OUTPUT 2: Grasses and legumes genotypes with known reaction to pests and diseases, and interaction with symbiont organisms are developed

Activity 2.1 Bioecology of spittlebug species in contrasting environments

Highlights

- Comparative biological studies completed for two spittlebug species (*Mahanarva andigena*, *Prosapia simulans*) and studies initiated to describe the population dynamics and phenology of *Prosapia simulans*, a newly detected *Brachiaria* pest in the Cauca Valley.
- Host plant water stress and age were found to have no detectable effect on the incidence and duration of diapause in *Aeneolamia varia* eggs.
- Documented an increase in the incidence of egg diapause towards the end of the dry season in field populations of spittlebugs from two seasonal sites with unimodal precipitation; incidence of diapause was minimal throughout the year in a site with bimodal precipitation.

Despite a high pest status and long history in the Neotropics, an effective and coordinated program for the integrated management of spittlebugs in forage grasses does not yet exist. Among the challenges are (1) poor understanding of the natural history of the family Cercopidae, (2) diversity of insect/host/habitat associations, (3) lack of biological information for the majority of economically important species, (4) scarcity of detailed site-specific studies on ecology that offer the resolution necessary to guide advances in pest management, (5) IPM tools that are rudimentary or absent, and (6) rapidly changing pest status.

In 2001 we continued studies to overcome these limitations, focusing on three entry points: (1) the acquisition of new bioecological information on this pest complex and the family Cercopidae, (2) development of five contrasting ecoregions in Colombia as model sites for advancing the diagnosis and management of spittlebugs, and (3) development and evaluation of research methodologies and technologies to promote higher quality research from NARS.

Progress towards achieving output milestones

- **Defined variation in the biology and abundance of spittlebug species in Colombia**
  Previously established research methodologies were implemented to continue characterizing the natural history of the family Cercopidae, the comparative biology of the major spittlebug pests in Colombia, and the population ecology of the spittlebug complex in contrasting sites. These five ecoregions are (1) the Interandean Region of the Cauca River Valley (Dept. Cauca and Valle del Cauca), highly seasonal, bimodal annual precipitation, (2) the Caribbean Coast (Dept. Córdoba and Sucre), highly seasonal, unimodal precipitation, (3) the Orinoquian Piedmont (Dept. Meta), intermediate seasonal, unimodal precipitation, (4) the Amazonian Piedmont (Dept. Caquetá), continuously humid and (5) the South Pacific Coast (Dept. Nariño), continuously humid.

Development of these sites is crucial for linking bioecological information to improvements in pest management. Our research on the spittlebug complex in each of these regions is establishing the patterns of variation in biology, behavior and ecology, fundamental for advancing management by tailoring control tactics to the diverse habitats, regions and production systems where spittlebugs are economically important.

2.1.1 Biology and habits of *Mahanarva andigena*

Contributors: Jairo Rodríguez, Ulises Castro, Oscar Yela, and Daniel Peck (CIAT)

Rationale

Mahanarva andigena was detected for the first time in Colombia in 1999, augmenting the known diversity of spittlebugs associated with graminoids. Up to now, this species is only known in Colombia from the south Pacific coast of Dept. Tumaco at C.I. El Mira of CORPOICA (1º33'10.001 N, 78º42'05.849 W, 50 m elev.). Hosts in that region are *Sorghum halepensis* (Johnson grass) and *Saccharum officinarum* (sugar cane). *Mahanarva andigena* is also known from sugar cane in Ecuador where this spittlebug species is of increasing concern in cane production in the coastal and interior regions of the country.

No biological, behavioral or ecological studies have yet been carried out on this economically important species. We therefore studied certain aspects of the basic biology including description and recognition of the life stages, duration of the life stages and oviposition sites to obtain information on the habits of this species and thereby guide advances in pest management.

Materials and Methods

Biological studies were carried out according to methodologies previously established at CIAT emphasizing morphological characterization of the life stages, duration of the life stages and reproductive biology. To have access to all life stages, a small colony was established in the greenhouse with eggs collected from field-caught adults during a visit to C.I. El Mira. With the aid of a stereoscope and ocular micrometer, certain aspects of the external morphology were measured for four developmental stages of the eggs, five nymphal instars, both sexes of late instar V (Vb) and both adult sexes. Adult specimens were obtained from the field, nymphs from the colony, and eggs from ovipositing adults in the colony.

To measure the duration of the life stages, field conditions were replicated in the screenhouse for controlled observations of adults and nymphs. For the adults, tenerals (<12 hours old) from the colony were confined in cohorts of four individuals under acetate sleeve cages over pots of *Brachiaria ruziziensis*; mortality was assessed daily. For the nymphs, recently emerged first instars (<12 hours old) were placed in individual pots of *B. ruziziensis* established with abundant surface roots required as feeding sites. Transformation from one instar to the next was determined by direct observation of the molted exuvia or the nymph itself. The mean longevity of each life stage was calculated from observations of 40 individuals. Duration of the egg stages was determined under controlled incubation conditions (27°C, 100% RH, total darkness). Recently laid eggs (<24 hours old) were maintained on moist filter paper in petri dishes and observed daily. The mean duration of each of the four generalized developmental stages was calculated from observations on 100 eggs.

To study oviposition sites as part of the description of reproductive biology, field conditions were replicated in the screenhouse. The soil surface was specially prepared with soil oviposition substrate dispersed on top with 2 g leaf litter. Each pot was infested with two females and two males from the colony and 10 days later eggs were recovered from four oviposition substrates: uncovered soil, soil covered by leaf litter, leaf litter and the plant surface.

Results

Mahanarva andigena eggs conformed to the four generalized developmental stages (S1, S2, S3, S4) established for *Aeneolamia varia* and other spittlebug species. Certain externally visible characteristics accompanied these stages. In S2 a spot of red pigment was visible. In S3 the chorion opened to expose the black operculum and the red spot was no longer visible. In S4 two pairs of red spots were visible, the posterior representing the Batelli glands of the abdomen and the anterior representing the eyes of the developing nymph. Each progressive stage was accompanied by a statistically significant increase in both

length and width (Table 13). Total development time was 16.4 days; S2 was the shortest development stage and S4 was the longest (Table 14).

Table 13. Width and length (mm) of development stages of *M. andigena* eggs (mean±S.E., range, n=93-100).

	Development stage			
Parameter	S1	S2	S3	S4
Length	1.22 ± 0.03 a (1.14-1.29)	1.24 ± 0.04 b (1.16-1.34)	1.26 ± 0.03 c (1.20-1.34)	1.30 ± 0.04 d (1.21-1.40)
Width	0.31 ± 0.01 a (0.29 -0.34)	0.33 ± 0.01 b (0.30-0.41)	0.35 ± 0.02 c (0.31-0.39)	0.39 ± 0.01 d (0.37-0.43)

For each parameter, means followed by different letters are significantly different (P<0.05).

Table 14. Duration (days) of *M. andigena* eggs by development stage (mean±S.E., range, n=96-105).

	Development stage				
	S1	S2	S3	S4	Total
Mean±S.E.	4.97 ± 0.46 c	1.57 ± 0.52 a	3.41 ± 0.61 b	6.44 ± 0.60 d	16.39 ± 0.85
Range	(4-6)	(1-3)	(2-5)	(5-8)	(15-19)

Means followed by different letters are significantly different (P<0.05).

Nymphs increased in size from one instar to the next for each parameter measured. There was no overlap in head capsule width or stylet length among the five instars confirming these to be the most useful measures for instar determination (Table 15). Sexual dimorphism was observed in instar Vb (nymphs within a few days of molting to adults) where females were larger than males in all four parameters. Total development time was 46.5 days; instar I was the shortest and instar V was the longest representing 32.4% of the entire nymphal stage (Table 16).

Table 15. Morphological characterization (mm) of nymphal life stages of *M. andigena* (mean±S.E., range, n=15-40).

Instar	Head capsule width	Body length	Anterior wing pad length	Stylet length
I	0.42 ± 0.03 a (0.36-0.46)	1.80 ± 0.30 a (1.21-2.19)	---	0.30 ± 0.02 a (0.29-0.34)
II	0.67 ± 0.02 b (0.61-0.71)	2.90 ± 0.30 b (2.22-3.47)	---	0.39 ± 0.02 b (0.36-0.43)
III	1.01 ± 0.04 c (0.94-1.07)	4.25 ± 0.44 c (3.04-5.09)	0.39 ± 0.03 a (0.34-0.44)	0.59 ± 0.03 c (0.53-0.64)
IV	1.53 ± 0.05 d (1.45-1.63)	6.93 ± 0.80 d (5.14-8.79)	1.05 ± 0.07 b (0.89-1.19)	0.87 ± 0.04 d (0.80-0.95)
Va	2.12 ± 0.10 e (1.96-2.37)	9.57 ± 0.72 e (7.93-11.0)	2.52 ± 0.14 c (2.14-2.79)	1.20 ± 0.04 e (1.13-1.27)
Vb Female	2.27 ± 0.05 f (2.19-2.37)	11.28 ± 1.16 g (8.79-13.71)	2.79 ± 0.16 d (2.36-3.07)	1.26 ± 0.04 f (1.16-1.33)
Vb Male	2.09 ± 0.08 e (1.93-2.28)	10.70 ± 0.84 f (9.29-12.64)	2.57 ± 0.16 c (2.14-2.86)	1.19 ± 0.03 e (1.13-1.30)

For each column, means followed by different letters are significantly different (P<0.05).

The behavior of nymphs differed from other Colombian species studied to date because nymphs of all age classes sought feeding sites in the upper portions of the plant such as leaf axils. This behavior resulted in large cohabited spittle masses and increased aggregation of individuals. The behavior in laboratory is

similar to observations on the two known hosts in the field and to reports from sugar cane studies in Ecuador.

Table 16. Duration (days) of *M. andigena* nymphs by instar (mean, n=40).

	Instar					
	I	II	III	IV	V	Total
Mean±S.E.	6.35 ± 1.03 a	8.64 ± 1.10 c	8.18 ± 1.30 b	10.14 ± 1.50 c	15.05 ± 3.80 d	46.52 ± 9.85
Range	(5-8)	(7-10)	(8-11)	(8-13)	(9-23)	(41-54)

Means followed by different letters are significantly different (P<0.05).

Adults were significantly larger than instar Vb nymphs of the same sex in terms of head capsule width and forewing length, but smaller in terms of body length without wings and stylet length (P<0.05). Sexual dimorphism was observed in the adults, expressed as the greater size of females in every parameter measured (Table 17). Overall adult longevity was 24.0 ± 11.1 days with 25.5 ± 12.9 (8-27) days for females and 20.6 ± 9.0 (8-27) for males (difference not statistically significant). Under the conditions of this study, duration of the life cycle of *M. andigena* was 74.9 d (=16.4+46.5+12.0, egg+nymph+½ adult).

Table 17. Morphological characterization (mm) of *M. andigena* adults by sex (mean±S.E., range, n=40).

Sex	Head capsule width	Stylet length	Body length with wing	Body length without wing	Anterior wing length	Body width
Female	2.54 ± 0.08 a	1.14 ± 0.06 a	10.97 ± 050 a	10.16 ± 0.92 a	8.61 ± 0.45 a	5.19 ± 0.28 a
	(2.29 – 2.64)	(1.05 – 1.28)	(9.71 –11.93)	(8.43 – 12.00)	(7.71 – 10.07)	(4.14 – 5.71)
Male	2.28 ± 0.09 b	1.03 ± 0.06 b	9.96 ± 0.46 b	9.05 ± 0.78 b	7.95 ± 0.32 b	4.72 ± 0.24 b
	(2.07 – 2.50)	(0.91 – 1.14)	(9.07 – 10.71)	(7.14 – 10.36)	(7.21 – 8.50)	(4.21 – 5.21)

For each column, means followed by different letters are significantly different (P<0.05).

Mahanarva andigena exhibited some flexibility in oviposition substrates. Most eggs (67.6%) were recovered from the soil with 51.2% from uncovered soil and 16.4% from soil under litter. Nearly a third of eggs (32.4%), however, was recovered from the plant surface. None were recovered from leaf litter.

Discussion

Like other graminoid spittlebugs studied to date, *M. andigena* eggs pass through four egg development stages that increase in size and are distinguished by externally visible characteristics; nymphs pass through five morphologically distinguishable instars best differentiated by width of the head capsule and stylet length; and adults exhibit sexual dimorphism expressed as greater size of females.

The life cycle of 74.9 d is longer than other spittlebugs studied to date with the same methodology, including *Aeneolamia* (45.3-52.6 d) and *Zulia* (61.5-69.6 d), and is 9.3 days longer than its congener *Mahanarva* sp. nov. *Prosapia simulans* is the only other described Colombian species with a life cycle >70 days.

A preference for laying eggs in the soil is common to most other species studied (*A. lepidior, A. reducta, A. varia, Mahanarva* sp. nov., *Z. carbonaria, Zulia* sp. nov.). The tendency of *M. andigena* to lay eggs (32.4%) on the surface of the plant stem is greater in *Z. pubescens* (59.2%) and *P. simulans* (82.6%).

2.1.2 Biology and habits of *Prosapia simulans*

Contributors: Jairo Rodríguez, Ulises Castro, Anuar Morales, and Daniel Peck (CIAT)

Rationale

The first detection of the Central American spittlebug, *Prosapia simulans*, in Colombia has serious economic ramifications for ranchers and cane producers of the Cauca River Valley. The insect has already reached economically damaging levels in *Brachiaria* pastures in the Dept. Valle del Cauca. In sugar cane, *P. simulans* represents a potential threat since it is the second most important spittlebug cane pest in Central America and since changing cultural practices in Cauca Valley cane production (prohibition of burning) may enable this species to get a foothold in cane fields. Up to now spittlebugs have not been present in sugar cane of this region.

We have previously documented that *P. simulans* occurs over a large elevation range, extending from the Cauca Valley floor (1100 m elev.) to just over the western cordillera of the Andes (1621 m elev.). Multiple visits to the same farms have shown *P. simulans* populations to be persistent especially in improved pastures of *Brachiaria decumbens*. Despite its importance in pastures and cane of Central America, little is known about this insect's biology and ecology. To support advances in management, we carried out initial biological studies of *P. simulans* in the Cauca Valley focusing on differentiation of the life stages, duration of the life stages and reproductive biology.

Materials and Methods

The biology of *P. simulans* was characterized using previously established methods. To differentiate among the life stages, these were characterized morphologically using different measures of body size. To quantify duration of the life stages, the development of individual eggs, nymphs and adults was observed under controlled conditions. To begin to describe the reproductive biology, oviposition site preferences were determined.

Results

The eggs conformed to the four generalized developmental stages (S1, S2, S3, S4) described in other spittlebug species. Both size and width of eggs increased from one stage to the next (Table 18). Total development time was 18.0 days; S2 was the shortest development stage and S1 the longest (Table 19). Diapause was not detected among individuals of the study population, however diapause during stage S2 was observed in eggs collected from a lower elevation site during the course of other studies (1100 m elev., Santa Helena, Dept. Valle del Cauca); maximum time to eclosion of these diapause eggs was 128 days.

Table 18. Width and length (mm) of development stages of *P. simulans* eggs (mean±S.E., range, n=75-100).

	Development stage			
Parameter	S1	S2	S3	S4
Length	1.16 ± 0.03 a (1.09-1.24)	1.18 ± 0.03 b (1.10-1.26)	1.21 ± 0.03 c (1.14-1.30)	1.25 ± 0.03 d (1.19-1.34)
Width	0.32 ± 0.02 a (0.29-0.36)	0.34 ± 0.01 b (0.31-0.37)	0.39 ± 0.03 c (0.30-0.47)	0.42 ± 0.01 d (0.39-0.46)

For each parameter, means followed by different letters are significantly different (P<0.05).

Table 19. Duration (days) of *P. simulans* eggs by development stage (mean±S.E., range, n=66-100).

	Development stage				
	S1	S2	S3	S4	Total
Mean±S.E.	6.90 ± 1.09 d	2.13 ± 1.69 a	3.98 ± 0.77 b	5.18 ± 0.58 c	17.99 ± 1.27
Range	(6-13)	(1-9)	(2-5)	(4-7)	(16-23)

Means followed by different letters are significantly different (P<0.05).

For the nymphs, each of the morphological parameters measured (head capsule width, body length, anterior wing pad length, stylet length) increased in size from one instar to the next (Table 20). There was no overlap in head capsule width among the five instars confirming this to be the most diagnostic character for instar determination. Total development time was 45.6 days; instar I was the shortest and instar V was the longest, representing 28.8% of the entire nymphal stage (Table 21).

Table 20. Morphological characterization (mm) of nymphal life stages of *P. simulans* (mean±S.E., range, n=40).

Instar	Head capsule width	Body length	Anterior wing pad length	Stylet length
I	0.45 ± 0.02 a (0.36-0.50)	1.66 ± 0.17 a (1.24-1.96)	-	0.36 ± 0.04 a (0.28-0.44)
II	0.65 ± 0.03 b (0.50-0.69)	2.33 ± 0.31 b (1.55-2.88)	-	0.47 ± 0.03 b (0.37-0.52)
III	0.96 ± 0.03 c (0.89-1.01)	3.16 ± 0.21 c (2.64-3.70)	0.32 ± 0.02 a (0.27-0.37)	0.62 ± 0.05 c (0.53-0.73)
IV	1.42 ± 0.06 d (1.28-151)	5.99 ± 0.57 d (4.91-7.52)	0.90 ± 0.08 b (0.71-1.10)	0.97 ± 0.04 d (0.89-1.04)
Va	1.92 ± 0.08 e (1.78-2.13)	7.79 ± 0.43 e (6.64-8.50)	2.23 ± 0.13 c (1.78-2.43)	1.16 ± 0.05 e (1.04-1.27)
Vb Female	2.01 ± 0.08 f (1.84-2.13)	8.12 ± 0.95 e (6.07-10.86)	2.27 ± 0.13 c (1.78-2.55)	1.30 ± 0.05 f (1.19-1.39)
Vb Male	1.92 ± 0.09 e (1.78-2.19)	7.99 ±0.65 e (6.43-9.43)	2.26 ± 0.09 c (1.99-2.43)	1.28 ± 0.06 f (1.04-1.42)

For each column, means followed by different letters are significantly different (P<0.05).

Table 21. Duration (days) of *P. simulans* nymphs by instar (mean, n=40).

	Instar					
	I	II	III	IV	V	Total
Mean±S.E.	6.75 ± 1.16 a	7.54 ± 2.16 a	9.30 ± 2.79 b	10.04 ± 2.26 b	13.14± 2.70 c	45.59 ±5.45
Range	(5-11)	(4-13)	(5-17)	(5-14)	(10-20)	(35-57)

Means followed by different letters are significantly different (P<0.05).

Adults were larger than instar V in all parameters with the exception of male body length and male and female stylet length (shorter in adults). Sexual dimorphism was observed in the adults, expressed as the greater size of females in every parameter measured with the exception of forewing length (Table 22). Overall adult longevity was 17.8 ± 8.2 days with 19.9 ± 8.6 (6-32) days for females and 14.5 ± 5.1 (5-21) days for males (difference not statistically significant). Under the conditions of this study, duration of the life cycle of *P. simulans* was 72.5 days (=18.0+45.6+8.9, egg+nymph+½ adult).

