1 we show a graphical representation of the results using data from representative genotypes from each of these groups.

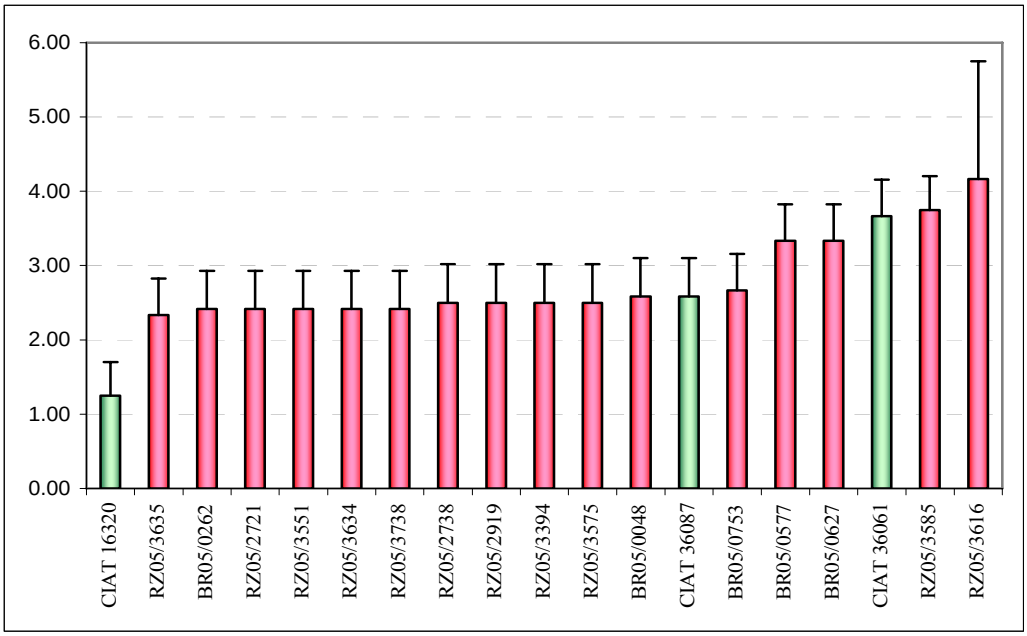

Figure 1. Ratings of *Brachiaria* genotypes for foliar blight disease reaction on a 1-5 scale 65 days after inoculations with sclerotia of *Rhizoctonia solani* under field conditions, Caquetá, Colombia. Bars indicate standard deviation.

2.6 Endophytes in tropical grasses

Highlights

- The transformation and expression of the GFP (green fluorescent protein)-encoding gene in an isolate of an endophytic plant growth promoting bacterium associated with species of *Brachiaria* is described. To the best of our knowledge, this is the first report on transformation of this endophytic bacterium. This is also the first report of a plant growth promoting endophytic bacterium associated with *Brachiaria* that contains nif-gene sequences.

- The introduction of three strains of endophytic bacteria, originally isolated from *Brachiaria* hybrid CIAT 36062, had a positive effect on plant growth and development in the recipient plant *B. brizantha* CIAT 6294. There was more tiller and root development in artificially inoculated plants than control plants. Results suggest a close and beneficial interaction between the introduced endophytic bacteria and *B. brizantha* CIAT 6294, resulting possibly in nitrogen fixation and enhancement of plant growth.

2.6.1 Bacterial endophytes in *Brachiaria*

Contributors: J. Abello, P. Fory and S. Kelemu (CIAT)

Rationale

Bacterial endophytes are known to reside in plant tissues without doing harm to their host. These bacteria are often isolated either from surface-sterilized tissues or extracted from internal plant parts. They can enter plants mainly through the root zone, although other plant parts such as stems, flowers and cotyledons can also be entry points. In general, many of the entry points for

pathogenic bacteria can serve the same purpose for the endophytic ones. Several different endophytic bacteria may reside within a single plant. These endophytes may either remain localized at their entry points or spread in other parts of the plant. Various bacterial endophytes have been reported to live within cells, in the intercellular spaces or in the vascular system of various plants. Although variations in the endophyte populations have been reported in various plants depending on a number of factors, generally bacterial populations are higher in roots and decrease in stems and leaves.