Prosapia simulans exhibited a marked preference for laying eggs on the surface of the plant stem; 82.6% of eggs were recovered from this substrate. Only 17.4% was recovered from the soil with 3.6% from uncovered soil and 13.8% from soil under litter. No eggs were recovered from leaf litter.

Table 22. Morphological characterization (mm) of *P. simulans* adults by sex (mean±S.E., range, n=40).

Sex	Head capsule width	Stylet length	Body length with wing	Body length without wing	Anterior wing length	Body width
Female	2.31 ± 0.06 a (2.21-2.43)	0.98 ± 0.33 a (0.89-1.16)	8.71 ± 0.33 a (7.29-9.29)	8.18 ± 0.61 a (7.29-9.29)	6.80 ± 0.22 a (6.36-7.21)	4.63 ± 0.15 a (4.36-5.07)
Male	2.04 ± 0.06 b (1.93-2.14)	0.89 ± 0.03 b (0.82-0.94)	8.52 ± 0.31 b (7.36-9.29)	7.23 ± 0.32 b (6.57-8.14)	6.84 ± 0.28 a (5.93-7.43)	4.16 ± 0.14 b (3.79-4.43)

For each column, means followed by different letters are significantly different (P<0.05).

Discussion

Prosapia simulans conforms to the developmental and morphological patterns established in graminoid spittlebugs (see section 2.1.1) including diapause expressed as an extended S2 egg stage. The life cycle of 72.5 days in the Cauca Valley is longer than what is reported from Central America (58.4 and 58.0 days). It is also longer than all other species studied to date in Colombia (*Aeneolamia lepidior, A. reducta, A. varia, Mahanarva* sp. nov., *Z. carbonaria, Zulia pubescens, Zulia* sp. nov.), comparable only to *M. andigena* from the south Pacific coast (74.9 days; see section 2.1.1). The marked preference for oviposition sites on the plant stem is different from other Colombian species studied to date which all prefer to oviposit in the soil. Only *Z. pubescens* has also been shown to lay a majority of eggs (59.2%) on the plant stem.

2.1.3 Population dynamics and phenology of *Prosapia simulans*

Contributors: Jairo Rodríguez, Ulises Castro, Anuar Morales, Oscar Yela, and Daniel Peck (CIAT)

Rationale

The graminoid spittlebug, *Prosapia simulans*, is a new arrival to the Cauca Valley of Colombia and has been detected in four municipalities to date: Santander de Quilichao (Dept. Cauca), Cerrito, Calima Darién and Yotoco (Dept. Valle del Cauca). In many of the sites where it has been detected, *P. simulans* shares pastures with *Zulia carbonaria* and *Zulia pubescens* particularly where *Brachiaria decumbens* is the dominant forage grass host.

In general, the management of graminoid spittlebugs has been compromised by a lack of bioecological information specific to the species and habitats of concern, and by a tendency to over generalize among the diverse insect/host/habitat associations in which these pests have economic impact. Advances in spittlebug management requires a detailed understanding of aspects such as differentiation and duration of the life stages, correspondence between population fluctuations and precipitation, habitat and host plant preferences, and the incidence of natural enemies.

Biological studies on *P. simulans* in the Cauca River Valley have been initiated. In this report we summarize recent phenological studies of *P. simulans*. This research was carried out to provide baseline data for field studies on fungal entomopathogens and assesses various components of population ecology including population fluctuation, diapause, natural enemies and precipitation.

Materials and Methods

Observation plots were established in *B. decumbens* pastures at Hacienda Piedechinche, Municipality El Cerrito, Dept. Valle del Cauca. Methods for surveying nymphs, adults, diapause eggs and natural enemies were modified from previously established protocols used in studies with similar objectives (CIAT Annual Report 1999, 2000).

Five 0.16-ha plots were established, each in a different paddock and under the same typical management regime of the farm in terms of grazing pressure, fertilization and weed suppression. To facilitate sampling, each plot was divided into 16 subplots in which nymphs were collected from two 0.0625m^2 quadrats to measure absolute density, and adults from two series of 10 sweeps of an insect net to measure relative density. All nymphs were determined to instar and adults to species and sex.

The abundance and incidence of natural enemies were measured as part of the same surveys. Surveys were carried out once weekly and were initiated 25 January 2001. The first 7 months (through 30 August 2001) are summarized in this report. Data were analyzed to determine patterns and variation at the farm level in population fluctuation, correspondence with precipitation, population synchrony, number of generations and incidence of natural enemies.

To help interpret phenology, parallel data were collected on the incidence and duration of egg diapause, a physiological condition that enables the insect to synchronize its life cycle with the humid environmental conditions necessary for development and reproduction. Every 15 days a group of females (1-10 individuals, depending on availability) was collected from each plot and allowed to oviposit for a period of 3 days in moist filter paper lining the bottom of a large petri dish. Petri dishes and their eggs were kept under incubation (27^{o}C, 100% RH, total darkness) and evaluated twice weekly for empty chorions (indicating nymphal emergence) and inviable eggs. The incidence and duration of diapause were quantified with eggs eclosing after 30 days considered diapausing.

Results

Over this 7-month period a total of 190 nymphs and 1465 adults were collected. Both life stages were found on every survey date until julian day 172 and 179 when nymphs and adults, respectively, were no longer detected. Abundance of these life stages coincided with the wet season, in particular March-May, historically the three wettest months of the year where 35.6% of the total annual precipitation falls. Nymphs and adults disappeared in the driest months of June-August when the insect presumably survives as diapausing eggs.

At the farm level (combined plot data), *P. simulans* exhibited one well-differentiated pair of nymph and adult population peaks (Figure 3). The major nymph peak occurred days 81-130 and was preceded and followed by periods of abundance days 25-67 and 158-164. The major adult peak occurred days 109-158 but was also preceded by the apparent tail end of a previous peak days 25-38.

These results suggest a pair of population peaks at the start of the new year just before initiation of the surveys. Although these population peaks probably represent discrete and consecutive generations a precise interpretation depends on future analysis according to nymphal life stages.

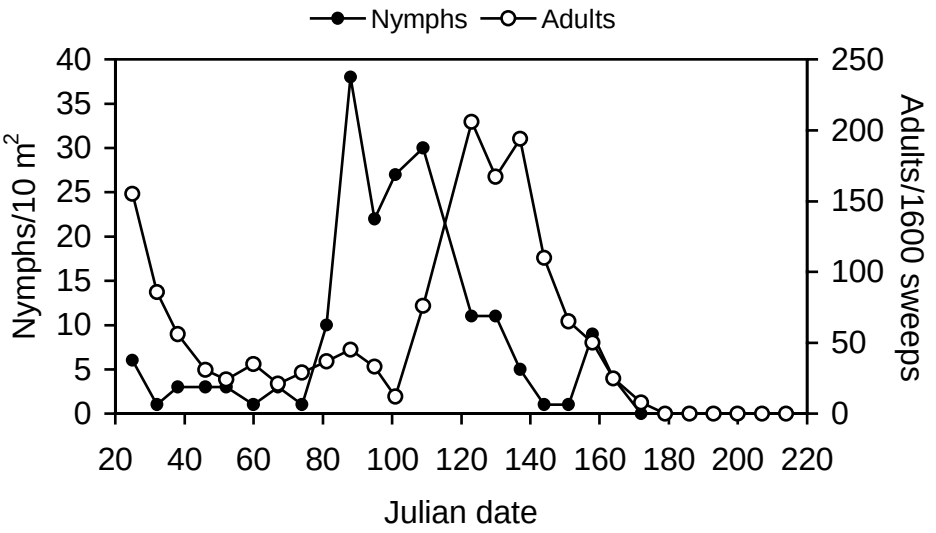

Figure 3. Population fluctuations of nymph and adult *P. simulans* populations in Piedechinche, Dept. Valle del Cauca in 2001.

Fluctuation curves for the five individual plots reveal the degree of on-farm variation in spittlebug phenology (Figures 4, 5). In terms of nymph abundance, the plot with the highest insect load (Plot 2) had 4.7 times more than the plot with the lowest (Plot 5), or 32.1% versus 6.8% of the nymph population. For the adult life stage Plot 1 ranked first and Plot 5 again ranked last with 7.4 times more adults in Plot 1, or 37.4 and 5.1% of the adults, respectively. All plots had the same general periods of peak abundance as in the overall farm fluctuation curves. However, some individual plots exhibited evidence of bimodal peaks in the period March-April. This included nymphs in Plots 2 and 4 and adults in Plots 1, 2 and 4. This suggests overlapping generations that are obscured in the overall farm fluctuation curve.

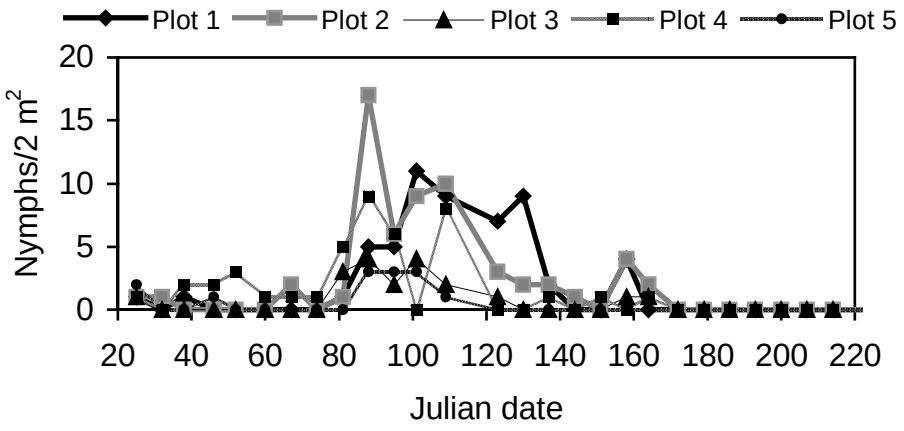

Figure 4. Population fluctuation of *P. simulans* nymphs in five *B. decumbens* pastures, Piedechinche, Dept. Valle del Cauca (2001).

Very few natural enemies were detected. Only parasitic mites (Acari: Erythraeidae) on adults were found over the survey period. Overall, 9.5% of adults had mites with a maximum of 10 mites per individual. For the entire population of adults mite load was 0.19 per adult or 0.20, 0.47, 0.21 and 0.40 per adult over

the months of March, April, May and June, respectively. Mite load according to sex was 0.19 per male and 0.16 per female.

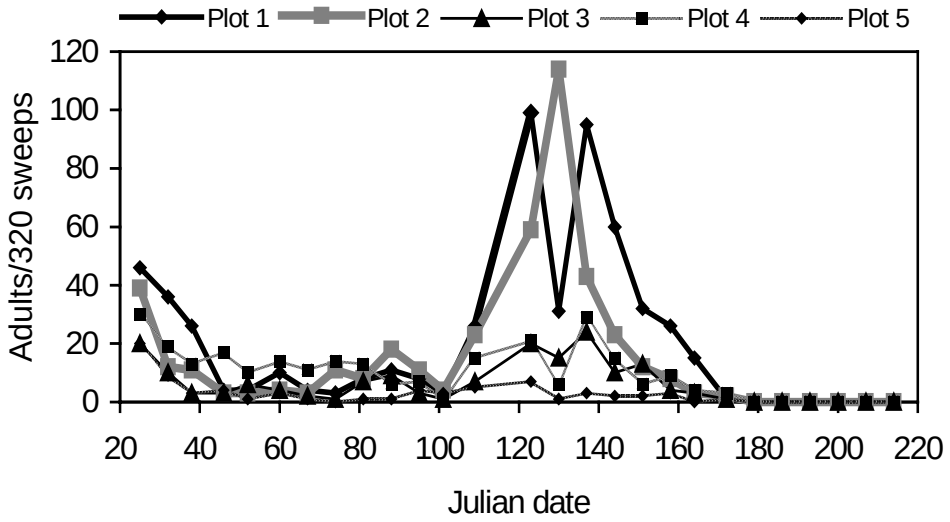

Figure 5. Population fluctuation of *P. simulans* adults in five *B. decumbens* pastures, Piedechinche, Dept. Valle del Cauca (2001).

For the 1249 eggs collected over eight dates during the first five months of the study (January-May), overall mortality was 5.5% and incidence of diapause eggs was 69.8%. With the exception of two dates (22 March, 31 May), the proportion of eggs in diapause exceeded 70% in each collection date (Table 23). There was a trend toward lower diapause incidence in the second half of the period. Eggs eclosed over a period of 18-128 days (Figure 6). Mean time to eclosion was 24.0 days for non-diapausing and 79.5 for diapausing eggs. There was a trend towards longer eclosion times for eggs in the second half of the period.

Table 23. Seasonal changes in the incidence and time to eclosion of nondiapausing and diapausing *P. simulans* eggs in Piedechinche, Dept. Valle del Cauca (2001).

Collection		Proportion (%)		Time to eclosion all eggs (days)	
Date	n	Nondiapause	Diapause	Mean ± S.E.	Range
22 Jan	204	6.4	93.6	43.0 ± 14.89	18-68
10 Feb	138	14.5	85.5	40.0 ± 11.98	20-60
23 Feb	84	3.6	96.4	53.0 ± 15.44	27-79
15 Mar	46	0.0	100.0	63.0 ± 2.16	60-66
22 Mar	69	100.0	0.0	23.0	---
5 Apr	190	29.5	70.5	74.5 ± 31.32	21-128
3 May	482	26.6	73.4	67.0 ± 28.72	18-116
31 May	36	61.1	38.9	48.5 ± 13.42	26-71
Overall	1249	30.2	69.8	18-29	30-128

Discussion

Prosapia simulans populations coincided with the wetter months of this initial survey period (January-May), and then declined and disappeared coincident with the dry season. The end of an initial generation and a complete second generation was documented based on nymph and adult peaks, but a precise

determination of generations depends on future analysis of the separate life stages of the nymphs. This further analysis will also shed light on apparent phenological differences observed among the five survey plots and help us measure the degree of on-farm variation.

The majority of eggs collected over this period were diapausing despite the apparently adequate humid conditions for population development. This differs from other species studied to date that exhibit very little diapause during the wet season. The relationship between the incidence of diapause and season for *P. simulans* is unclear and requires continued studies.

It is expected, for example, that we will document an even higher incidence of diapause in eggs collected from the field in June, start of the dry season. These methods should prove adequate for documenting the phenology of *P. simulans* populations in the field for the first time in Colombia. The new information should help us interpret the relationship between habitat and spittlebug presence and lead to predictions of the spatial and temporal arrival of outbreaks.

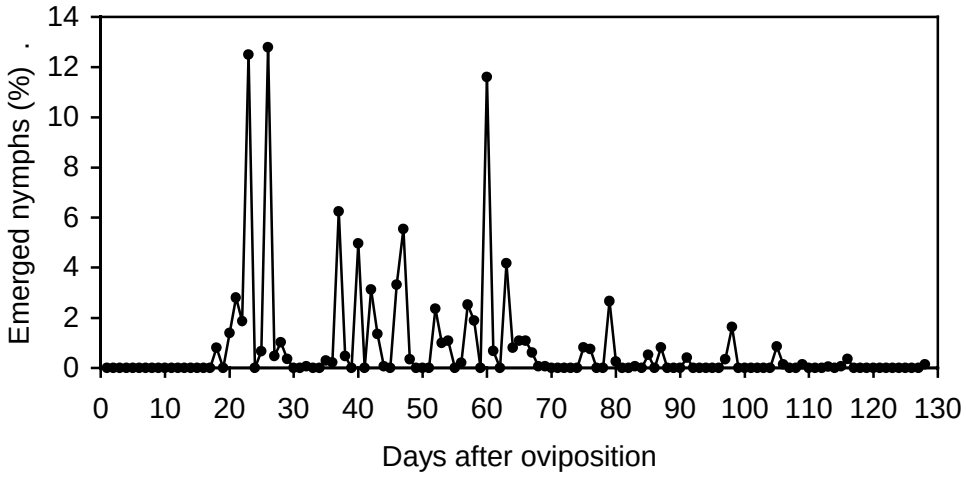

Figure 6. Pattern of eclosion of *P. simulans* eggs summed over eight collection dates (every two weeks) from January to May 2001 in Piedechinche, Dept. Valle del Cauca.

2.1.4 Population dynamics and phenology of *Zulia carbonaria*

Contributors: Ulises Castro, Anuar Morales, and Daniel Peck (CIAT)

Rationale

Over the past several years the impact of spittlebugs has apparently increased in forage grasses of the Interandean valleys and hillsides of Colombia, such as pastures of *Brachiaria* spp. in the Cauca River Valley. This area has a bimodal precipitation pattern and thereby represents an environment for studying spittlebug seasonality that is distinct from previously studied lowland sites of the highly seasonal Caribbean coast, intermediate seasonal Orinoquian Piedmont, and the continuously humid Amazonian Piedmont.

The first information on the phenology of the spittlebug complex in the Cauca Valley was presented in 2000 (CIAT Annual Report). In this report we summarize results from detailed population surveys of the spittlebug *Zulia carbonaria* over two complete years.

Materials and Methods

This study was carried out on a representative farm of the Cauca River Valley, Hacienda Las Palmas, Municipality Santander de Quilichao, Dept. Cauca. This site featured pastures of *Brachiaria decumbens* in association with the forage legume *Centrosema* sp. The methods were the same as in previously established protocols. Three 0.5-ha plots were established in separate pastures and divided into four subplots (0.125 ha) to facilitate sampling. Nymph surveys comprised counts in two 0.25m^2 quadrats in each subplot while adult surveys comprised 50 sweeps of an insect net in each subplot. Nymphs were counted and classified to life stage while adults were counted and classified to sex and species. Natural enemies were also recorded and identified.

Surveys were carried out weekly during two years (20 January 1999 to 19 January 2001). Data were analyzed to determine patterns and variation at the farm level in population fluctuation, correspondence with precipitation, population synchrony, number of generations and incidence of natural enemies.

Results

A total of 10,546 nymphs and 2,247 adults were collected during the course of this study. With the exception of one female *Prosapia simulans*, all adults were *Zulia carbonaria*. The abundance, or insect load, of *Z. carbonaria* varied greatly from one year to the next; there were 6.2 and 3.0 times more nymphs and adults, respectively, in 1999 compared to 2000 (Table 24).

Table 24. Variation in insect load of *Z. carbonaria* between years and among plots in Santander de Quilichao, Dept. Cauca.

		Insect load[1]	
Year	**Plot**	**Nymphs**	**Adults**
1999	Plot 1	760	253
	Plot 2	5704	754
	Plot 3	2612	676
	Sum	9076	1683
2000	Plot 1	703	251
	Plot 2	218	106
	Plot 3	549	207
	Sum	1470	547
Overall		10,546	2,247

[1]Measured as total number of individuals collected in surveys

There was also significant variation among the individual plots in total abundance. Insect load was 7.5 and 3.0 times greater for nymphs and adults, respectively, between the plot of lowest (Plot 1) and highest (Plot 2) abundance in 1999, and 3.2 and 2.4 times for 2000 (Plot 2 versus Plot 1). The plots of highest and lowest abundance were not consistent from one year to the next, in fact their ranking switched between 1999 and 2000.

Nymph and adult populations were most abundant during the first half of each year, coincident with the wettest months. *Z. carbonaria* essentially disappeared the second half of the year in 1999 after the two extremely dry months of June and July (Figure 7, Table 25).

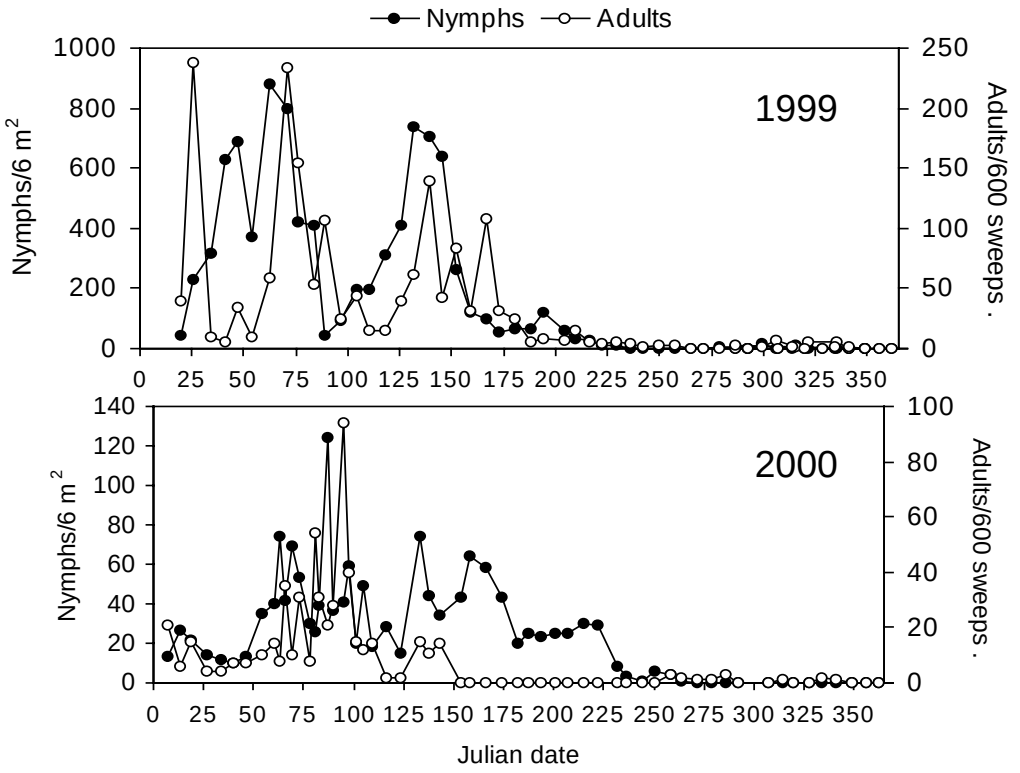

Figure 7. Population fluctuation of nymph and adult *Z. carbonaria* populations in Santander de Quilichao, Dept. Cauca over two years.

Table 25. Monthly precipitation in survey sites, Santander de Quilichao, Dept. Cauca.

	Precipitation (mm)			
Month	Mean (11 years)	1998	1999	2000
Jan	155.9	20.0	305.0	241.7
Feb	141.8	126.0	345.0	179.8
Mar	216.9	125.2	265.0	327.3
Apr	251.0	304.0	236.3	319.9
May	163.7	210.0	170.7	230.6
Jun	83.6	32.0	120.2	148.0
Jul	51.4	76.0	21.5	71.5
Aug	56.1	128.0	53.2	72.2
Sep	135.9	277.0	202.6	173.1
Oct	181.6	248.0	153.0	121.0
Nov	238.6	414.0	183.7	159.0
Dec	150.0	70.0	147.8	163.0

Populations did not recover until early 2000. Populations again declined severely after the dry months of June and July in 2001.

In 1999, population fluctuation curves revealed three well-defined peaks for each plot with a correspondence between nymph peaks and the subsequent adult peaks (Figure 8). In 2000, peaks were much less defined (Figure 9). To more precisely interpret these data and resolve different generations, population data were analyzed according to nymphal life stage. Recruitment patterns from one life stage to the next revealed three generations of *Z. carbonaria* in 1999 and four in 2000. Cumulative insect day calculations were used to quantify the arrival of discrete generations of nymphs and adults for each plot. Peak abundance was designated as the date of 50% accumulation of the insect days or the area under the population fluctuation curve. In 1999 there was little variation in the timing of generations across plots. The three generations of nymphs peaked at a mean of julian day 52.7, 132.0 and 194.0 while adults peaked at 66.0, 144.0 and 207.0 (Table 26). The time between subsequent nymph peaks and adult peaks is the generation time calculated as a mean of 70.6 (n=12, range 45-89) for the farm in 1999 with little variation among plots (Table 27).

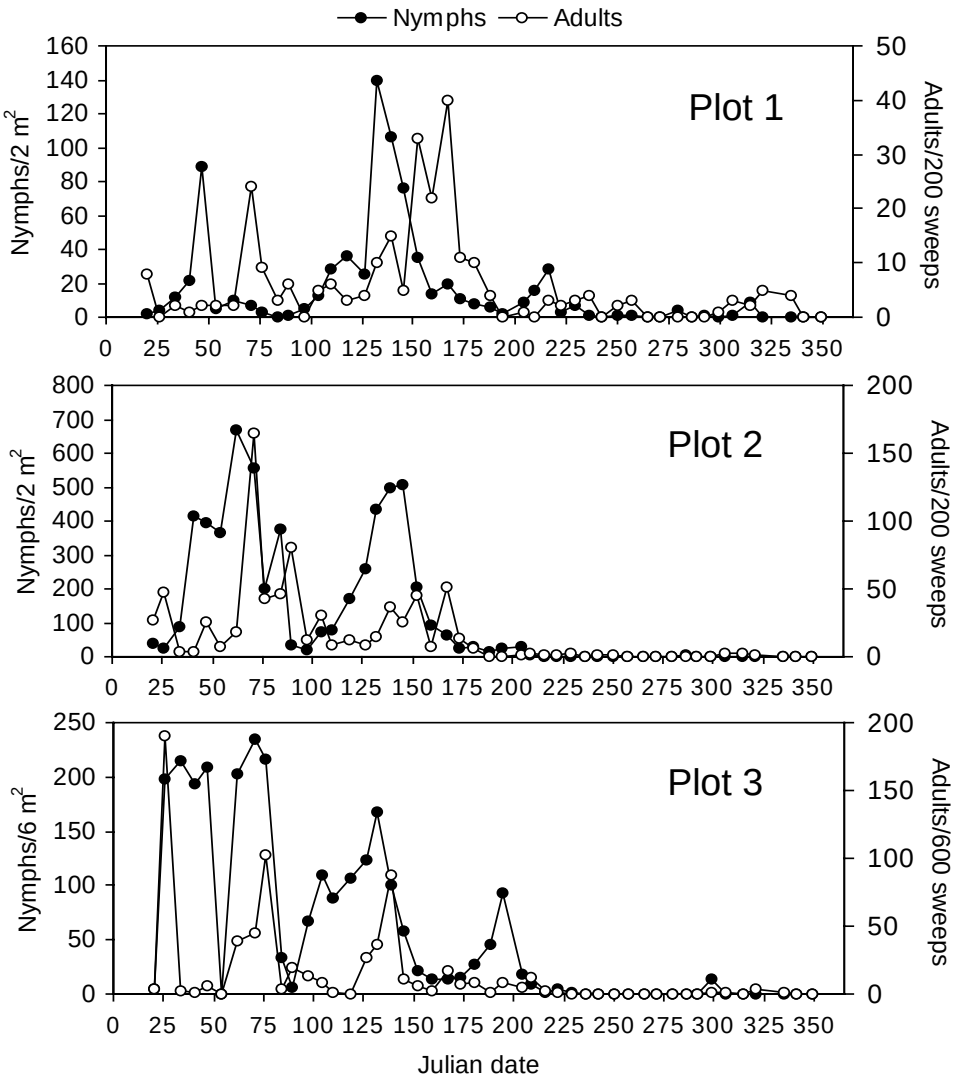

Figure 8. Population fluctuations of *Z. carbonaria* nymphs and adults in three survey plots in Santander de Quilichao, Dept. Cauca (1999).

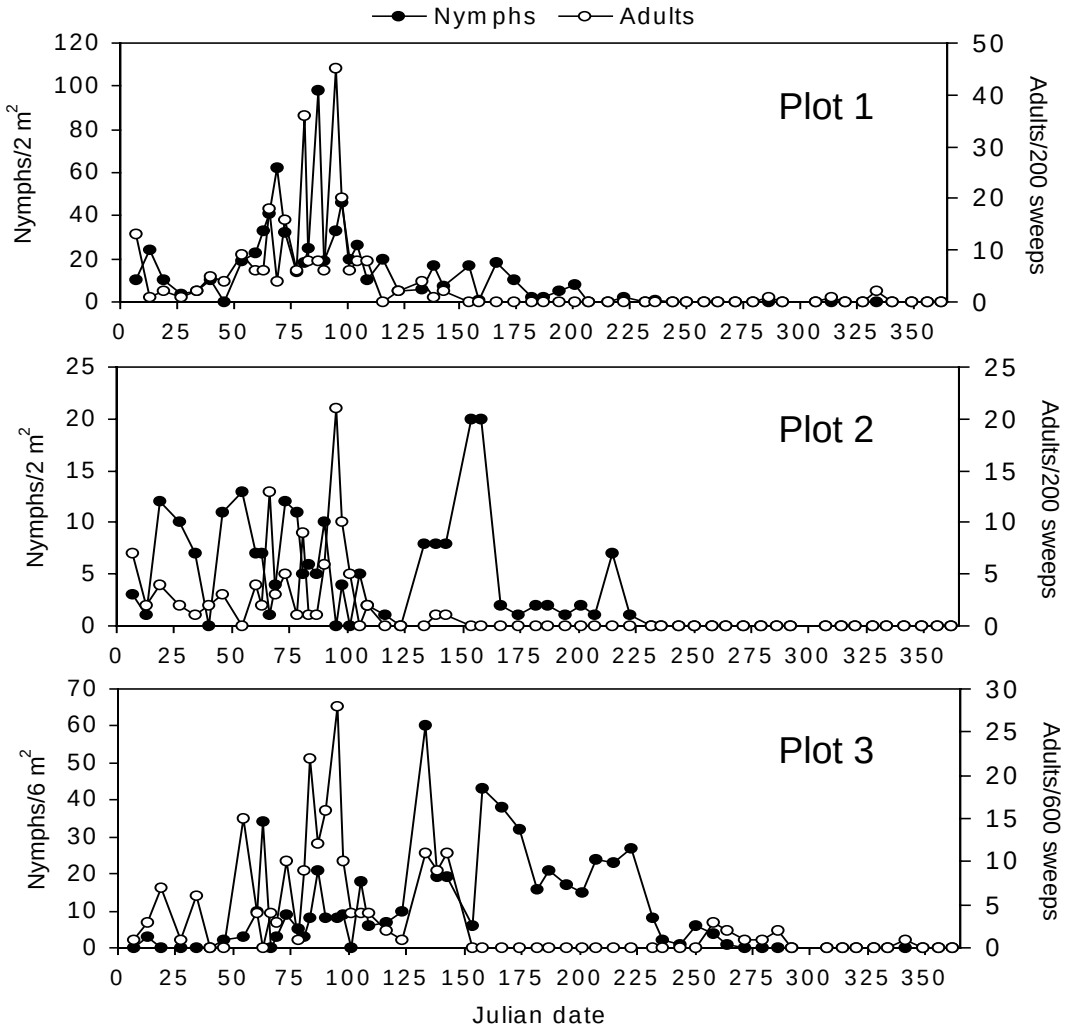

Figure 9. Population fluctuations of *Z. carbonaria* nymphs and adults in three survey plots in Santander de Quilichao, Dept. Cauca (2000).

In 2000, the phenology of spittlebug populations was best interpreted as two overlapping periods of emergence of the initial generation. The first outbreak of nymphs was day 17 leading to adults day 37. The next peak of nymphs was day 61, too early to represent progeny of the previous adult generation, and thereby probably representing the eclosion of an additional group of eggs that were late in contributing to the first generation. These dual peaks led to early and late groups of a second and third generation, and then lost any detectable separation in the fourth generation. With this interpretation, the mean generation time for 2000 was calculated as 63.9 (n=18, range 45-83) days (Table 27).

Over both years (1999 and 2000), mean generation time was 67.3 days (n=31), corresponding very well with the time determined from greenhouse biology studies (69.6 days).

Table 26. Time of arrival (calculated as 50% cumulative insect days) of *Z. carbonaria* populations in three survey plots, Santander de Quilichao, Dept. Cauca.

			50% cumulative insect days (julian date)			
Year	Generation	Life stage	Plot 1	Plot 2	Plot 3	Mean
1999	1	Nymph	47	60	51	52.7
		Adult	70	71	57	66.0
	2	Nymph	136	136	124	132.0
		Adult	156	140	136	144.0
	3	Nymph	205	185	192	194.0
		Adult	201	215	205	207.0
2000	1 a	Nymph	17	24	10	17.0
		Adult	48	38	25	37.0
	1 b	Nymph	67	53	63	61.0
		Adult	77	66	61	68.0
	2 a	Nymph	94	77	93	88.0
		Adult	97	94	92	94.3
	2 b	Nymph	142	106	133	127.0
		Adult	140	107	138	128.3
	3 a	Nymph	168	132	170	156.7
		Adult	---	140	---	140.0
	3 b	Nymph	195	154	215	188.0
		Adult	---	---	---	---
	4	Nymph	229	205	255	217.0
		Adult	---	---	267	---

Table 27. Generation time of *Z. carbonaria* calculated from population dynamics studies in three survey plots, Santander de Quilichao, Dept. Cauca.

	Generation time (days)			
Year	Plot 1	Plot 2	Plot 3	Overall
1999	72.3	67.3	72.3	70.6
N	4	4	4	12
Range	45-89	49-76	68-79	45-89
2000	65.2	50.4	76.0	63.9
N	6	7	6	19
Range	49.1-77.7	40.7-55.7	66.9-82.8	44.7-82.8
Overall	68.7	58.8	74.15	67.3
N	10	11	10	31

Discussion

Zulia carbonaria achieves 3-4 generations a year in *Brachiaria* pastures of the Cauca River Valley, increasing in abundance at the onset of the wet season, and decreasing with the dry season. Small populations were still detectable during the driest months indicating that the insect is capable of finding microhabitat suitable for maintenance of nymphs and adults despite the dry pasture conditions. The dramatic decline in population between 1999 and 2000 may have been caused by habitat alterations. Grazing bouts were more frequent and heavy in 2000 compared to 1999, degrading much of the pasture to turf-like conditions and offering poor spittlebug habitat.

Zulia carbonaria populations were shown to be highly synchronous, indicating a response to environmental variables such as mass eclosion of eggs upon return of the wet season rains. The very low frequency of diapause in eggs, however, means that *Z. carbonaria* may rely on quiescence to synchronize life cycle with humid conditions. These data will be analyzed further for the correspondence between precipitation and phenology and begin to clarify how *Z. carbonaria* phenology tracks bimodal precipitation in the Cauca River Valley.

2.1.5 First generation population phenology in two lowland sites

Contributors:: Ulises Castro, Daniel Peck (CIAT), Antonio Pérez (Universidad de Sucre) , Rafael Mora, Andrea Báez (Universidad de los Llanos), Juan Carlos Campo, Guillermo León (CORPOICA C.I., La Libertad)

Rationale

In seasonal pasture systems, the first generation of spittlebugs is of critical importance to forage production because it leads to subsequent generations (as many as six generations per year) and is the source of infestation of previously unaffected areas of the farm. Suppressing this initial outbreak depends on our ability to predict when and where focal populations of nymphs and adults will manifest on the farm and thereby more effectively target application of available control tactics. With information on environmental factors and population fluctuation of the first generation, we hope to generate a predictive model correlating the timing of initial outbreaks with precipitation patterns at the onset of the rainy season. In this report we summarize results of the first generation population phenology at two lowland sites of Colombia over two years.

Materials and Methods

The first generation population dynamics were documented in three contrasting ecoregions of Colombia over two years (2000, 2001). Survey methods were similar to those used in previous studies to document population fluctuations in forage grasses. The survey period was limited to two months starting at the beginning of the rainy season. Surveys were carried out twice weekly in three 0.5 ha focal plots each established in a separate pasture. These studies were carried out on the Caribbean Coast (pronounced seasonality, unimodal precipitation) at Finca Tarapacá, Corozal, Dept. Sucre with the collaboration of the Universidad de Sucre; in the Orinoquian Piedmont (intermediate seasonality, unimodal) at C.I. La Libertad, Villavicencio, Dept. Meta with the collaboration of CORPOICA; and the Cauca River Valley (pronounced seasonality, bimodal) at Hacienda Las Palmas, Santander de Quilichao, Dept. Cauca.

Results

The results from Cauca are summarized elsewhere.

In Meta, 64 nymphs and 566 adults were captured and assessed in 2000, 698 and 1883 in 2001 (Table 28). Populations of nymphs were very low in Plots 2 and 3 because no nymphs were detected there in 2000 and 97% of total nymphs came from Plot 1 in 2001. Adults were also much more abundant in Plot 1 where 77 and 76% of adults were recovered in 2000 and 2001, respectively. *Aeneolamia varia* comprised 75.5 and 97.8 of adult populations in the two years while *Aeneolamia reducta* comprised 22.0 and 2.2%, and *Zulia pubescens* 2.5 and 0.0%. The date of first detection of nymphs and date of peak abundance of the first nymph generation (date of 50% accumulated insect days) was julian day 108 and 117 in 2000, and ranged from 92-99 and 104-108 in 2001 across the three plots (Table 29, Figure 10). The corresponding dates for adults were 101-104 and 121-122 in 2000, and 99 and 115-117 in 2001. At the farm level over the two years, the first adult generation reached its peak 5 and 7 days, respectively, after the nymph generation.

Table 28. Comparative abundance of first generation spittlebugs surveyed in two regions over two years.

Site	Year	Life stage	Plot 1		Plot 2		Plot 3		Total
			No.	%	No.	%	No.	%	
Meta	2000	Nymphs	64	100.0	---	---	---	---	64
		Adults[1]	436	77.0	65	11.5	65	11.5	566
	2001	Nymphs	650	93.0	32	5.0	16	2.0	698
		Adults[2]	1423	76.0	270	14.0	190	10.0	1883
Sucre	2000	Nymphs	658	60.0	64	6.0	375	34.0	1097
		Adults[3]	20065	55.0	7318	20.0	9013	25.0	36396
	2001	Nymphs	157	41.0	123	32.0	104	27.0	384
		Adults[3]	7121	36.0	8418	43.0	4099	21.0	19638

[1] *A. varia+A. reducta+Z. pubescens*
[2] *A. varia+A. reducta*
[3] *A. reducta*

Spittlebug populations were much higher in Sucre where 1097 nymphs and 36,396 adults were captured and assessed in 2000, 384 and 19,638 in 2001. There were large populations across the three plots over each year. *Aeneolamia reducta* was the only species detected. The date of first detection of nymphs and date of peak abundance of the first nymph generation (date of 50% accumulated insect days) was julian day 132-143 and 145-152 in 2000, and 137-140 and 147-154 in 2001 across the three plots (Table 29, Figure 10). The corresponding dates for adults were 143-148 and 155-161 in 2000, and 140-148 and 158-162 in 2001. At the farm level over the two years, the first adult generation reached its peak 10 and 13 days, respectively, after the nymph generation.