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

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

The green fluorescent protein (GFP) gene, isolated from the jellyfish *Aequorea Victoria*, or its derivatives have been expressed in a wide array of organisms including plants and microbes. This work describes the establishment of a transformation protocol and expression of the green fluorescent protein (GFP) gene in an isolate of a bacterial endophyte associated with species of *Brachiaria*. The purpose of this study is to evaluate the use of GFP in host-parasite interactions.

Material and Methods

Bacterial isolate and growth conditions*:* a bacterial isolate designated as CIAT 36062R2 (IP5 Annual Report 2005), isolated from roots of *Brachiaria* hybrid CIAT 36062, was marked for antibiotic resistance (rifampicin, rif^r). This isolate tested positive for *nif*H gene (the gene that encodes nitrogenase reductase) sequences (IP-5 Annual Report 2005; Kelemu *et al*., 2006, Phytopathology 96:S59) Bacterial cells were collected from a single colony and cultured on Luria agar medium containing rifampicin (LB; tryptone 10 g/l, NaCl 5g/l, yeast extract 5 g/l and agar 15 g/l; rifampicin 50 µg/ml) and incubated at 28 ^{o}C for 24 hours in darkness.

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

Transformation of the bacterial endophyte CIAT 36062R2: *E. coli* strain DH5á was electrotransformed with the plasmid pGT-kan for maintenance of the plasmid. CIAT 36062R2/rif^r was electrotransformed using a protocol described by Dulk-Ras and Hooykaas (1995, Methods Molecular Biology. 55: 63-72) with some modifications. To prepare competent bacterial cells, the cells were grown in LB medium at 28^{o}C with shaking at 250 rpm for 16 hours till a growth density of OD_{600}= 0.5. The cells were collected after centrifugation at 4,000 rpm, 4^{o}C for 15 minutes. The cells were rinsed three times with 20 ml solution of 10% glycerol

and 1mM HEPES (pH: 7.0). They were then resuspended in 3 ml of 10% glycerol, 200 µl aliquots were made and stored at -80°C for subsequent use. Electroporation was conducted using a BIO-RAD® gene pulser at 12,5 Kv/cm, 200 Ω of resistance and 25 µF of capacity. Forty µl of competent cells were mixed with 100ng/µl of plasmid pGT-kan and electric pulse was applied to the mixture. The cells were then transferred to a 1 ml LB medium and incubated for 3 hours at 28°C. One hundred µl of this culture was plated on Luria agar plates containing 50 µg/ml rifampicin and 15 µg/ml of gentamycin for selection of transformants. Putative transformants appeared on the selection plates after 48 hours of incubation.

PCR analysis of bacterial transformants*:* Genomic DNA was isolated from putative transformants using a protocol described by Cheng *et al.* (2006, Biotechnology Letters. 28: 55-59.). Identification of GFPmut1 gene in transformants was conducted using specific primers T14GFP5′ (5′ATTCCCTAACTAATAA-TGATTAACTTTATAAGGAGGAAAAAC 3′) and T1GFP3′ (5′ GATGCCTGGA-ATTAATTCCTATTTGTATAGTTCATCC 3′) (Miller *et al*., 2000, Molecular Plant-Microbe Interactions. 13: 1243-1250). Amplifications were carried out in a Programmable Thermal Controller (MJ Research, Inc) programmed to 30 cycles comprised of I minute denaturation step at 95°C (3 minutes for the first cycle), followed by 2 min at 50°C, and primer extension for 3 minutes (10 minutes in the final cycle) at 72°C. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad Laboratories), stained with ethidium bromide, and photographed under UV lighting.

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

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

Microscope examination: The putative GFP-expressing transformants were examined under a LEICA fluorescence microscope fitted with a Leica D filter with an excitation range between 355 and 425 nm, and an H3 filter with an excitation range between 420 and 490 nm. For observations of GFP expressions inside plant tissues, young roots and leaves were sectioned with diameters of approximately 0.5-1.5 mm.

Results and Discussion

Transformation of endophytic bacterium CIAT 36062R2/rif^r*:* Putative transformants appeared on selection plates after 48 hours of incubation. Colonies with a diameter of approximately 1-mm were isolated for analysis. Bacterial cells grown to an optical density (OD_{600}) = 1.0 were examined for green fluorescence. All cells examined demonstrated strong fluorescence indicating successful expression of gfp. Control colonies showed no fluorescence. The GFP protein (27 kDa) is a spontaneously fluorescent protein that absorbs light at maxima of 395 and 475 nm and emits at a maximum of 508 nm. This protein is a success as a reporter because it requires only UV or blue light and oxygen, but requires no cofactors or substrates as many other reporters do for visualization.