Table 29. Time of arrival (calculated as 50% cumulative insect days) of the first spittlebug generation in two regions over two years.

Site	Year	Life stage	Date first detected			Date of abundance peak			
			Plot 1	Plot 2	Plot 3	Plot 1	Plot 2	Plot 3	Sum
Meta	2000	Nymphs	108	---	---	117	---	---	117
		Adults	104	101	101	121	123	122	122
	2001	Nymphs	92	99	99	108	104	104	108
		Adults	99	99	99	115	117	116	115
Sucre	2000	Nymphs	143	143	132	148	152	145	147
		Adults	143	143	143	155	161	158	157
	2001	Nymphs	137	140	140	147	154	154	147
		Adults	140	148	144	158	161	162	160

Discussion

In both survey sites there was little variation in the timing of the first generation among plots and between years. In Meta, the difference in arrival of nymph and adult populations between years was only 9 and 6 days. In Sucre the difference was only 8 and 5 days. From these results we predict that the timing of the return of the wet season rains was similar in 2000 and 2001 since post-diapause quiescent eggs in the soil continue their development and hatch in direct response to the return of humid conditions. These population data will be combined with data from previous years (1997-1998 in Meta and 1997-1999 in Sucre) as repetitions to establish a predictive model, based on precipitation patterns, of when the first generation of spittlebugs is expected to appear in pastures of these regions. Predicting when and where the first outbreaks occur is critical information for targeting spittlebug management tactics in highly seasonal ecosystems.

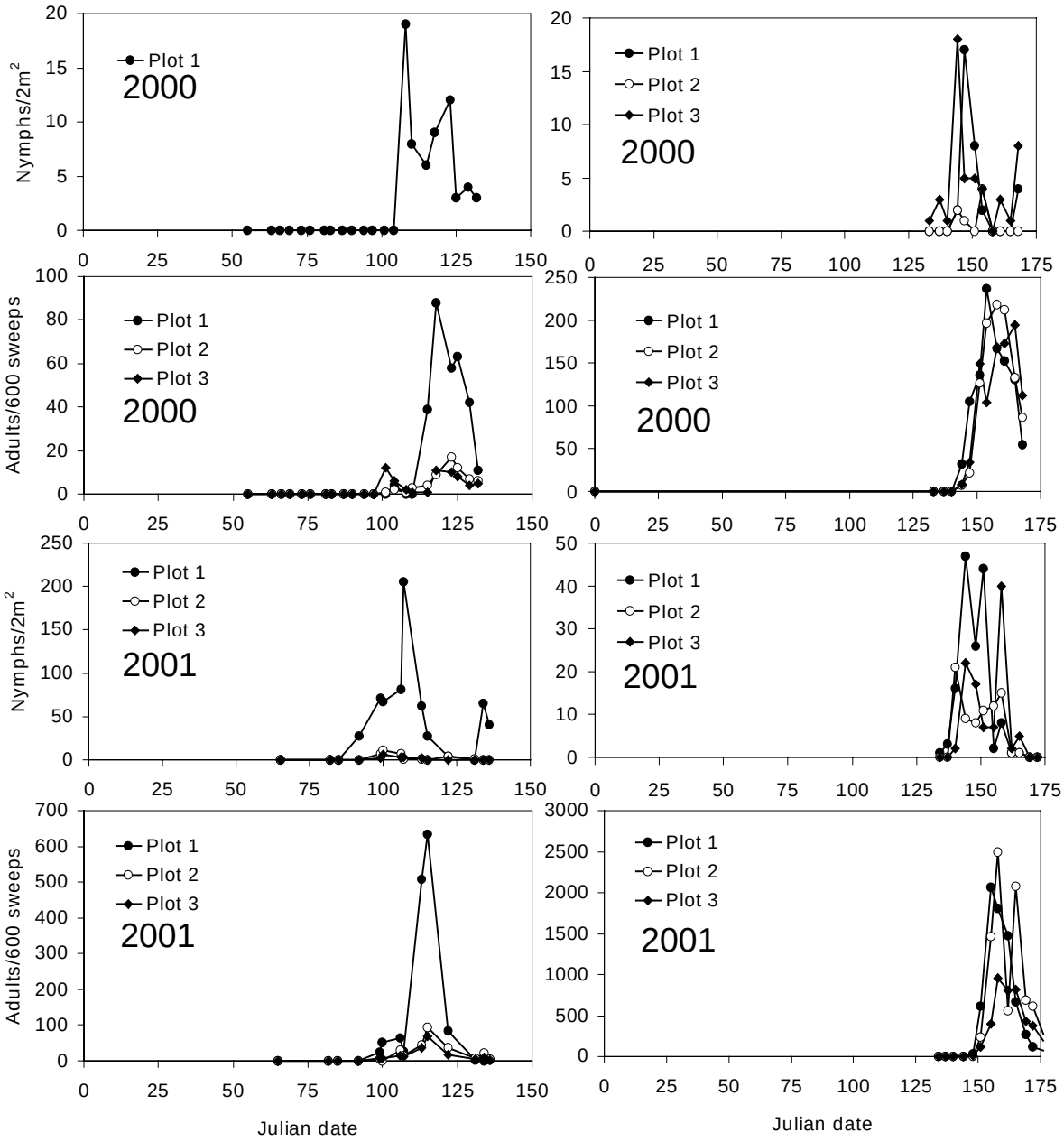

Figure 10. Population fluctuations of the first generations of spittlebug nymphs and adults in Meta (left column) and Sucre (right column) over two years.

2.1.6 Preoviposition determinants of egg diapause

Contributors: Ulises Castro, Oscar Yela, and Daniel Peck (CIAT)

Rationale

Female spittlebugs generally lay an increased proportion of diapausing eggs in response to the approaching dry season and the conditions unfavorable for spittlebug development and reproduction. The pest survives these adverse conditions of drought and high temperature as diapausing eggs that hatch upon

return of the rains in the subsequent wet season. The immature stage (nymph) is responsible for predicting the extreme conditions of the future by perceiving token environmental stimuli that induce diapause in the adult stage. In temperate zones, photoperiod and temperature are dominant stimuli involved in the induction and regulation of diapause in many insects. In the tropics, however, the precise token stimuli that induce diapause in graminoid spittlebugs remain unknown. Photoperiod probably does not play a role in Colombia due to its proximity to the equator. In this report we summarize advances in assessing the role of plant age, water stress and their combination in inducing diapause in *Aeneolamia varia*.

Materials and Methods

Plants of *Brachiaria ruziziensis* were established in wooden boxes (1.4 x 0.6 x 0.1 m) with a proliferation of surface roots required as feeding sites by the nymphs. This arrangement was described previously as a component of an improved mass rearing design (CIAT Annual Report 2000). Boxes served as units of repetition for four treatments based on combinations of two factors: host plant age (4 and 8 weeks after transplanting) and water stress (field capacity and stressed). Boxes at field capacity were watered daily at the rate of 6 l/m^2 while stressed plants were watered as the rate of 3 l/m^2 every 3 days. Each box was infested with eggs of *A. varia* collected from adults in the field in the Orinoquian Piedmont (C.I. La Libertad, Villavicencio, Dept. Meta). The CIAT colony was not used as the source for these eggs because the insects were not regarded as fully receptive to token stimuli; colony management selects strongly against diapause eggs and the most recent genetic addition to the colony were individuals from the Amazonian Piedmont (Dept. Caquetá) where conditions are continuously humid and diapause may not be important to species survival.

Each treatment had three repetitions. Boxes were infested with 1600 eggs and water treatments initiated one week later once first instars had emerged and established spittle masses. Once the adults began to appear the box was covered with an emergence cage (1.4 x 0.6 x 0.9 wooden frame covered in mesh) and individuals were collected with an aspirator and transferred to a separate small oviposition cage assigned to each repetition. After enough adults had emerged they were allowed 3 days to oviposit on fresh substrate following which eggs were extracted, disinfected and stored in petri dishes on humid filter paper under controlled conditions (27^oC, 100% RH, darkness). As more females emerged or stayed alive, a second batch of eggs was collected. Eggs were evaluated twice weekly to score chorions (emerged nymphs) and inviable eggs. Viable eggs remaining after 30 days were classified as diapausing. To confirm that treatments had an affect on the quality of the host plant, plant material in each repetition was assessed for dry weight and dry matter digestibility.

Results

Of a total 9277 eggs evaluated, 12.2% were considered diapausing. Among the four treatments, the incidence of diapause varied from 8.3-13.8 % and the time to eclosion 33.2-37.0 days (Table 30). For non-diapause eggs time to eclosion was 20.4-21.6 days and for diapause eggs 32.7-37.0 days. Analysis of variance did not detect an effect of water stress or plant age on diapause incidence. Mean dry matter (g), percent dry matter, and digestibility were measured to gauge differences in expression of the treatments on plant quality (Table 31). These means have not yet been statistically tested for differences.

Discussion

Under the conditions of these experimental treatments, no effect of host plant age or water stress was detected on the incidence or duration of diapause eggs in *A. varia*. Other factors are probably responsible for the documented increase in diapause incidence at the end of the wet season. The incidence of diapause of eggs from Meta populations was far higher than those previously examined in females from Caquetá

(0.24%), a continuously humid site. This reinforces the idea that diapause is more expressed in seasonal sites and that studies should use insects originating directly from these populations.

Table 30. Influence of host plant age and water stress on the incidence and duration (mean±S.E., range) of diapause eggs in *A. varia*.

	Eggs	Mean proportion (%)		Mean time to eclosion (days)	
Treatment	observed	Non-diapause	Diapause	Non-diapause	Diapause
Field capacity	2183	86.8 ± 0.5	13.2 ± 0.5	21.6 ± 1.4	37.0 ± 0.3
4-wk old		(86.5-87.2)	(12.8-13.5)	(20.5-226)	(36.8-37.2)
Field capacity	5017	86.5 ± 8.3	13.5 ± 8.3	20.6 ± 0.8	34.1 ± 3.5
8-wk old		(71.5-95.8)	(4.2-28.5)	(19.4-21.7)	(30.4-37.7)
Water stress	964	86.5 ± 4.7	13.5 ± 4.7	20.6 ± 1.4	33.2 ± 3.3.
4-wk old		(82.6-91.8)	(8.2-17.4)	(19.8-22.2)	(31.2-37.0)
Water stress	1113	91.7 ± 5.5	8.3 ± 5.5.	20.4± 1.9	34.9 ± 3.7
8-wk old		(83.7-96.1)	(3.9-16.3)	(18.3-22.7)	(31.4-38.2)

Table 31. Influence of host plant age and water stress on percent dry matter and in vitro digestibility of *B. decumbens*.

	Field capacity		**Water stress**	
Measure	**4-wk old**	**8-wk old**	**4-wk old**	**8-wk old**
Dry weight (g)	0.043	0.048	0.044	0.044
Dry weight (%)	60.8	63.8	64.4	54.0
Digestibility (%)	61.0	59.4	64.5	64.0

2.1.7 Seasonal changes in the incidence and duration of egg diapause

Contributors: Ulises Castro, Daniel Peck (CIAT), Antonio Pérez (Universidad de Sucre), Rafael Mora, Andrea Báez (Universidad de los Llanos), Juan Carlos Campos, Guillermo León (CORPOICA C.I. La Libertad)

Rationale

For various spittlebug species in seasonal environments it has been shown that the incidence of diapause eggs increases at the end of the wet season in anticipation of the unfavorable dry season. To complement studies on the population dynamics of spittlebug nymphs and adults in contrasting regions of Colombia, changes in the incidence and duration of diapause eggs was documented over the season in three contrasting ecoregions: the Caribbean Coast, the Orinoquian Piedmont and the Cauca River Valley.

Materials and Methods

One year of data has been analyzed for *Aeneolamia reducta* (Dept. Sucre, Caribbean Coast), *Aeneolamia varia* (Dept. Meta, Orinoquian Piedmont) and *Zulia carbonaria* (Dept. Cauca, Cauca River Valley). These data were collected from the same focal paddocks established in these sites for population dynamics studies. In each of the three plots per farm, two groups of females (1-5 for Cauca, 1-25 for Meta and Sucre, depending on availability) were caught with sweep nets, brought to the laboratory and confined to large petri dishes (2 cm tall, 15 cm diameter) lined on the bottom with moist filter paper that served as oviposition substrate. Females laid eggs over 3 days, the filter paper was disinfected for 2-3 min with Clorox and washed with distilled water, and petris were express mailed to CIAT for incubation under controlled conditions (27^{o}C, 100% RH, darkness). Collections were made every 2 wk over the season

when females were available in the field. Petri dishes were evaluated twice weekly for chorions (emerged nymphs) and inviable eggs.

Results

Of 38,088 eggs collected and evaluated from January to December 2000, 12.95% were diapausing (Table 32). The incidence of egg diapause in *Z. carbonaria* was extremely low throughout the year (Figure 11). Diapausing eggs were only detected on one date (July) and at very low incidence. The six diapause eggs eclosed 36, 36, 46, 46, 53 and 57 days after oviposition. In Sucre and Meta the incidence of diapause increased at the end of the year at the start of the dry season achieving rates as high as 85.0 and 49.5%, respectively. Overall, the time to eclosion for non-diapause eggs was 22.7 ± 5.5 for *A. reducta*, 18.2 ± 4.6 for *A. varia* and 18.0 ± 1.9 for *Z. carbonaria* (Table 32). The time to eclosion for diapausing eggs was 96.9 ± 43.6, 71.5 ± 32.7 and 48.2 ± 12.5 d, respectively. In Meta the time to eclosion of diapause eggs did not vary over the season, however in Sucre there was a noticeable increase in duration toward the end of the season.

Table 32. Seasonal changes in diapause incidence and time to eclosion of spittlebug eggs in three ecoregions of Colombia (bars indicate periods where no females were available).

Month	Incidence of diapause (%)			Time to eclosion (days)			n (eggs examined)		
(2000)	Sucre	Meta	Cauca	Sucre	Meta	Cauca	Sucre	Meta	Cauca
Jan	---	---	---	---	---	---	0	0	0
Feb	---	---	---	---	---	---	0	0	0
Mar	---	---	---	---	---	---	0	0	0
Apr	---	0.0	0.0	---	---	---	0	305	183
May	---	0.0	0.0	---	---	---	0	645	315
Jun	0.01	0.0	0.0	55	---	---	3781	1986	531
Jul	2.0	0.1	3.3	50	---	48	4274	2651	174
Aug	2.0	0.6	0.0	55	74	---	1916	6244	110
Sep	---	8.1	---	---	43	---	0	2991	0
Oct	57.0	11.2	---	137	89	---	1419	4953	0
Nov	72.0	2.7	0.0	127	53	---	3692	513	289
Dec	85.0	49.4	0.0	134	68	---	914	166	0

Discussion

In the Caribbean Coast and the Orinoquian Piedmont the incidence of diapause increased from zero at the beginning of the wet season to 85 and 50%, respectively, at the start of the dry season. In both of these sites the rainy season is highly seasonal with 2-4 months of extremely dry months annually and in accordance with our expectations, the principal spittlebug species in these regions use diapause as a means to survive the adverse conditions. In the Cauca River Valley, precipitation is bimodal and although dry periods are severe, they are comparatively brief.

These seasonality conditions may allow for different mechanisms for surviving the adverse conditions because no seasonal changes in the incidence of diapause or the time to egg eclosion was detected. In this region, *Z. carbonaria* may adopt a different strategy, such as maintenance of low population levels in localized humid areas with subsequent recolonization of pastures. Alternatively, instead of diapause *Z. carbonaria* could depend on drought-tolerant quiescent eggs where development is temporarily delayed in direct response to adverse conditions, and reinitiated once adequate humid conditions return. These possibilities will be explored in future studies.

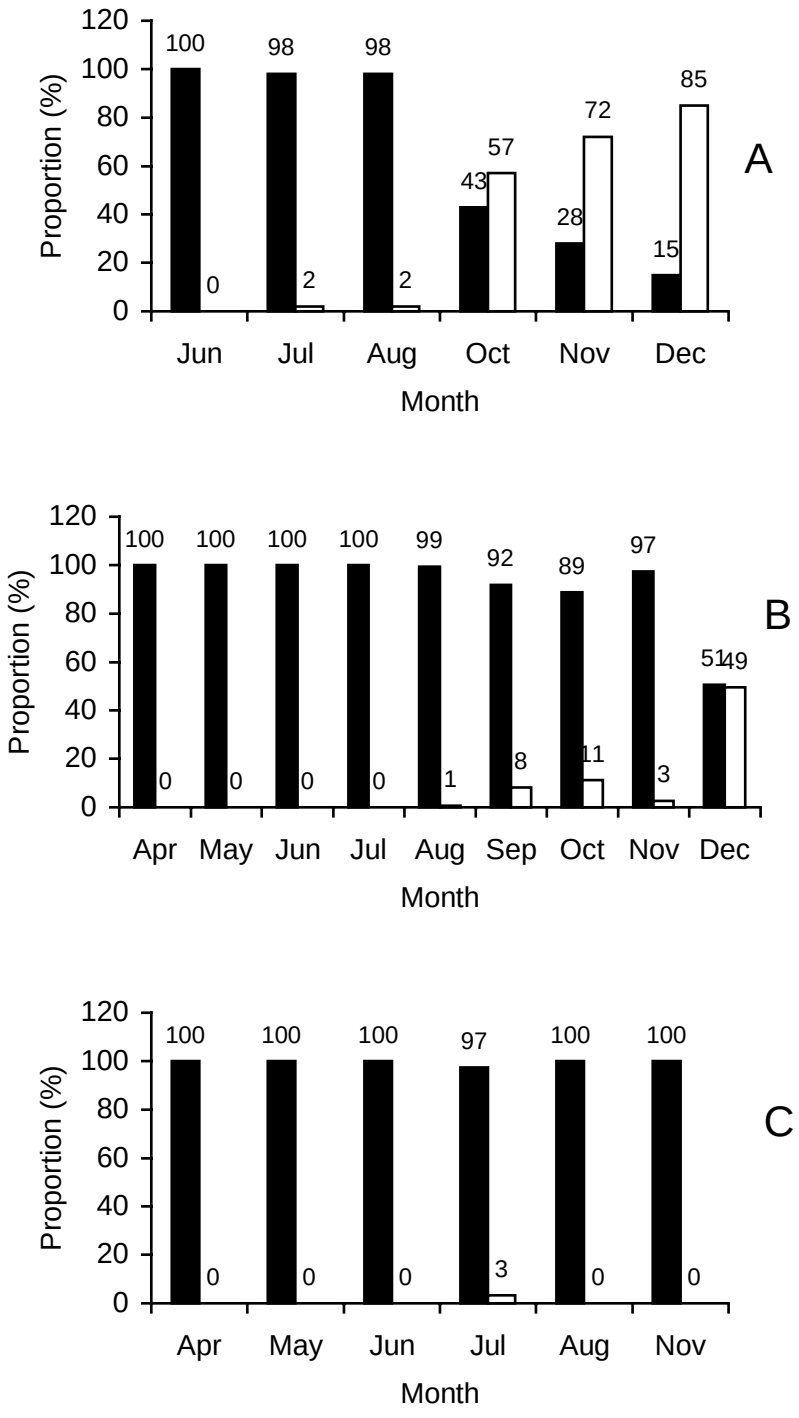

Figure 11. Seasonal changes in the incidence of spittlebug egg diapause in (A) the Caribbean Coast with *A. reducta*, (B) the Orinoquian Piedmont with *A. varia*, and (C) the Cauca River Valley with *Z. carbonaria* during 2000. Black bars are non-diapause and open bars are diapause eggs.

Activity 2.2 IPM components for spittlebug management

Highlights

- An artifical diet validated as effective for maintaining adult *Aeneolamia varia*.
- Strengthened the collection of fungal entomopathogens of major insect pests which now includes 73 isolates from cassava pests (burrower bugs, stem borers, whiteflies) and 77 from forage grass pests (spittlebugs)
- Eighteen fungal entomopathogen isolates were screened for virulence to burrower bug nymphs and adults showing high levels of virulence in certain strains.
- Determined that virulence of fungal entomopathogen isolates varies among spittlebug species.
- Protocols established for determining LC_{50} and LC_{90} of fungal entomopathogen isolates to spittlebug nymphs.
- Field studies initiated in two contrasting regions to test the number and timing of applications of a formulated fungal entomopathogen product to suppress spittlebug populations.

Efforts to control spittlebugs in forage grasses and other graminoid crops have been compromised by difficult access to the literature, inappropriate research methodologies, and rudimentary decision-support tools and other components of IPM. In addition, there is lack of a model system for tailoring IPM to the contrasting ecoregions and agricultural production systems where spittlebugs occur.

Results from CIAT's group on Spittlebug Bioecology and IPM over the period 1997-2001 offer the most detailed information on this pest complex for any country. Through the development of contrasting ecoregions as model sites for advancing the diagnosis and management of this pest complex, these studies will serve as a template for other regions or countries confronting their own problems with this pest. Linking these results to advances in spittlebug IPM will depend on the transfer and diffusion of new information, diagnostic tools, and research methodologies and technologies.

Progress towards achieving output milestones

- **IPM components relevant to spittlebug management in forage grasses and other graminoids better understood**
  In 2001 we continued to advance the management of spittlebugs through studies on diverse components of IPM. This research included evaluating artificial diets for the maintenance of spittlebug adults; strengthening and maintaining a ceparium of diverse fungal entomopathogen isolates; screening this entomopathogen collection for the most virulent strains; evaluating the variation in virulence across spittlebug life stages and species; and evaluating and deploying the most promising isolates in field trials. These and other IPM tools will be assessed withinin contrasting ecoregions serving as model systems where bioecological information is been acquired concurrently, ultimately leading to recommended IPM programs for field testing, followed by modification and impact assessment.

2.2.1 Artificial diet for maintenance of spittlebug adults

Contributors: Ulises Castro, Claudia Flores, Rosalba Tobón, and Daniel Peck (CIAT)

Rationale

The development of an artificial diet for maintenance of spittlebug adults would permit the evaluation of feeding deterrents or deleterious compounds such as lectins that could be incorporated into *Brachiaria*

though genetic transformation. Here we summarize results of three trials of a particular formula chosen as the best option from among a series of diets developed and assessed in 2000.

Materials and Methods

Trials consisted of three treatments: vial, plant and diet. In the plant treatment, adults were enclosed inside cylindrical acetate cages (40 cm tall, 15 cm diameter) over potted *Brachiaria ruziziensis* plants that provided a food source. In the other treatments adults were held in large petri dishes (2 cm tall, 15 cm diameter). In the vial treatment, the food source was stems of *B. ruziziensis* kept with their base in a vial of water. In the diet treatment, 500 µl of the liquid diet was sealed in parafilm sachets (3 x 3.5 cm). Thirteen repetitions were carried out over three trial periods. Each of the three trials and all their treatments were performed in an insect growth chamber with mean (± S.E.) temperatures of 23.8 ± 1.5, 22.5 ± 3.2 and 23.21 ± 1.4°C, respectively. Each treatment repetition had four adult *Aeneolamia varia* taken as tenerals (<12 hours old) from the CIAT colony (Table 33).

Table 33. Results from Probit analysis for three trial of three diets.

Treatment	Trial	n	X^2	Prob. X^2	b	L_{50}	L_{90}
Vial	1	585	7.6377	0.6642	6.7316	7.0661	10.9537
	2	780	30.0965	0.0027	7.6551	8.6623	12.7364
	3	728	44.5463	0.0000	5.1588	6.5599	11.6229
Diet	1	784	15.5965	0.2716	8.3733	9.9825	14.2001
	2	780	6.7797	0.8718	8.1406	8.7894	12.6295
	3	888	215566	0.0882	6.0587	7.5096	12.2219
Plant	1	550	43.3962	0.0000	7.0250	6.0452	9.2011
	2	714	9.4180	0.5834	14.7956	10.4654	12.7753
	3	901	5.2907	0.9813	10.6372	10.9781	14.4879

The composition of the diet was modified from Hagley 1967. To make the diet easier and cheaper to prepare, yeast and hydrolyzed casein were substituted as the sources of amino acids and p-aminobenzoic acid substituted as the source of vitamin B_{12}. The list of ingredients in the diet were (1) amino acids: yeast extract, hydrolyzed casein, (2) vitamins: biotin, calcium pantothenate, choline chloride, folic acid, inositol, nicotinic acid, pyridoxine, thiamine, riboflavin, ascorbic acid, ρ-aminobenzoic acid, (3) carbohydrates: sucrose, (4) salts: $MgCl_2$, KH_2PO_4, Wesson's' salts, and (5) lipids: cholesterol benzoate. Adult mortality was assessed daily. Mean adult longevity was determined with Probit analysis and differences among treatments were tested with an ANOVA.

Results

Mean longevity among repetitions varied from 6.0-11.0 days. Analysis of variance detected no significant differences among the three diet treatments with mean longevity of 7.4, 8.7 and 9.2 days for vial, diet and plant treatments, respectively. Probit analysis, however, indicated that more trials will have to be carried out due to low X^2 values in several individual trials.

Discussion

Replacement of certain components in the original diet (Hagley 1967) did not affect the maintenance and longevity of adults *A. varia*. Use of yeast extract and hydrolyzed casein as the sources of amino acids made preparation easier and could significantly reduce costs. Whether the diet is effective for maintenance of nymphs remains to be tested; Hagley's original diet was designed and tested only for

adults. Regardless, the current formulation should be adequate for future studies on substances of interest in adult feeding or toxicity.

2.2.2 Maintenance of a ceparium for fungal entomopathogens of major forage grass and cassava pest

Contributors: Anuar Morales, Rosalba Tobón, Mauricio Rendón, Irina Alean, and Daniel Peck (CIAT)

Rationale

Ongoing field studies on the major insect pests in diverse regions of Colombia have allowed us to collect, isolate, propagate and store a diverse collection of fungal entomopathogens. This ceparium was established last year (CIAT Annual Report 2000) and is designed to serve as a source of pathogenic material for studies on biological control. Maintaining and strengthening this collection is of utmost importance for advancing non-toxic alternatives to insecticides and other effective tactics as components of integrated pest management. In this report we summarize maintenance and diversity of the ceparium with a particular focus on the fungal entomopathogens of forage grass pests (spittlebugs) and cassava pests (burrower bugs, stem borers, whiteflies).

Materials and Methods

There are two main activities related to the ceparium. The first consists of the isolation, maintenance, propagation and storage of isolates based on previously established protocols (see CIAT Annual Report 2000). The second is the multiplication of isolates for reactivation and studies on virulence and pathogenicity.

Results and Discussion

The CIAT ceparium now houses a total of 150 different isolates of fungal entomopathogens. In the area of cassava pests, 34 new isolates of fungal entomopathogens were added to the 39 strains already purified and stored on filter paper (Table 34). Of the 73 isolates, 28 were reactivated on the burrower bug *Cyrtomenus bergi* (Heteroptera: Cydnidae) and 18 of these have been evaluated for virulence to nymphs and adults in laboratory studies. Once the efficiency of these isolates is calculated in comparison to mortality in the controls, the five most virulent isolates will be selected for future studies. The levels of control are highly promising with up to 100% mortality in nymphs and 58% in adults (Table 35).

Seven other isolates of diverse fungi including *Paecilomyces* spp. (CIAT 210, 211, 212, 216), *Verticillium lecani* (CIAT 215), *Beauveria bassiana* (CIAT 217) and *Cladosporium* sp. (CIAT 272) were reactivated on nymphs and adults of the whitefly *Aleurotrachellus socialis*. These isolates are currently in the multiplication phase to provide material for the first applications to determine pathogenicity and virulence.

In the area of spittlebug pests of forage grasses, the main activities were related to selection and characterization of isolates for field trials (see section 2.2.5) including (1) multiplication for virulence studies on different species of adult spittlebugs (see section 2.2.3), (2) multiplication for determination of LC_{50} and LC_{90} in nymphs (see section 2.2.4), (3) and quality control studies of formulated material developed in collaboration with BioCaribe, S.A. with the goal of achieving a product of higher quality.In terms of ceparium maintenance, viability tests of stored material are continuously carried out with the goal of reactivating on culture media the isolates that have lost vigorous growth characteristics. Three new isolates from spittlebugs were incorporated into the collection this year: CIAT 076 isolated from a nymph

collected in C.I. Macagual, Dept. Caquetá; and CIAT 077 and 078 isolated from an adult of *Mahanarva andigena* collected in Tumaco, Dept. Nariño.

Table 34. Accession, host and origin of fungal isolates entomopathogenic to different cassava insects.

Host species	CIAT accession numbers	Origin	
		Department	Municipality
Aleurotrachellus socialis	CIAT 215-217	undet.	undet.
Brassoly sp.	CIAT 246	Casanare	Villanueva
Chilomima klarkei	CIAT 249, 252-257, 263-267, 269	Tolima	Espinal
Chilomima klarkei	CIAT 274	Tolima	Ibague
Chilomima klarkei	CIAT 277	Tolima	Nataima
Coleoptera	CIAT 262	Cauca	undet.
Corinus sp.	CIAT 219	Valle	La Cumbre
Cosmopolites sordidus	CIAT 247	Valle	Jamundi
Cyrtomenus bergi	CIAT 200	Cauca	Timbio
Cyrtomenus bergi	CIAT 214, 224, 225	undet.	undet.
Cyrtomenus bergi	CIAT 226-243	Cauca	Popayan
Cyrtomenus bergi	CIAT 250, 251, 258-261, 268, 275, 276	Risaralda	Pereira
Erinnys ello	CIAT 218	undet.	undet.
Galeria melonella	CIAT 208, 213	Valle	Pradera
Galeria melonella	CIAT 270	Risaralda	Pereira
Galeria melonella	CIAT 271, 273	Tolima	Guamo
Galeria melonella	CIAT 278, 279	Cauca	Cajibio
Hymenoptera	CIAT 248	Valle	Palmira
Trialeurodes vaporariorum	CIAT 210-212	Valle	Pradera
Trialeurodes variabilis	CIAT 272	Tolima	Espinal
Whitefly	CIAT 244	undet.	Imbabura
undet.	CIAT 209	Valle	Palmira
undet.	CIAT 220-222	undet.	undet.

Table 35. Virulence (% mortality) of 18 fungal entomopathogen isolates to nymphs and adults of the burrower bug *C. bergi*.

Accession	Nymphs	Adults	Accession	Nymphs	Adults
CIAT 227	66.0	56.0	CIAT 230	89.0	53.0
CIAT 231	53.0	48.3	CIAT 237	81.0	50.0
CIAT 233	67.0	53.3	CIAT 261	74.0	49.0
CIAT 234	58.0	31.7	CIAT 224	100.0	47.0
CIAT 241	30.0	58.3	CIAT 245	100.0	47.0
CIAT 242	55.0	50.0	CIAT 239	76.0	33.0
CIAT 250	52.0	56.7	CIAT 228	55.0	23.0
CIAT 258	58.0	55.0	CIAT 238	50.0	20.0
CIAT 259	51.0	65.0	CIAT 240	74.0	21.0

In addition to these activities, "Access" software was used to establish a database to manage all information related to ceparium isolates. This program allows easy consultation of the information by interested scientists.

2.2.3 Variation in the virulence of fungal entomopathogens among spittlebug species

Contributors: Anuar Morales, Rosalba Tobón, Jairo Rodríguez, Ulises Castro, Oscar Yela, and Daniel Peck (CIAT)

Rationale

A major challenge for the implementation of an integrated management plan for graminoid spittlebugs is the taxonomic diversity of species that contribute to this pest complex. In Colombia, for instance, 15 species from six genera have been identified with graminoid host plants. Management is limited by the extent that a particular control tactic can be tailored to different species, further complicated by the presence of 2-3 species in the same local pastures. We are obtaining new information on the biology and ecology of major species in contrasting ecoregions of Colombia, and this information is broadening our understanding of the variation across this group and of the different strategies for their management in different habitats. From these results we predict that particular control tactics will also need to be tailored to the particular spittlebug species and habitat in which control is required. In the specific case of fungal entomopathogens as biological control agents, effectiveness of a given isolate may also vary across species. Advancing the use of fungal entomopathogens in an IPM program for spittlebug management will therefore depend on gauging the variation in virulence across different spittlebug species.

Materials and Methods

Methods were based on protocols established and described in 2000 (CIAT Annual Report). Evaluation units were 30-day old plants (7-10 stems) of *Brachiaria ruziziensis* (CIAT 654) in pots (15 cm diameter) covered by acetate cylinders (40 cm tall, 15 cm diameter). These plants were infested with 10 adult tenerals (<24 hours old) of *Aeneolamia reducta*, *Aeneolamia varia, Zulia carbonaria* and *Zulia pubescens* obtained from colonies maintained at CIAT. Two to three hours after infestation plants were sprayed with 5 ml of a concentrated conidial suspension (10^8 conidia/ml) with an airbrush and compressor (10 PSI). Four isolates were evaluated: CIAT 007C, CIAT 009, CIAT 054 and CIAT 055, identified as *Metarhizium anisopliae*, *Paecilomyces farinosis Metarhizium* sp. and *Metarhizium* sp. respectively. These isolates were selected from among 48 strains as the most virulent to *A. varia* adults (see CIAT Annual Report 2000).

For each spittlebug species, 10 repetitions (pots) were evaluated for each isolate and a control (water with tween at 0.05%). After spraying, plants and insects were maintained in a growth chamber (27°C ± 2°C, RH 80% ± 10%). Virulence was evaluated 5 days later when all insects were scored as alive, dead, and dead with evidence of mycosis. Dead insects with no visible signs of fungus attack were stored in petri dishes with moist filter paper for 3-4 days to ascertain whether they were infected with fungus. Differences were evaluated with an ANOVA and Tukey multiple range test.

Results

Mortality in the control varied from 3.9-28.5 among the four spittlebug species. The lowest mortality was experienced by *Z. pubescens* and the highest by *A. reducta*, corresponding to the species of longest and shortest adult longevity according to greenhouse biology studies.

Virulence of isolates varied significantly among species with *A. varia* being most susceptible, followed by *A. reducta*, then *Z. carbonaria* and *Z. pubescens* (Figure 12). As expected, control for all four isolates was significantly higher on *A. varia* since this species was used for preselecting the most virulent strains used in this study. Mortality ranged from 62.8-95.1%. Mortality in *A. reducta* ranged from 42.5-61.9%.

For *Z. carbonaria* and *Z. pubescens*, mortality ranged from 20.2-33.6 and 16.1-30.4%, respectively, and in most cases this was not significantly different than the control. In the case of *Z. carbonaria*, none of the isolates achieved higher mortality than the control. The *Paecilomyces* isolate (CIAT 009) achieved relatively higher mortality (33.6%) in *Z. carbonaria* compared to the *Metarhizium* isolates.

Control was also significantly higher in the genus *Aeneolamia* compared to *Zulia* for all four isolates evaluated. For instance, CIAT 054 achieved a mortality of 56.8 and 95.1% for *A. reducta* and *A. varia*, respectively but only 31.4 and 16.1 for *Z. carbonaria* and *Z. pubescens*. This suggests that some of the variance in virulence among spittlebug species may be expressed as differences at the genus level.

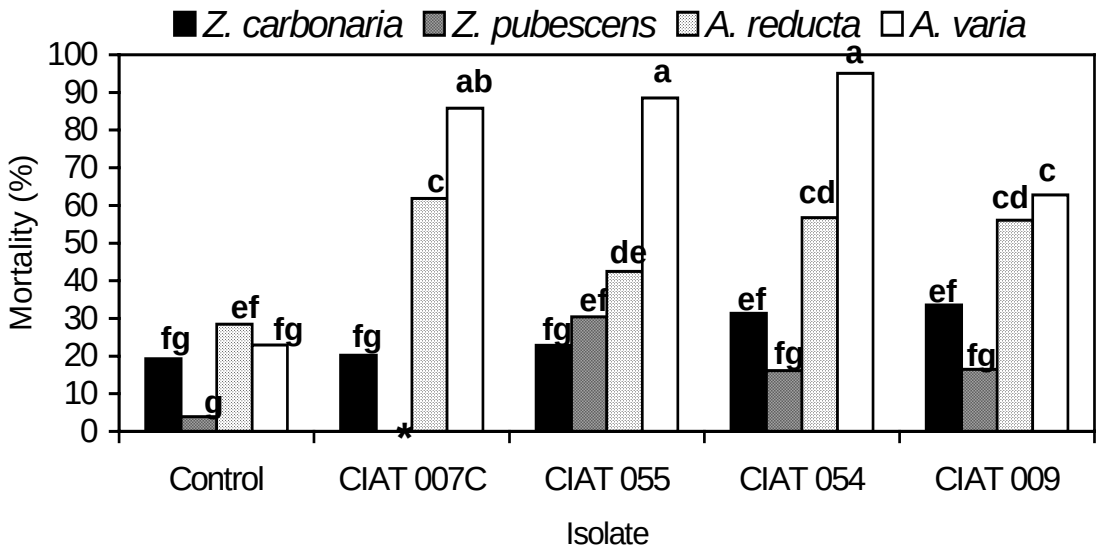

Figure 12. Mortality (absolute percent) of four isolates of fungal entomopathogens on four spittlebug species. Means followed by different letters are significantly different at P<0.05. * This isolate was not evaluated on *Z. pubescens*.

Discussion

Virulence of fungal entomopathogen strains varies among spittlebug species. Deploying these pathogens as agents of biological control therefore depends on an understanding of the species complex in the area where control is desired, selecting isolates specific to spittlebug species, and reassessing the broad effectiveness of commercial products. On the other hand, results indicate that the diverse collection of isolates in CIAT's ceparium probably has strains highly virulent to species other than *A. varia*, which up to this point has been used as the model species for developing evaluation methodologies. The most efficient screening process might therefore be evaluating a diversity of isolates to the particular spittlebug species of interest, rather than using preselection (with a model species such *A. varia*) with subsequent confirmation of high control on other species. One particular screening focus should be *Paecilomyces* isolates with *Z. carbonaria* since this fungus was relatively more virulent against this species than *Metarhizium*. At present there are three *Paecilomyces* strains in the ceparium that were originally isolated from *Z. carbonaria*.

These results confirm the need to continue documenting the patterns of variation among graminoid spittlebugs given that effectiveness of control tactics such as insect pathogens may be species specific.

Significant variation in host plant resistance among spittlebug species is further corroboration of this observation. Studies are under way to continue evaluating variation in virulence. Adults of *P. simulans* are under evaluation and variation between adults and nymphs are being explored with *A. varia*, *P. simulans*, *Z. carbonaria* and *Z. pubescens*.