PCR analysis of putative transformants*:* The putative bacterial transformants selected on the selection media were further examined using

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

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

Microscopic examination: Microscopic examinations of selected bacterial transformants demonstrated strong expression of *gfp* as evidenced by the intense fluorescence emission at a range of wavelength (Photos 1and 2). The strongest emission was observed at a 355-425 nm range with Leica D filter. The emission intensity was somewhat lower when a filter Leica H3 was used with a 420-490 nm range.

Root and leaf tissues from *Brachiaria* plants inoculated with endophytic bacterial cells

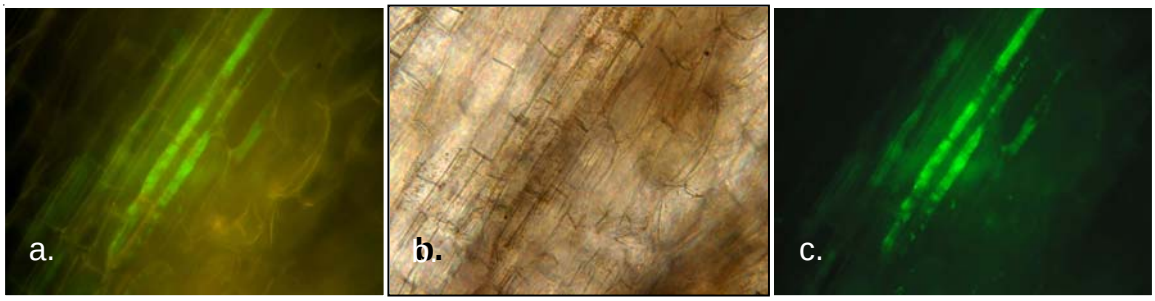

Photo 1. *Brachiaria* tissues from plants inoculated with bacterial endophyte transformed with green fluorescent protein gene *egfp*). a) fluorescence emission under UV light with Leica H3 filter, b) under normal lighting, c) fluorescence emission under UV light with Leica D filter.

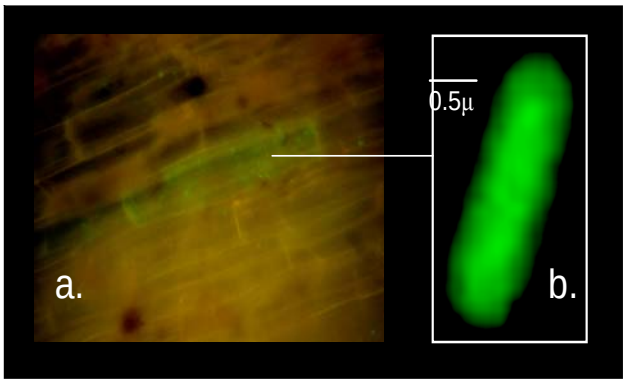

Photo 2. Endophytic bacterial cells transformed with a gfp gene. a) initial colonization of *Brachairia* roots inoculated with transformed bacterial cells one month after inoculation; b) a single bacterial cell (average length of 2,5 ìm). Photographed with Leica D filter.

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

Although the transformation protocol functioned well for the endophytic bacteria, the recombination of the introduced gene to that of the bacterial genome was not evident, as the transformed bacteria lost their green fluorescence with time. Preliminary data showed that *Brachiaria* tissues taken from plants inoculated with GFP-transformed bacterial endophytes expressed fluorescence emission (Photo 1)**.** This will allow us to study the endophyte-*Brachiaria* interaction, endophyte distribution within the plant tissue, and the correlation of endophytic bacterial colonization with *Brachiaria* plant growth and other related benefits.

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

2.6.2 Endophytic plant growth promoting bacteria associated with *Brachiaria*

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

Rationale

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

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

In 2005 Annual Report, we demonstrated the effect of endophytic bacteria on the growth of *Brachiaria* hybrid CIAT 36061 (cv. Mulato). *Brachiaria* hybrid CIAT 36061 had indigenous endophytic bacteria that are difficult to eliminate. Because of the difficulty to eliminate these

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

In nitrogen- and other nutrient-deficient conditions, *Brachiaria* plants inoculated with the three bacterial strains had significantly higher average values in all evaluated parameters (with the exception of soluble proteins in leaves) than those control plants containing just indigenous bacteria (IP-5 Annual Report 2005).