2.2.4 Characterization and formulation of select fungal entomopathogen isolates for field evaluation

Contributors: Anuar Morales, Rosalba Tobón, Oscar Yela, and Daniel Peck (CIAT)

Rationale

Four isolates have been selected from CIAT's fungal entomopathogen collection for experimental field trials designed to test application techniques. These isolates are the three *Metarhizium* and one *Paecilomyces* strains screened from 49 isolates (see CIAT Annual Report 2000) as the most virulent to adults of *Aeneolamia varia* (Table 36). Before deploying in the field, these isolates must be characterized for their biological and virulence activity on different species and life stages of spittlebugs. Variation in virulence among adults of four species was described elsewhere. Here we summarize results of studies to determine the LC_{50} and LC_{90} on nymphs of *A. varia*.

Table 36. Identification and origin of fungal entomopathogen isolates selected for field trials.

	CIAT accession number			
	CIAT 054	CIAT 055	CIAT 007C	CIAT 009
Fungal isolate :				
Genus	*Metarhizium*	*Metarhizium*	*Metarhizium*	*Paecilomyces*
Species	sp. 1	sp. 2	*anisopliae*	*farinosis*
Spittlebug host:				
Genus	*Aeneolamia*	*Aeneolamia*	*Zulia*	undet.
Species	*Varia*	*varia*	*pubescens*	
Sex	Male	undet.	female	undet.
Life stage	Adult	nymph	adult	nymph
Department	Valle del Cauca	Valle del Cauca	Caquetá	Caquetá
Municipality	Palmira	Palmira	Albania	Florencia

Materials and Methods

Evaluation methods for nymphs were based on previously established protocols (see CIAT Annual Report 2000). Evaluation units were the same small-scale PVC tubes (1.5" diameter) now standard for host plant resistance screening. At 6 weeks after planting with *Brachiaria ruziziensis* (CIAT 654), surface roots were sufficiently established for nymph development and egg infestation. Eggs of *Aeneolamia varia* about to hatch were prepared for treatments and infestation by placing 10 on each of 10 small pieces of filter paper in a petri dish that corresponded to one treatment. Nine different concentrations of conidial suspensions ($1x10^4$, $5x10^4$, $1x10^5$, $5x10^5$, $1x10^6$, $5x10^6$, $1x10^7$, $5x10^8$, $1x10^9$ conidia/ml) were prepared for three isolates (CIAT 007C, CIAT 054, CIAT 009) with a control (water and tween at 0.05%) (Table 37).

Applications were made on the substrate before infestation and on the eggs in petri dishes before infestation. An airbrush and compressor (10 PSI) were used at a volume of 1 ml for substrate and <1 ml for direct egg application. Plants were maintained in the greenhouse until evaluation of mortality 30-32 days after infestation. During this period, plants were fertilized twice (just before and 15 days after

infestation) with urea at 2g/l. There were ten repetitions per treatment. Mortality data were analyzed with Probit (SAS).

Table 37. Probit analysis of mortality caused by three fungal entomopathogen isolates to nymphs of *A. varia*.

Isolate	N	LC_{50} (95% CI)	LC_{90} (95% CI)	X^2	Prob X^2	B (S.E.)
CIAT 054	900	$8.0x10^6$ ($3.7x10^6$-$1.3x10^7$)	$8.9x10^7$ ($5.2x10^7$-$2.0x10^8$)	7.3	0.290	1.2 (0.19)
CIAT 009		-	-	22.3	0.66	2.4 (0.25)
CIAT 007C	900	$4.6x10^5$ ($1.6x10^4$-$3.0x10^6$)	$3.6x10^8$ ($6.7x10^7$-$4.4x10^9$)	12.2	0.057	0.44 (0.06)

Results

For CIAT 054 and CIAT 007C, *A. varia* nymph mortality increased with increasing conidial concentration as expected (Figure 13). For CIAT 009, however, the relationship between mortality and concentration was not clear, showing irregular activity along the concentration gradient.

Probit analysis showed low X^2 values and acceptable X^2 probability values for CIAT 054 and CIAT 007C. The LC_{50} and LC_{90} were $8.0x10^6$ and $8.9x10^7$ conidias/ml for CIAT 054, and $4.6x10^5$ and $3.6x10^8$ for CIAT 007C. Given the high X^2 value for CIAT 009, the calculated concentrations are inaccurate and the trial must be repeated.

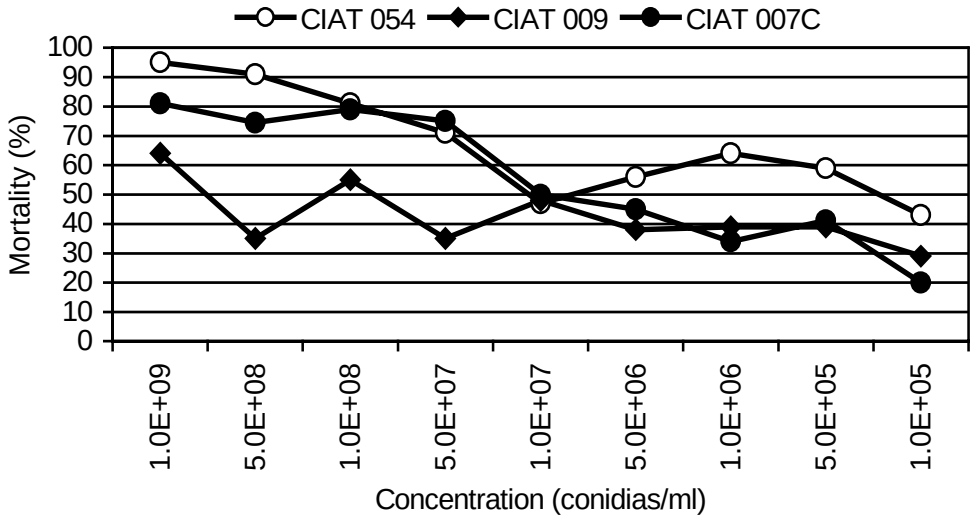

Figure 13. Mortality in *A. varia* nymphs caused by three fungal entomopathogen isolates at different concentrations.

Comparative studies are currently underway with the same three isolates and nymphs of *Prosapia simulans*, the major spittlebug species in one of the two field evaluation sites. An additional phase before field evaluation is the formulation of strains. This is being carried out by BioCaribe, S.A. in a formal agreement with CIAT. To confirm the quality of this material, studies are underway to compare the effectiveness of formulated versus unformulated product.

Discussion

Even though identical methodologies were used for the isolates, different LC_{90} were expected given the different origin of the strains. Applications of fungal entomopathogens in upcoming field trials will be based on the LC_{90} determined here to avoid the situations where too little material is applied to have an effect, or too much is added and material is wasted. Ongoing studies will corroborate this information and establish whether formulation has altered virulence. Plans are also underway to evaluated LC_{50} and LC_{90} on adults of *A. varia*.

2.2.5 Field evaluation of fungal entomopathogens in two contrasting regions

Contributors: Anuar Morales, Jairo Rodríguez, Ulises Castro, Oscar Yela, Daniel Peck (CIAT), Daniel Corradine, German Chacón, Orlando Narváez, Fabio Obregón (Universidad de la Amazonia)

Rationale

In general, previous attempts to evaluate the efficiency of fungal entomopathogens as biological control agents of spittlebugs in pastures have been focused on laboratory assays. The few that have gone to the field have demonstrated highly variable and low levels of control due to a variety of factors including poor evaluation and applications techniques. Aspects such as the number of applications and the timing of applications in relation to phenology of the life stages have received no attention. To seriously evaluate the potential of fugal entomopathogens as an alternative for managing pasture spittlebugs, we are combining a detailed knowledge of the biology and phenology of spittlebugs with a series of studies to collect, screen, characterize, and formulate select isolates for deployment in field trials. In this report, we summarize the field trials established in two contrasting ecoregions of Colombia, the Amazonian Piedmont and the Cauca River Valley.

Materials and Methods

The Amazonian Piedmont ecoregion is continuously humid, corresponding to presence of spittlebug nymphs and adults throughout the year with little population synchrony. In this site the number of applications required to achieve an effect will be evaluated. The Cauca River Valley ecoregion is a highly seasonal site with bimodal precipitation and here spittlebug nymphs and adults are present only during the rainy months and have a high population synchrony. In this site the timing of the applications in relation to the insect's life cycle will be evaluated. The premise is that the diverging environmental conditions of these two ecoregions will require different strategies and control tactics for management of spittlebugs in pastures.

Five plots each were established in Hacienda Piedechinche, Santa Helena, Dept. Valle del Cauca (1600 m^2) and C.I. Macagual of CORPOICA, Florencia, Dept. Caquetá (1200 m^2). Each plot is located in a separate pasture under the same fertilization, grazing and weed management regime established for the rest of the farm. Each plot was subdivided into subplots (100 m^2) for application of treatments. Applications began 7 September 2001 in Macagual and will begin 15 days after the start of the next wet season in Piedechinche, predicted to be around the third week of September. Treatments are summarized in Tables 38 and 39 and are in a completely randomized block design with 5 repetitions. The isolates CIAT 054 (*Metarhizium* sp.) and CIAT 007C (*Metarhizium anisopliae*) were selected from among 49 isolates as the most virulent to adult *A. varia*.

Weekly population surveys are being carried out before the treatments to establish baseline data that verifies and gauges presence of the insect, and for six months after application to measure treatment effect. Surveys consisted of nymph counts in two 0.0625 m^2 quadrats and adult counts in two series of 10

sweeps with an insect net. In the laboratory nymphs were determined to instar and adults to species and sex. Natural enemies were also collected as part of the spittle mass and sweep net surveys. Pretreatment surveys began 25 January 2001 in Piedechinche and 23 March 2001 in Macagual. The start of the experiment in Macagual was postponed due to a long delay in delivery of the formulated product based on technical difficulties related to production by our commercial collaborator BioCaribe, S.A. The start of the experiment in Piedechinche depends on start of the wet season.

To gauge field mortality due to entomopathogens, 5 adults will be collected from each plot repetition with a sweep net and confined to petri dishes lined with moist filter paper to assess mycosis after 5 days.

Table 38. Field treatments applied in Macagual, Dept. Caquetá.

Product	No. applications per month for six months
Entomopathogen (CIAT 054)	0.5
	1
	2
Entomopathogen (CIAT 007C)	0.5
	1
	2
Insecticide (Malathion)	0.5
	1
	2
Control	0

Treatment effects on spittlebug nymph and adult populations will be tested by measuring insect load, or the number of insects under the population fluctuation curves, using cumulative insect days analysis.

Table 39. Field treatments applied in Piedechinche, Dept. Valle del Cauca.

Product	Weeks after appearance of first generation outbreak
Entomopathogen (CIAT 054)	1
	2
	3
	4
	5
	6
Insecticide (Malathion)	1
	2
	3
	4
	5
	6
Control	0

Results

As confirmed in previous studies, three species occur in Macagual: *Aeneolamia varia*, *Zulia pubescens* and *Mahanarva* sp. nov. Of 1207 adults collected to date, 97.0% were *A. varia*, 2.6% *Z. pubescens* and

0.4% *Mahanarva* sp. nov. at overall mean relative densities of 2.87, 0.07 and 0.01 adults/10 sweeps, respectively. Over this same period a total of 795 nymphs and 8 adult tenerals were collected (Table 40).

Table 40. Number of nymphs per life stage and teneral adults collected in spittle mass surveys at two contrasting field evaluation sites.

Instar	Macagual	Piedechinche
I	151	10
II	190	25
III	167	52
IV	114	31
Va	113	29
Vb	60	38
Tenerals	8	5
Total	803	190

In Piedechinche, only *Prosapia simulans* (1465 adults) has been detected to date, although previous populations surveys before the start of this experiment detected the presence of *Zulia carbonaria* and *Z. pubescens* at lower abundance. Phenological analysis of these data is summarized elsewhere.

Discussion

Initial population data confirm that the selected sites have sufficient populations of nymph and adult spittlebugs for this field experiment. Application of treatments has only just begun in Macagual and will begin in Piedechinche at the start of the next rainy season.

Activity 2.3 *Brachiaria* genotypes resistant to spittlebug and other biotic stresses

Highlights

- For the first time selected *Brachiaria* hybrids that showed high levels of antibiosis resistance to *Aeneolamia reducta*
- New *Brachiaria* hybrids with high levels of resistance to one or more spittlebug species were identified
- New *Brachiaria* hybrids with resistance to Rhizoctonia foliar blight were identified.

Progress towards achieving milestones

- **Produced new *Brachiaria* hybrids with selected sexual clones and different pollen parents**
  A total of 15 crossing blocks, each including from 25 to 41 selected sexual clones were successfully established and F1 seed is currently being harvested.

- **Identified new *Brachiaria* hybrids for spittlebug screening based on field performance**
  After rigorous culling on poor seed fill, it is likely that fewer than 1000 clones will be judged to merit further testing for spittlebug reaction.

- **Identified parental clones for field evaluation of recombined progeny**
  Field evaluation of open pollinated progenies of 41 clones selected on resistance to *A. varia* is in progress. Only a small number of these clones is highly resistant to two or more spittlebug species. Final selections from the progeny population will be conditioned by additional information on maternal parents reaction to other species of spittlebug.

- **Identified *Brachiaria* hybrids with resistance to *Rhizoctonia* foliar blight**
  A total of 13 (out of 108 entries) *Brachiaria* hybrids were identified as resistant to *Rhizoctonia* foliar blight using as selection of criteria proportion of infected leaves and upward disease progress from the plant inoculation.

2.3.1 Development of new hybrid population for spittlebug screening using pollen from a resistant parent (AP) and selected clones (SX) as maternals

Contributors: J. W. Miles (CIAT)

Establishment of crossing blocks at CIAT-Popayán. Collaborative research agreements to develop new *Brachiaria* hybrids for spittlebug resistance and adaptation to drought stress required the generation of large populations of diverse hybrid genotypes formed by crossing sexual (SX) clones selected from our tetraploid, sexual breeding population with elite apomictic (AP) hybrids and accessions. A total of 41 SX clones, selected on agronomic performance and spipttlebug (*A. varia*) resistance (damage and nymphal survival as good, or better, than *B. brizantha* cv. Marandu) were identified in the 1999 cycle of the tetraploid, sexual breeding population (Table 41). Compact blocks of 15 different AP genotypes were available (as seed multiplication areas) or were established, at CIAT-Popayán. Vegetative propagules of the SX clones were transplanted into established field plots of each of the AP selections at approximately 3-5 m between SX plants; i.e., each SX plant was surrounded by plants of the respective AP genotype in each of the crossing blocks. (Some of the crossing blocks were not large enough to accomodate all 41 SX clones at sufficient distance between SX plants, and fewer SX clones were planted in these: A complete listing of the SX x AP combinations established is given in Table 1.) A space approx. 1 m diameter around each SX plant was maintained clear to reduce competition. By judicious defoliation of both SX and AP genotypes, synchronous flowering was sought, but was not always achieved owing to the wide differences in flowering date (or even failure to flower) among the SX clones.

Seed from open pollination is being harvested individually from each of the SX plants in each crossing block (Table 41). An inventory of hybrid seed harvested and processed is being prepared.

Propagation of plant material for spittlebug evaluations. In 2000, over 1,700 SX x AP seedlings were evaluated as unreplicated spaced plants in duplicate field trials established at CIAT-Quilichao and at the Matazul farm in Puerto López (Llanos Orientales). Following periodic visual assessment, 121 genotypes were pre-selected on vigor, leafiness, and general freedom from disease or nutrient deficiency symptoms. These plants were propagated for evaluation of spittlebug reaction and have been tested with three different spittlebug species. A very small set of new hybrids exhibit antibiotic resistance to more than one spittlebug species.

Preselect new sexual clones for spittlebug screening in 2002. Over 4,300 sexual progenies (obtained from the random intercrossing of 41 selected sexual clones included in the 2000 recombination block) were established during first semester 2001 as unreplicated spaced plants in duplicate field trials established at CIAT-Quilichao and at the Matazul farm in Puerto López (Llanos Orientales). Clones are being culled based on periodic visual assessment (vigor, leafiness, disease or nutrient deficiency symptoms). In addition, at CIAT-Quilichao, spikelet fill (caryopsis formation) is being assessed in some detail. Caryopsis formation will receive heavy weight in final selection. Preliminary observation suggests that, in general, the sexual breeding population has poor spikelet fill (as expected), but a small number of plants with good seed formation are being identified. Final "pre-selections" from this tetraploid, sexual breeding population will be identified before 01 December 2001, when propagation for subsequent evaluations (spittlebug, Rhizoctonia, Al tolerance, etc.) will begin.

Table 41. New *Brachiaria* hybrid cross combinations being formed at Popayán during 2000.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
SX99NO/	**606**	**6387**	**16113**	**16121**	**16212**	**16315**	**16316**	**16320**	**16322**	**16467**	**16488**	**26124**	**26318**	**26556G**	**26562**
29				√	√	√	√	√	√		√	√	√	√	√
164				√	√	√	√		√		√	√	√	√	
236	√			√	√	√	√	√	√		√	√	√	√	√
246				√	√	√	√	√	√		√	√	√	√	√
275	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
497	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
574	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
711	√				√	√	√		√		√	√	√	√	
731	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
823	√			√	√	√	√		√		√	√	√	√	
835		√	√	√	√	√	√	√	√	√	√	√	√	√	√
1145	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
1260	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
1345	√			√	√	√	√		√		√	√	√	√	
1370	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
1513				√	√	√	√	√	√	√	√	√	√	√	√
1616	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
1622		√	√	√	√	√	√	√	√	√	√	√	√	√	√
1630	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
1805		√	√	√	√	√	√	√	√	√	√	√	√	√	√
1833	√	√	√	√	√	√	√		√	√	√	√	√	√	√
2030	√				√	√	√		√		√	√	√	√	
2115		√	√	√	√	√	√	√	√	√	√	√	√	√	√
2162			√	√	√	√	√	√	√	√	√	√	√	√	√
2173				√	√	√	√	√	√		√	√	√	√	√
2200		√	√	√	√	√	√	√	√	√	√	√	√	√	√
2280		√	√	√	√	√	√	√	√	√	√	√	√	√	√
2341	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
2349	√			√	√	√	√	√	√		√	√	√	√	√
2354	√				√	√	√		√		√	√	√	√	
2514				√	√	√	√	√	√		√	√	√	√	√
2606		√	√	√	√	√	√	√	√	√	√	√	√	√	√
2621		√	√	√	√	√	√	√	√	√	√	√	√	√	√
2663	√			√	√	√	√	√	√	√	√	√	√	√	√
2822	√				√	√	√		√		√	√	√	√	
2857	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
2927	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3488	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3564	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3690		√	√	√	√	√	√	√	√	√	√	√	√	√	√
3770	√			√	√	√	√	√	√		√	√	√	√	√

Sexual females are selections from tetraploid sexual breeding population. Apomictic males are CIAT accessions.
√ = Cross combination does exists. Other cross combinations not available.