Analysis of variance showed that the total biomass production (leaf, stem and root) collected from control *Brachiaria* CIAT 36061 plants was significantly ($P< 0.05$) less than that from inoculated ones (IP-5 Annual Report 2005). The data presented indicate that a close and beneficial interaction existed between the introduced as well as indigenous endophytic bacteria and *Brachiaria* hybrid CIAT 36061, resulting possibly in nitrogen fixation and enhancement of plant growth.

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

Materials and Methods

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

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

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

Plant evaluations*:* Sixty days after inoculations of *B. brizantha* CIAT 6294, the following measurements were taken in control and treated plants: 1) plant growth and development based on plant height, number of tillers, number of leaves and leaf area, 2) leaf chlorophyll content 3) leaf and stem nitrogen content, and 4) soluble protein content in leaves. Plant height was measured in centimeters from stem base to the highest part of the plant. Number of leaves per plant and the number of tillers were determined. Leaf area was determined in cm^2/plant and measured using a LI-300 leaf area meter (LI-COR, inc., Lincoln,

NE). In addition, dry matter distribution among leaves, stems and roots was determined after drying each tissue separately in an oven at 70ºC for 48 hours. Leaf chlorophyll content was measured with a chlorophyll meter SPAD 502 (Minolta), taken across the third fully developed leaf as an average of 6 measurements. Soluble leaf protein was measured as described by Rao and Terry (Plant Physiol 90: 814-819). Nitrogen content in leaves and stems was determined using methods described by Salinas and García (1985, CIAT, Working document 83 p).

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

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

Results and Discussion

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

Analysis of variance showed that the total biomass production (leaf, stem and root) collected from control *Brachiaria* CIAT 6294 plants was significantly ($P< 0.05$) less than that from inoculated ones (Figure 2). The data presented indicate that a close and beneficial interaction existed between the introduced bacteria and *B. brizantha* CIAT 6294, resulting possibly in nitrogen fixation and enhancement of plant growth. These results are consistent with the results reported in IP-5 Annual Report 2005 with *Brachiaria* hybrid CIAT 36061 (cv. Mulato).

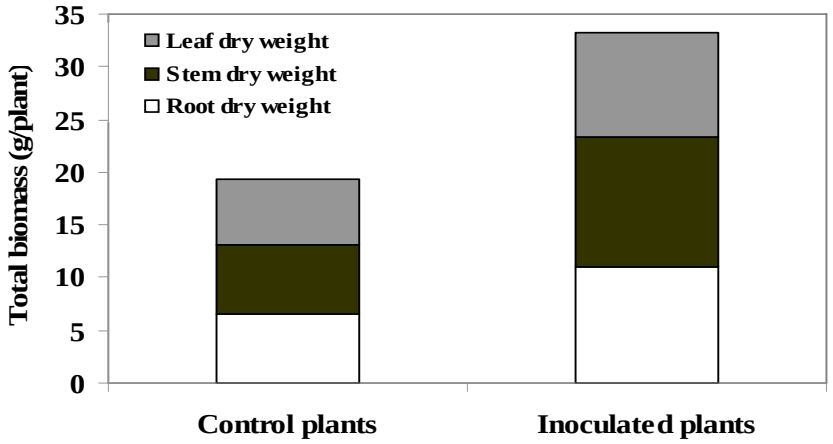

Figure 2. Total tissue biomass production in *Brachiaria brizantha* CIAT 6294 control plants, and inoculated with a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062), 60 days after inoculations and maintained under greenhouse conditions with no nutrients. Values are average of 6 plants per treatment.

In nitrogen- and other nutrient-deficient conditions, *Brachiaria* plants inoculated with the three bacterial strains had significantly higher average values in all evaluated parameters, plant height, number of tillers, number of leaves, and leaf area than those control plants (Figure 3).

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

These data strongly suggest that endophytic bacteria have a direct beneficial effect on plant growth and development, and possibly on associated nitrogen fixation in *Brachiaria*. The possibility that this plant growth is through associated nitrogen fixation is further corroborated by the endophytic bacteria sequence data described in the report in section 2.6.3.

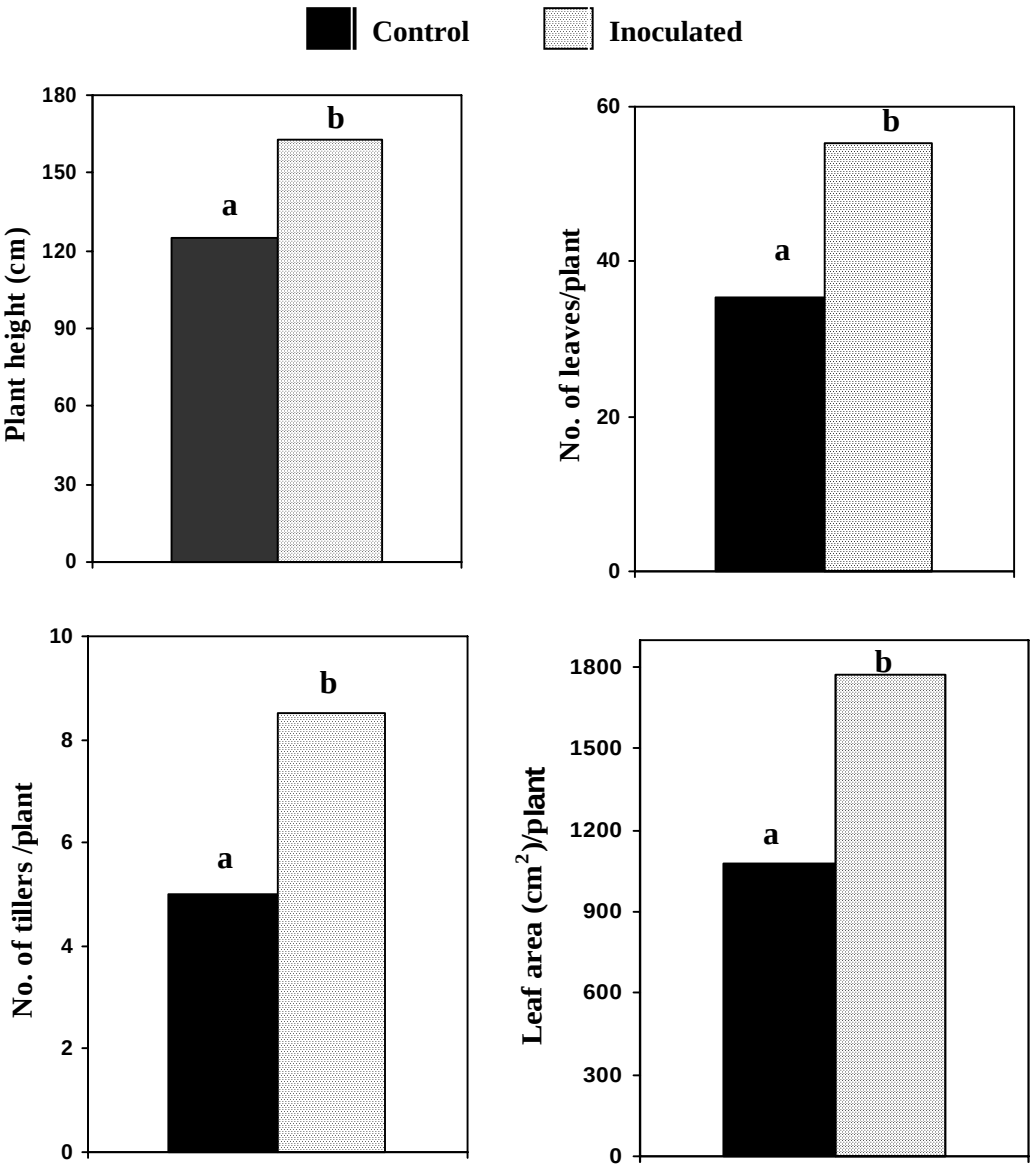

Figure 3. Effect of bacterial isolates (a mixture of 3 bacterial strains 01-36062-R2, 02-36062-H4, and 03-36062-V2 (originally isolated from *Brachiaria* CIAT 36062) on the growth of *Brachiaria brizantha* CIAT 6294 at 60 days after inoculations. Plants were grown under greenhouse conditions with no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

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

Contributors: P. Fory and S. Kelemu (CIAT)