2.3.2 Identify *Brachiaria* genotypes resistant to different species of spittlebug

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

Rationale

Recent evidence suggests that a significant (and important) genotype-species interaction exists for reaction to different spittlebug pest species. Hence, evaluation of plants artificially inoculated with only one species (*A. varia*) is not sufficient completely to characterize a host genotype's possible performance in the field when exposed to different species, alone or in combination. Forty-one sexual clones selected on reaction to *A. varia* are being tested with additional insect species (*A. reducta, Zulia carbonaria*, and *Z. pubescens*). In 2001, intensive screening of selected hybrids was conducted under greenhouse and field conditions.

Materials and Methods

Screenings for resistance were conducted with *Aeneolamia varia, Zulia carbonaria, Z. pubescens, Mahanarva* sp., and for the first time, *A. reducta*. Test materials were compared with five checks fully characterized for resistance or susceptibility to *A. varia*. Plants were infested with a known number of eggs of the respective spittlebug species and the infestation was allowed to proceed without interference until all nymphs were mature or adult emergence occurred. Plants (usually 10 per genotype) were scored for symptoms using a damage score scale. 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 those susceptible were discarded.

Results and Discussion

As reported last year (see p. 55 of the 2000 Annual Report), a set of 41 hybrid-derived, sexual clones equally or more resistant to *A. varia* than the resistant cv. Marandú was identified in 2000. As planned, the reaction to *A. varia* was reconfirmed between August 2000 and January 2001. Subsequently, these clones were also tested for resistance to *Z. carbonaria* and *Z. pubescens* in order to detect materials combining resistance to two or more species. Some of these hybrids were outstanding for resistance to spittlebug showing low levels of damage and reduced nymph survival, in many cases outperforming the resistant cultivar 'Marandú' (Table 42).

Table 42. Levels of resistance to three species of spittlebug in a set of 41sexual *Brachiaria* hybrids previously selected for resistance to *Aeneolamia varia*

Genotype	Mean damage scores[a]			Mean percentage nymph survival		
	Aeneolamia varia	*Zulia carbonaria*	*Zulia. Pubescens*	*Aeneolamia varia*	*Zulia carbonaria*	*Zulia pubescens*
BRX 44-02[b]	4.8	3.7	3.0	88.3	78.3	63.3
CIAT 0606[b]	4.3	4.2	4.1	95.0	76.7	91.7
CIAT 6294[c]	1.2	2.0	2.0	18.3	56.7	53.3
Resistant hybrids	1.3	1.6	1.6	15.7	26.9	23.3
Intermediate hybrids	2.3	2.3	2.3	42.3	40.0	41.1
Susceptible hybrids	-	3.7	3.2	60.0	63.5	57.8

[a] On a 1 - 5 damage score scale (1, no damage; 5, severe damage, plant killed)
[b] Susceptible check
[c] Resistant check.

A number of materials combined resistance to two or more species of spittlebug. Thus, 15 were resistant to all three species tested (Table 43) in terms of reduced damage levels. More important, three hybrids (SX99NO/0164, SX99NO/0236, and SX99NO/0823) showed antibiosis resistance (reduced nymph survival) to all species tested. This is important because it means that the breeding process has been successful in recombining genes for antibiosis resistance to *A. varia* and to other species like *Z. carbonaria* and *Z. pubescens*.

Progenies derived from crosses between some of the best sexual hybrids and apomictic parents will be evaluated for spittlebug resistance early next year.Another nursery evaluated this year was a set of 121 hybrids coded BR developed in 2000. The 32 hybrids that were initially selected for resistance to *A. varia* were then screened for resistance to both *A. varia* and *Z. carbonaria*. As shown in Table 44, high levels of resistance to *A. varia* were present in this group of materials, but none of them combined antibiosis resistance to both spittlebug species (Table 45).

Table 43. Frequency distribution of resistance reactions to three species of spittlebug in a set of 41sexual *Brachiaria* hybrids previously selected for resistance to *Aeneolamia varia.*

Category	On the basis of damage scores				On the basis of percentage survival			
	A. varia	*Z. carbonaria*	*Z. pubescens*	All three species	*A. varia*	*Z. carbonaria*	*Z. pubescens*	All three species
Resistant	38	21	28	15	32	8	9	3
Intermediate	3	19	13	1	5	9	15	0
Susceptible	0	1	0	0	4	24	17	0

Table 44. Levels of resistance to two species of spittlebug in a set of 32 *Brachiaria 2*hybrids initially selected for resistance to *Aeneolamia varia* in 2000.

Genotype	Mean damage scores[a]		Mean percentage survival	
	Aeneolamia varia	*Zulia carbonaria*	*Aeneolamia varia*	*Zulia carbonaria*
BRX 44-02[b]	4.3	4.4	61.7	72.9
CIAT 0606 [b]	4.6	4.1	83.3	85.4
CIAT 6294[c]	1.6	2.9	20.8	75.0
Resistant hybrids	1.6	1.8	24.9	35.6
Intermediate hybrids	2.4	2.5	41.5	45.0
Susceptible hybrids	3.2	3.5	64.8	67.8

[a] On a 1 - 5 damage score scale (1, no damage; 5, severe damage, plant killed)
[b] Susceptible check
[c] Resistant check.

Table 45. Frequency distribution of resistance reactions to two species of spittlebug in a set of 32 *Brachiaria* hybrids initially selected for resistance to *Aeneolamia varia*

Category	On the basis of damage scores			On the basis of percentage survival		
	Aeneolamia varia	*Zulia carbonaria*	Both species	*Aeneolamia varia*	*Zulia carbonaria*	Both species
Resistant	26	8	5	7	0	0
Intermediate	5	18	4	14	5	1
Susceptible	1	6	0	11	27	-

2.3.3 Greenhouse screening of *Brachiaria* accessions and hybrids for resistance to *Aeneolamia* species

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

For the first time we conducted studies on the resistance of *Brachiaria* genotypes to *Aeneolamia reducta*, the most important spittlebug species affecting grasses in the Caribbean region of Colombia. This is an aggressive species that causes important losses in native savannas and that may have a significant impact on any new cultivar released by the project. In a split-plot design in which the spittlebug species was the main plot and the genotypes were sub-plots, we compared the resistance to *A. varia* and *A. reducta* of four well known checks and two hybrids previously selected for resistance to *A. varia*.

As shown in Table 46, *A. reducta* caused significantly higher levels of damage on all genotypes tested, irrespective of their resistance or susceptibility to *A. varia*. More important, survival of *A. reducta* was significantly higher on all *A. varia*-resistant genotypes, suggesting that there is no antibiosis to *A. reducta* in these genotypes. These are important results that should be taken into account in the development of

future breeding strategies. Further studies with *A. reducta* were conducted with a set of 41 hybrid-derived, sexual clones equally or more resistant to *A. varia* than the resistant cv. Marandú. This had been identified in 2000.

Table 46. Comparative levels of resistance to *Aeneolamia varia* and *Aeneolamia reducta* in six selected *Brachiaria* genotypes.

Genotype	Mean damage scores[a]		Mean percentage nymph survival	
	Aeneolamia varia	*Aeneolamia reducta*	*Aeneolamia varia*	*Aeneolamia reducta*
BRX-44-02[b]	3.5aB	4.6aA	55.8aA	65.8bA
CIAT 0606[b]	3.7aB	4.6aA	66.7aA	69.2bA
CIAT 6294[c]	1.3cB	3.0cA	13.4cB	60.8bcA
CIAT 36062[c]	1.3cB	3.0cA	1.2dB	58.3cA
FM9503/4624[d]	1.2cB	3.9bA	25.8bB	81.7aA
BR99NO/4132[d]	2.1bB	4.3abA	24.2bB	58.3cA

For each variable, means within a column followed by the same lower case letter are not significantly different. Means within a row followed by the same upper case letter are not significantly different; Fischer's protected LSD ($P<0.001$)

[a] On a 1-5 damage score scale (1, no damage; 5, severe damage, plant killed)

[b] Susceptible check

[c] Resistant check

[d] Previously selected as resistant to *A. varia.*

Surprisingly, five of the hybrids showed high levels of antibiosis resistance to *A. reducta* that are comparable to those detected with *A. varia* in this and previous trials (Table 47). Dry weight losses in resistant genotypes were low, a reflection of the high levels of antibiosis present in them. As in the previous trial (Table 46), CIAT 6294 ('Marandú') did not exhibit antibiosis resistance to *A. reducta*. These are important results as they show that combining resistance to *A. reducta* and *A. varia* is feasible.

Table 47. Comparative levels of resistance to *Aeneolamia varia* and *Aeneolamia reducta* in selected sexual *Brachiaria* hybrids previously identified as resistant to *A. varia.*

Genotype	Mean damage scores		Mean percentage survival		Mean percentage dry weight loss	
	Aeneolamia varia	*Aeneolamia reducta*	*Aeneolamia varia*	*Aeneolamia reducta*	*Aeneolamia varia*	*Aeneolamia reducta*
			Best hybrids			
SX99NO/0029	2.0	2.5	1.7	21.7	11.8	19.2
SX99NO/0164	2.8	2.7	10.0	17.0	34.7	23.9
SX99NO/0236	1.4	1.9	0.2	10.0	0.02	0.0
SX99NO/0823	1.8	1.9	15.0	29.9	3.2	6.5
SX99NO/2606	2.8	2.2	15.0	24.8	18.3	4.5
			Susceptible checks			
BRX-44-02	4.7	4.3	88.3	68.3	45.8	35.4
CIAT 0606	4.3	4.6	94.9	80.0	28.0	42.3
			Resistant check			
CIAT 6294	2.1	3.3	18.3	53.3	16.5	6.4

2.3.4 Field screening of *Brachiaria* accessions and hybrids for resistance to spittlebug

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

Field screening for resistance to spittlebug continued in 2001. The methodologies have been described in previous reports. We have reported before on the reliability of the system as judged by the high correlation between greenhouse and field resistance ratings. We have also reported on the possibility of adapting the methodology to all spittlebug species. Seven major screening trials (three with *A. varia*, three with *Z. pubescens*, and one with *Z. carbonaria*) were set up in 2001. In Table 48 we highlight the results of evaluating 50 genotypes (41 sexual hybrids, 3 apomictic hybrids, 4 resistant checks, and 2 susceptible checks). On average, resistant hybrids performed as well as the resistant checks and significantly outperformed the susceptible checks.

In general, resistance levels to *Z. carbonaria* were lower than those encountered with the other two species tested. Damage scores between trials correlated well ($r= 0.841$; $P<0.001$; $n= 1500$). There was a significant, positive correlation ($r = 0.876$; $P<0.001$) between damage scores and percentage tillers killed by the nymphs.

Table 48. Field resistance to three spittlebug species in selected sexual *Brachiaria* hybrids and checks. Means of three trials with *Aeneolamia varia*, three trials with *Zulia pubescens* and one trial with *Zulia carbonaria.*

Genotype	Mean visual damage scores[a]			Tiller ratio[b]		
	A. varia	*Z. pubescens*	*Z. carbonaria*	*A. varia*	*Z. pubescens*	*Z. carbonaria*
			Best hybrids			
SX99NO/2173	1.5	2.0	2.1	1.96	1.30	0.92
SX99NO/1630	1.5	2.1	2.1	1.96	1.43	0.96
SX99NO/2115	1.5	2.0	2.0	1.96	1.12	1.38
SX99NO/2663	1.5	2,1	2.1	1.80	1.59	1.11
SX99NO/0029	1.7	2.2	2.1	1.89	1.48	1.30
SX99NO/1370	1.4	2.1	2.2	1.85	1.17	1.11
SX99NO/2857	1.5	2.1	2.0	1.58	1.48	1.66
SX99NO/2822	1.4	2.2	2.1	1.40	1.35	1.18
SX99NO/3690	1.5	2.1	2.0	1.45	1.29	0.89
SX99NO/0835	2.1	2.2	2.0	1.89	1.55	1.49
SX99NO/0246	2.0	2.0	2.0	1.67	1.28	1.32
SX99NO/2349	1.5	2.1	2.2	2.23	1.70	1.02
Mean	1.6ab	2.1b	2.1ab	1.80a	1.39a	1.19a
			Susceptible checks			
CIAT 0606	4.1	4.1	3.7	0.39	0.37	0.52
CIAT 0654	3.5	4.1	3.3	0.62	0.54	0.54
Mean	3.8a	4.1a	3.5a	0.50b	0.44b	0.53
			Resistant checks			
CIAT 6133	1.7	2.0	2.0	1.61	1.25	1.44
FM9503/4624	1.4	1.9	2.0	1.79	1.20	1.02
CIAT 36062	1.1	1.3	1.7	1.72	1.72	1.15
CIAT 6294	1.0	1.4	1.1	1.67	1.63	1.31
Mean	1.3b	1.6b	1.7b	1.69a	1.40a	1.23a

Means within a column followed by the same letter are not significantly different ($P<0.05$) by Scheffe's F analysis of arbitrary linear contrasts

[a] On a 1-5 damage score scale (1, no damage; 5, severe damage, plant killed)

[b] Number of tillers per plant at the end of the infestation process/ Number of tillers per plant at the beginning of the infestation process.

This means that field resistance expressed as reduced damage to the leaves may serve to predict losses due to nymph feeding. The most susceptible genotypes tested, the checks CIAT 0606 and CIAT 0654 lost 48-63% and 38-48% of their tillers, respectively. In contrast, tiller mortality in the resistant checks CIAT 6294 ('Marandú') and CIAT 36062 was negligible. Most important, as shown in Table 48, a number of sexual hybrids showed high levels of resistance to all three spittlebug species studied.

2.3.5 Screening of *Brachiaria* hybrids for *Rhizoctonia* foliar blight

Contributors: C. Zuleta and S. Kelemu (CIAT)

Rationale

Foliar blight disease caused by *Rhizoctonia solani* can result in substantial foliar damage on a wide range of plants. Fungal sclerotia are formed as white masses on infected tissues which later turn brown as they mature. These sclerotia easily shed forming the primary source of inoculum. The use of resistant plant materials (when available) remains the cheapest and most effective method of managing the disease. Good levels of resistance have been identified in at least one accession of *B. brizantha* (CIAT 16320). In this study, we used a uniform and reproducible inoculation and screening method to assess the reactions of *Brachiaria* hybrids to *R. solani* including CIAT 16320 as a positive control.

Materials and Methods

A fresh mycelial disc of *R. solani* AG-1, removed from a 4-day-old potato dextrose agar (Difco) culture, was added to each of several 250-ml Erlenmeyer flasks containing 30 ml PSY broth (20 g peptone, 20 g sucrose, 5 g yeast extract and 1 l deionized water). The flasks were wrapped with aluminum foil and incubated as still culture at room temperature for 10 days. Sclerotia were collected using sterile forceps. They were air-dried overnight on sterile filter papers in a laminar flow hood. Dry sclerotia were stored in sterile containers at 4 C for further use as inocula.

Plantlets were separated from tillers of each *Brachiaria* genotype to be evaluated. Individual plantlets were planted in small pots. Two weeks after planting, each plantlet was inoculated with one sclerotium placed on the soil surface in contact with the plantlet's stem. These plantlets were placed in a plastic box with one side made of cheesecloth immersed in water, in order to maintain humidity at approximately 100%. Plants were evaluated for their disease reactions 2 weeks after inoculation.

Results and Discussion

One hundred eighty hybrids of *Brachiaria* provided by the *Brachiaria* Improvement Program were evaluated for their reactions to *R. solani* in two batches. Foliar blight symptoms are shown in Photos 3, 4.

Using percentage of infected leaves and upward disease progress from the plant inoculation point as evaluation criteria, *Brachiaria* hybrids with the following codes were identified as resistant materials: 36, 42, 49, 92, 221, 228, 599, 1227, 1501, 289, 49, 93, 172, and 1098 (Figure 14). These materials had up to 5-cm upward disease symptom progress from the inoculation point of the plant and up to 10 % infected leaves.

The materials identified as intermediate resistant are: 59, 144, 235, 438, 755, 1032, 1407, 1418, 1600, 17, 861, 519, 1278, 783, 1281, 1590, 1598, 1165, 1250. These had up to 5-cm upward disease symptom progress from the inoculation point of the plant and 10-20 % infected leaves (Figure 14). All remaining materials were rated as susceptible.

Photo 3. *Rhizoctonia* foliar blight disease symptom in *Brachiaria*

Photo 4. *Brachiaria* genotypes with resistant (left) and susceptible (right) reactions

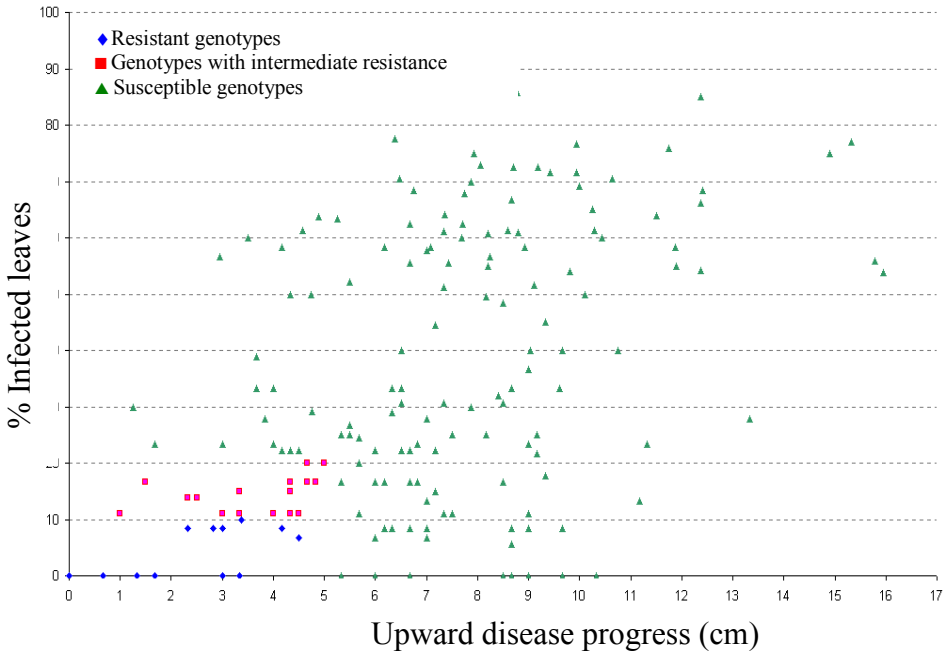

Figure 14. Evaluation of *Brachiaria* genotypes for their reactions to *Rhizoctonia* foliar blight using percentage of leaves infected and the upward progress of the disease from the inoculation points of the plants.

Activity 2.4 Identify host mechanisms for spittlebug resistance in *Brachiaria*

Highlights

- Progress was made in defining the mechanisms of resistance of *Brachiaria* to four species of spittlebug.

Progress towards achieving milestones

- **Defined reaction of new *Brachiaria* hybrids to different species of spittlebug**
  We have identified new *Brachiaria* hybrids with multiple resistance to two or more species of spittlebug. In addition, it would now seem that the main mechanisms responsible for resistance to *A. varia* and *Mahanarva* sp. in *Brachiaria* spp. is antibiosis. The resistance observed to *Z. pubescens* could be explained by a combination of tolerance with moderate levels of antibiosis. Resistance to *Z. carbonaria* seems to ne mostly tolerance.