Rationale

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

We have reported the isolation of three strains of bacteria from *Brachiaria* hybrid CIAT 36062 (BR97-1371) from roots, leaves and stems that were designated 01-36062-R2, 02-36062-H4, and 03-36062-V2, respectively. Using nested PCR and three primers designed for the amplification of the *nifH* gene sequences, amplified products were generated with template DNA from these bacterial strains. We have also reported previously (IP-5 Annual Report 2004) that fatty acid analysis conducted on these 3 strains resulted in matching them with various bacteria that are known to be nitrogen fixers and/or plant growth promoters (for example with *Flavimonas oryzihabitans*). We reported (IP-5 Annual Report 2005) the cloning and sequencing of a 371 bp nested PCR amplified product (with *nif*H gene specific primers) isolated from an endophytic bacterium strain 01-36062-R2 associated with *Brachiaria* hybrid CIAT 36062. Using this sequence data, specific primers were designed and synthesized in order to develop a simple diagnostic tool that enables us to do one step PCR analysis and avoiding nested PCR methods, that will eventually allow us to detect, evaluate and select *Brachiaria* genotypes that harbor *nif*H gene containing endophytic bacteria (IP-5 Annual Report 2005). In this study, we continued to clone and analyze the sequences of *nif*H gene in other native strains of endophytic bacteria associated with *Brachiaria* genotypes.

Materials and Methods

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

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

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

Amplification of DNA inserts for sequencing: PCR reactions (25-µl) contained 20 ng/µl plasmid DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTPs, primers T7 (5'-GTAATACGACTCACTATAGGGC-3') and

Sp6 (5' –TATTTAGGTGACACTATAG-3') each at 0.1 M concentration, 0-5U Taq polymerase and amplified in a programmable thermal controller (MJ Research, Inc, MA) programmed with 35 cycles of a 30 sec (2 min for the first cycle) denaturation step at 94°C, annealing for 30 sec at 50°C, and primer extension for 1 min (4 min in the final cycle) at 72°C. Samples of amplified products were separated on a 2% agarose gel by electrophoresis for further confirmation of the expected size insert.

Cloning and digestion of amplified DNA fragments: Amplified products were eluted from agarose gel using Wizard ® PCR Preps DNA Purification System (Promega) according to instructions supplied by the manufacturer. The purified fragments (size 320-322 bp) were ligated to the cloning vector PCR ® 2.1 (Invitrogen, Carlsbad, CA, USA) [Figure 4] and used to transform *E. coli* DH5á.using standard procedures (Sambrook et al., 1989. Molecular Cloning: a laboratory manual. 2nd ed. Cold spring harbor laboratory, USA)

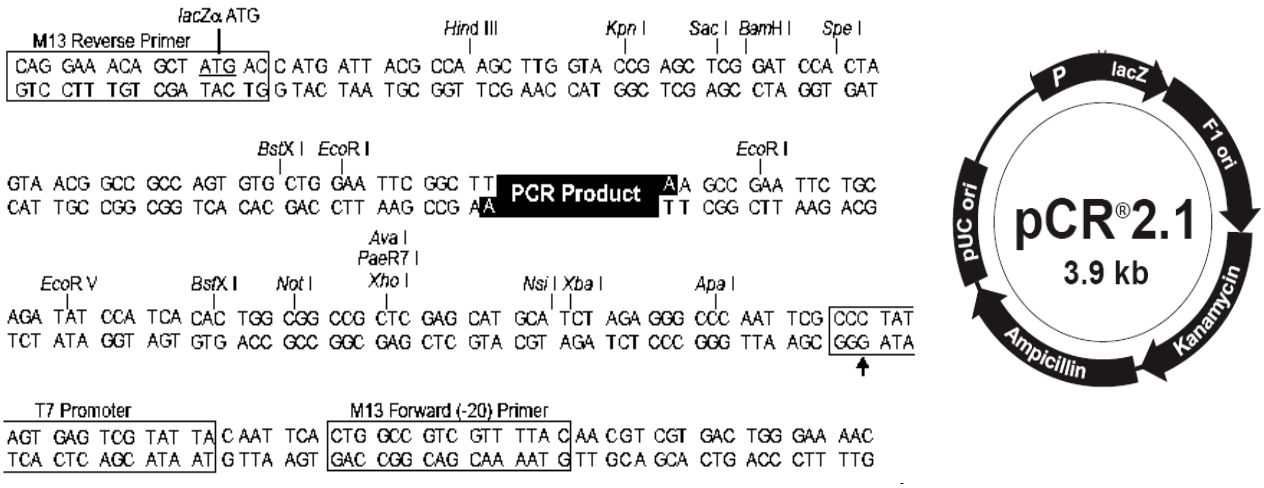

Figure 4. Cloning vector PCR ® 2.1. The vector has genes for resistance to the antibiotics ampicillin (Amp^r), kanamycin (Km^r), and *lacZ*.

Transformed colonies of *E. coli* DH5á were selected on Luria agar supplemented with n 1-mM of Isopropyl â-D-1-thiogalactopyranoside (IPTG) and 40-µg of X-Gal. Plasmids were extracted from transformed *E. coli* DH5á cells using a Wizard® Plus Mini-preps DNA Purification System (Promega) using the protocol supplied by the manufacturer. To confirm whether the transformants contained the desired size of insert, the plasmid DNA was digested to completion with the restriction enzyme *Eco*RI (Gibco/BRL). The digested products were separated by electrophoresis on a 1% agarose gel (Bio-Rad, NJ), stained with ethidium bromide and photographed under UV light. The ABI prism BigDye terminator Cycle sequencing kit was used to further prepare the samples for sequencing. Sequencing was conducted using ABI PRISM™ 377 DNA sequencer. The sequence data were compared with sequences in databases using the program BLAST version 2.0 or 2.1 (http://www.ncbi.nlm.nih.gov/BLAST/-). The program compares nucleotide sequences to databases and calculates the statistical significance of matches.

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

Results and Discussion

Nucleotide similarity comparison: The sequences corresponding to *nif*H gene sequence

were edited, cleaned and assembled using the program Secuencher v 3.0 (Sequencher 3.0 User Manual, 1995). The fragments that showed homology were aligned using the program Clustal version W (1.8). The sequences that correspond to strains designated as 01-36062-R2; 02-36062-H4 and 03-36062-V2, isolated from roots, leaves and stems, respectively, were identical to each other. The sequence analysis demonstrated the presence of *nif*H gene sequences in these sequenced clones, with a similarity of 89% in 283 bp with the *nif*H gene sequence of *Klebsiella pneumoniae* with a GenBank as AF303353.1. Further more the sequences had an 88% similarity with *Klebsiella* sp. Y83 (DQ821727.1) and *Enterobacter* spp. (Y79DQ821726.1) The clone from the endophytic bacterium isolated from *Brachiaria* hybrid CIAT 36061 had a 97% sequence similarity in 290 bp with three accessions registered in the GenBank, designated as DQ982313.1, DQ982300, and DQ982299.1. These sequences correspond to clones isolated from uncultured diazotrophes (nitrogen fixing organisms) isolated from roots and stems of maize plants. Nif genes that encode the nitrogenase complex and other enzymes involved in nitrogen fixation have consensus sequences identical in various nitrogen-fixing bacteria

Sequence comparison: The strains 01-36062-R2; 02-36062-H4, 03-36062-V2, and 36061 were compared with 16 nucleotide sequences that were selected with a maximum identity, Score and E-value, registered in the *GenBank* (Table 16). Furthermore, 18 nucleotide sequences of nitrogen-fixing organisms used in the studies by Franke *et al*., (1998, Lett. Appl. Microbiol. 26:12-16). Reiter *et al*., (2003, Can J. Microbiol. 49: 549-55). Bacteria with other characteristics were also included in these comparisons. Figure 5 clearly shows the sequences analyzed are phylogenetically grouped in three groups, A, B, and C, with high bootstrap values of 80, 82, 100 %, respectively. Group A contained 20 accessions, 8 of these belonging to protobacteria, one actinobacteria, one unidentified bacterium, 6 clones from uncultured organisms, and the four endophytic bacterial strains used in this study.

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

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

Group B consists of 15 accessions subdivided into three subgroups B-1, B-2, B-3. The sub-group B-1 contains 2 species of *Frankia* (a soil-inhabiting nitrogen-fixing bacterium) and one betaproto-bacteria. The subgroup B-2 consists of three accessions that belong to the genus *Azotobacter*,. Sub-group B-3 consists of 9 accessions. The group C consists of 2 species of the genus *Clostridium.* For a better understanding of the endophytic microbial diversity and identity associated with species of *Brachiaria*, it is important to extend the work to include more enophytic bacterial isolates from a number of *Brachiaria* hybrids. This work further complements the results reported in IP-5 Annual Report 2005 on the role of these bacterial endophytes in *Brachiaria* plant growth possibly through nitrogen-fixation.

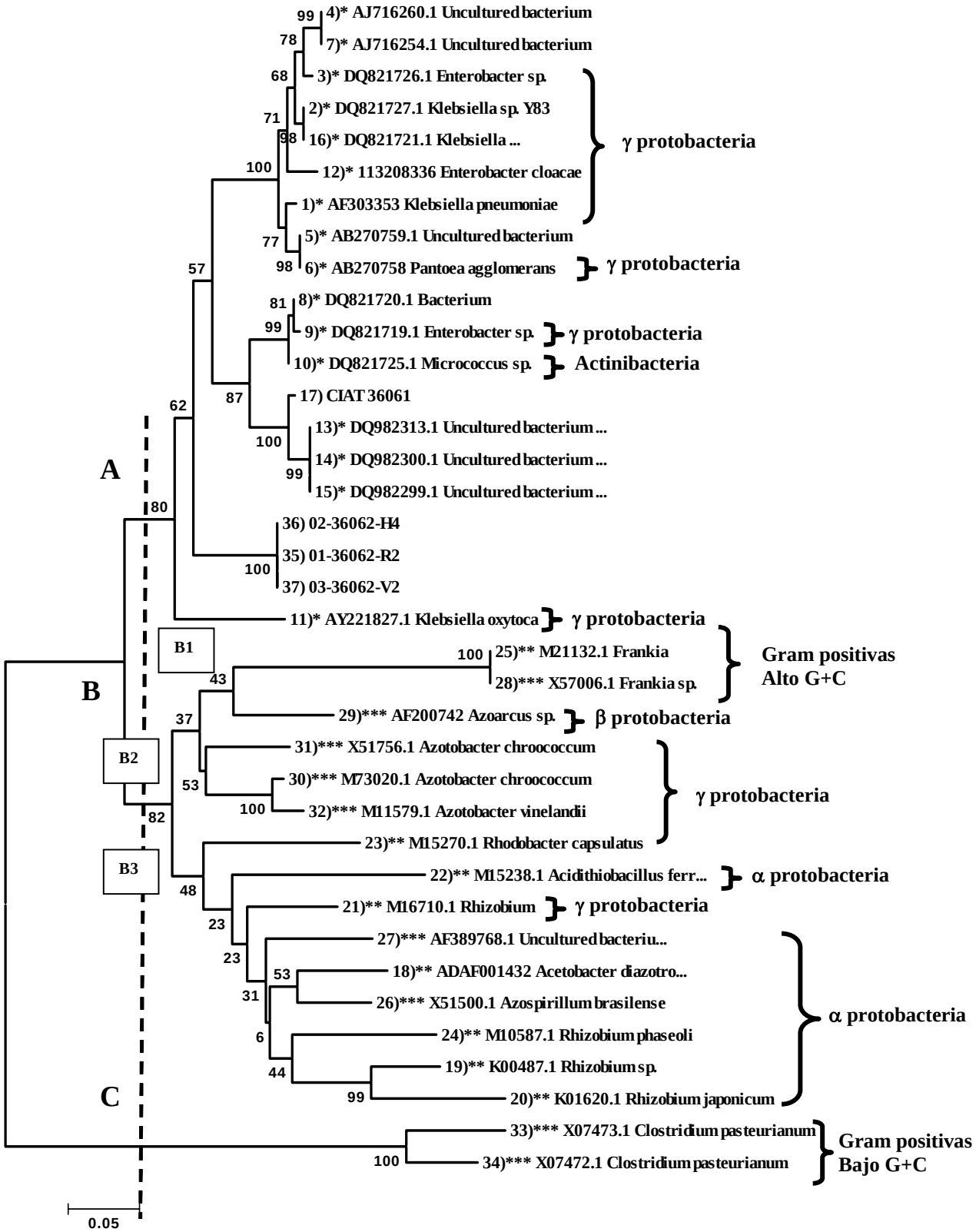

Figure 5. Phylogenetic tree generated for nucleotide sequences evaluated using Neighbor Joining analysis. The values represent 1,000 replications in bootstrap method.

Table 16. Nucleotide sequences and values generated using *GenBank* data for four endophytic bacterial isolates associated with Brachiaria hybrids in relation to other microbes.

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

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