2.4.1 Studies on resistance to spittlebug species

Collaborators: C. Cardona, G. Sotelo, P. Fory, and J. W. Miles

Rationale

There is need to ascertain that new *Brachiaria* hybrids produced by the Project do possess resistance to as many spittlebug species as possible. As reported previously, high levels of antibiosis resistance to *Aeneolamia varia* and to *Mahanarva* sp. have been detected in the resistant accession CIAT 6294, in the hybrid CIAT 36062, and in several other resistant hybrids. Although *Zulia carbonaria* and *Zulia pubescens* cause relatively little damage to these resistant genotypes, there are no indications of high

levels of antibiosis to the *Zulia* species. Tolerance might be an explanation for the lower levels of damage caused by the *Zulia* complex but we needed to ascertain this. Full characterization of the mechanisms of resistance is important in the formulation of appropriate breeding strategies. That is why a detailed study on the nature of resistance to four major spittlebug species was initiated in 2000. The main results are reported herein.

Materials and Methods

Studies on mechanisms of resistance were conducted with genotypes previously characterized for their resistance or susceptibility to *A. varia*. A susceptible check (CIAT 0654) and a resistant hybrid (CIAT 36062) were used to study antibiosis. These materials were infested following the methodologies described in previous annual reports. To determine mortality levels and the stage in the life cycle affected by the resistant hybrid CIAT 36062, 120 plants of each of the resistant and the susceptible genotypes were infested each with 6 eggs of each the spittlebug species per plant.

After egg hatching, a sample of 12 nymphs per species was taken daily and examined under a stereoscopic microscope in the laboratory. The fate of each individual (survival) was recorded. Nymphal instars and their duration were determined by measuring the widths of the head capsules of all nymphs recovered (dead or alive). This process continued until all surviving nymphs reached the adult stage.

We conducted two consecutive tests. To measure tolerance, we used a susceptible check (CIAT 0654) and two resistant genotypes (CIAT 36062 and CIAT 6294). For each spittlebug species we studied the effect of increasing levels of infestation: 2, 3, 5, 7, and 10 nymphs per plant. Uninfested controls were used for comparison and to calculate dry weight losses. Infestation was allowed to proceed until all nymphs were fully mature or adult emergence occurred. At this point plants were scored for damage, and survival rates and dry weight losses were calculated.

Results and Discussion

A very high level of antibiosis resistance to *Mahanarva* sp. was detected in the resistant hybrid CIAT 36062. All of more than 1,000 individuals studied failed to reach the second instar and the colony died out (Figure 15). The life cycles of *A. varia*, *Z. carbonaria* and *Z. pubescens* were delayed to a lesser extent by the resistant genotype.

Mortality of *Mahanarva* sp. on the resistant hybrid was so high that the population collapsed (Figure 16). The median survival time of the population on this genotype was 17 days, significantly lower than that calculated for the susceptible check (56 days). Mortality of *A. varia* was not so high, but still significantly different from that on the susceptible check. Apparently, the *Zulia* species were less affected.

Further analysis of the data by means of four different survival tests (Table 49) indicated that indeed the resistant variety CIAT 36062 had a significant impact on the survival of three of the species studied. The positive sign of Z, C, L, and Z (test statistics) in Table 49 indicate that the susceptible genotype (CIAT 0654) favors the survival of all spittlebug species.

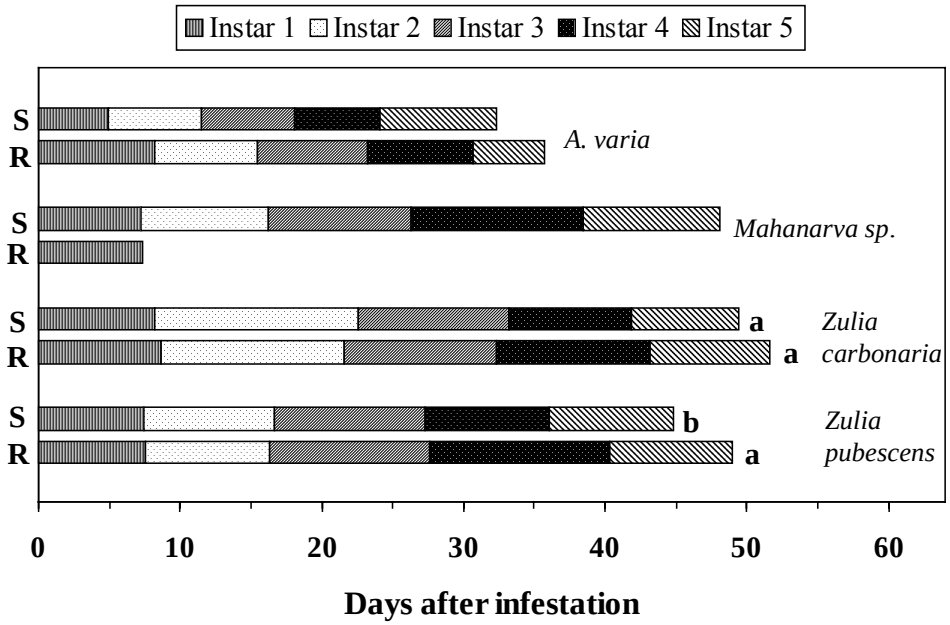

Figure 15. Duration of nymphal instars of four spittlebug species reared on a susceptible (S, CIAT 0654) and a resistant (R, CIAT 36062) *Brachiaria* genotype. Bars with the same letter are not significantly different (P<0.05). Pairwise comparison by *t* test within species. *Mahanarva* sp. was not analyzed due to total mortality of second instars. *A. varia* was not analyzed due to very high mortality of fifth instars in the resistant genotype.

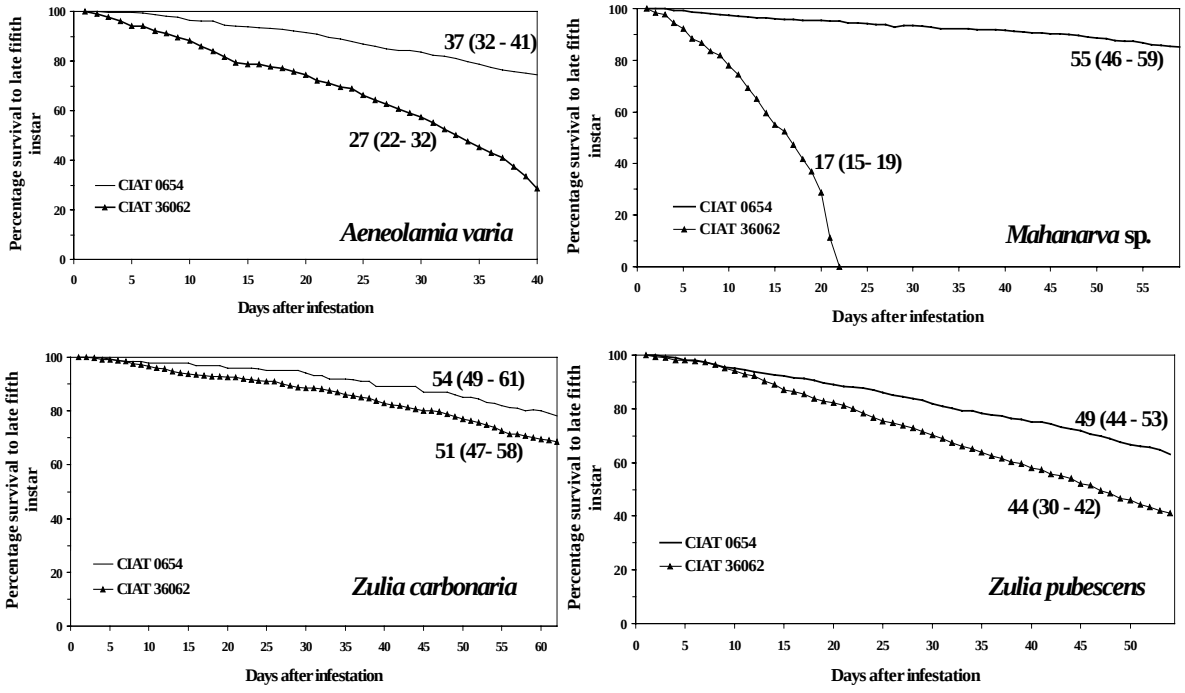

Figure 16. Survivorship curves for four species of spittlebug reared on susceptible (CIAT 0654) and resistant (CIAT 36062) *Brachiaria* genotypes. Values for median survival times (95% Confidence Intervals) are shown and were determined using the Kaplan-Meier survivorship test (Lee 1992). Values with overlapping confidence intervals are considered equal.

Table 49. Survivorship parameters for four spittlebug species reared on susceptible (CIAT 0654) and resistant (CIAT 36062) *Brachiaria* genotypes

Species	No. tested per trial per genotype	Two-sample survival tests			
		Z[a]	C[b]	L[c]	Z[d]
Aeneolamia varia	480	4.0**	4.8**	4.8**	4.3**
Mahanarva sp.	708	8.0**	9.7**	9.3**	8.2**
Zulia carbonaria	720	1.3ns	1.4ns	1.4ns	1.5ns
Zulia pubescens	648	1.8ns	2.2*	2.2*	2.2*

**Significant at the 1% level; *, significant at the 5% level; ns, not significant
[a]Gehan-Wilcoxon test; [b] Cox-Mantel test; [c] Logrank test; [d] Peto-Wilcoxon test

The higher the value of the coefficients, the higher the difference between the susceptible and the resistant genotype, that is to say the higher the level of antibiosis (mortality) caused by the resistant genotype. Highest values of the coefficients were calculated for *Mahanarva* sp. This means that this species is the one most affected by antibiosis, followed by *A. varia*. There was significance at the 5% level for *Z. pubescens*, suggesting low levels of antibiosis to this species. The lack of significance in the case of *Z. carbonaria* is a clear indication that there is no true antibiosis to this species. Furthermore, when we compared the survival of *Z. carbonaria* and *Z. pubescens* on the resistant genotype, we found that all test statistics were positive, meaning that CIAT 36062 was more favorable to *Z. carbonaria* than to *Z. pubescens*.

Antibiosis effects were also detected in terms of reduced dry weight of nymphs and adults. As shown in Figure 17, *A. varia* and *Z. pubescens* nymphs and adults reared on CIAT 36062 had significantly lower weights than those reared on the susceptible genotype. Antibiosis to *Mahanarva* sp. was so high that no nymphs developed beyond the third instar. There was no effect of the resistant genotype on the weight of *Z. carbonaria*. Further proof of differential antibiosis effects was obtained when the susceptible and the resistant genotypes were infested with increasing numbers of nymphs per plant (Table 50).

At all levels of infestation, survival of *Mahanarva* sp. nymphs was very low and significantly different from that of all other species studied. Significant differences in survival of nymphs at all levels of infestation also detected high and moderate levels of antibiosis to *A. varia* and *Z. pubescens*, respectively. No significant differences were found in the case of *Z. carbonaria*, meaning that antibiosis is not a mechanism of resistance to this species.

Highly significant differences were also found in terms of percentage dry weight losses (Table 51). Lower dry weight losses caused by *A. varia* and *Mahanarva* sp. are a reflection of high levels of antibiosis to these species; lower plant losses due to *Z. pubescens* may be due to a combination of moderate levels of antibiosis coupled with tolerance, whereas the only plausible explanation for resistance to *Z. carbonaria* is tolerance.

Similar results were obtained with the resistant accession CIAT 6294. At three levels of infestation highly significant differences among genotypes and spittlebug species were found for both percentage survival and percentage dry weight reduction (Table 52). CIAT 6294 was antibiotic to *A. varia* and to *Mahanarva* sp. but not to any of the *Zulia* species. Lower losses in CIAT 6294 due to *Z. carbonaria* and to *Z. pubescens* can only be explained as a manifestation of tolerance to the *Zulia* complex in this genotype.

Definite proof of tolerance as the main mechanism of resistance to *Z. carbonaria* was obtained when Functional Plant Loss Indexes for five levels of infestation were calculated (Table 53). Clearly, at all

levels of infestation tested, *Z. carbonaria* caused lower losses on the resistant genotypes CIAT 36062 and CIAT 6294 than on the susceptible genotype CIAT 0654. We conclude that the main mechanism responsible for resistance to *A. varia* and *Mahanarva* sp. in *Brachiaria* spp. is antibiosis. The resistance to *Z. pubescens* can be explained by a combination of tolerance with moderate levels of antibiosis. Tolerance is the mechanism underlying resistance to *Z. carbonaria*.

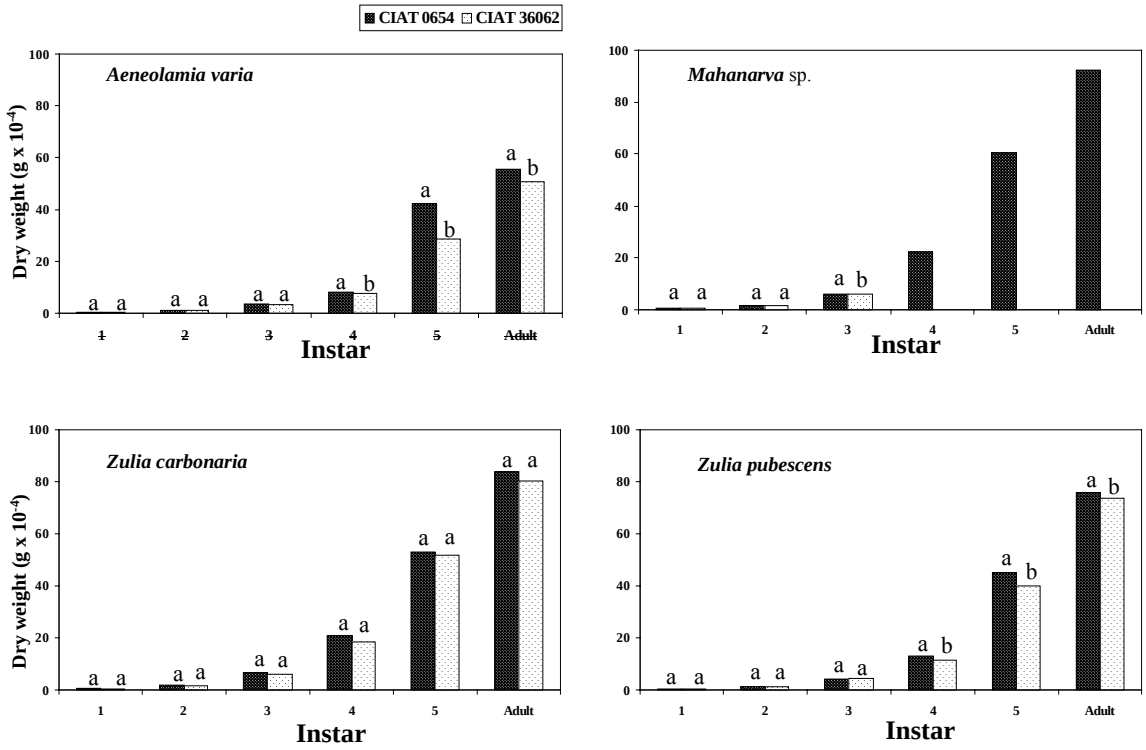

Figure 17. Effect of a susceptible (CIAT 0654) and a resistant (CIAT 36062) *Brachiaria* genotype on the dry weight of nymphs of four spittlebug species. Bars with the same letter are not significantly different ($P < 0.05$). Pairwise comparison by *t* test within instar.

Table 50. Percentage nymph survival[a] of four species of spittlebug reared at varying levels of infestation on a susceptible (CIAT 0654) and a resistant (CIAT 36062) *Brachiaria* genotype

Genotype	Species	Level of infestation (nymphs per plant)				
		2	3	5	7	10
CIAT 0654	*Aeneolamia varia*	86.0 ± 5.7ab	77.3 ± 6.5bc	74.1 ± 5.1b	85.0 ± 3.3a	51.9 ± 4.9b
	Mahanarva sp.	97.5 ± 2.5a	95.0 ± 2.7a	94.0 ± 2.6a	74.5± 3.9ab	80.5± 5.0a
	Zulia carbonaria	84.2 ± 5.5ab	85.0 ± 4.5ab	70.0 ± 8.8b	78.0 ± 3.6ab	65.5 ± 3.7b
	Zulia pubescens	79.2 ± 5.8b	65.0 ± 7.4cd	64.0 ± 5.1b	66.7 ± 4.3b	56.5 ± 4.2 b
CIAT 36062	*Aeneolamia varia*	17.5 ± 6.6d	26.3 ± 5.6f	19.0 ± 4.8cd	1.4 ± 0.8d	14.6 ± 3.6d
	Mahanarva sp.	0e	1.7 ± 1.7g	0d	0d	3.6 ± 2.0e
	Zulia carbonaria	77.5 ± 5.7b	55.5 ± 7.9de	61.0 ± 6.2b	62.8 ± 4.9b	57.5 ± 5.7b
	Zulia pubescens	47.5 ± 9.9c	38.3 ± 8.5ef	28.0 ± 6.4c	37.7 ± 5.7c	20.5 ± 4.6c

[a] Means ± SEM of two trials, 10 replications per level of infestation per trial. Means within a column followed by the same letter are not significantly different by LSD. Each level of infestation analyzed separately.

Table 51. Percentage dry weight loss caused by four species of spittlebug reared at varying levels of infestation on a susceptible (CIAT 0654) and a resistant (CIAT 36062) *Brachiaria* genotype

Genotype	Species	Level of infestation (nymphs per plant)				
		2	3	5	7	10
CIAT 0654	*Aeneolamia varia*	21.6 ± 3.2a	23.8 ± 4.3ab	38.6 ± 6.2a	49.9 ± 5.4a	41.7 ± 3.7a
	Mahanarva sp.	26.1 ± 3.7a	30.6 ± 4.9a	25.7 ± 5.5bc	35.3 ± 3.1b	32.2 ± 3.6ab
	Zulia carbonaria	23.8 ± 3.4a	25.3 ± 3.1a	37.8 ±3.0ab	33.0 ± 3.3b	35.7 ± 2.7ab
	Zulia pubescens	24.3 ± 3.6a	28.2 ± 3.4a	27.6 ± 3.7ab	32.1 ± 3.5b	29.4 ± 4.3b
CIAT 36062	*Aeneolamia varia*	9.9 ± 2.8b	11.7 ± 3.1c	7.5 ± 2.5ef	3.5 ± 1.4d	11.6 ± 3.6de
	Mahanarva sp.	5.6 ± 1.9b	6.6 ± 2.0c	2.7 ± 1.3f	5.5 ± 1.9d	6.0 ± 2.3e
	Zulia carbonaria	12.6 ± 3.2b	12.7 ± 2.6bc	17.9 ±3.9cd	15.5 ± 2.7c	19.6 ± 3.2c
	Zulia pubescens	9.2 ± 2.6b	10.9 ± 1.6bc	14.8 ± 3.6de	17.1 ± 3.1c	13.0 ± 2.0cd

Means ± SEM of two trials, 10 replications per level of infestation per trial. Means within a column followed by the same letter are not significantly different by LSD. Each level of infestation analyzed separately.

Table 52. Response of susceptible (CIAT 0654) and resistant (CIAT 6294) *Brachiaria* genotypes to three levels of infestation with four species of spittlebug.

Genotype	Species	Percentage nymph survival			Percentage dry weight loss		
		3[a]	5	7	3	5	7
CIAT 0654	*A. varia*	81.0 ± 9.3ab	80.0 ± 6.7ab	79.1 ± 8.1a	27.5 ± 5.2a	36.3 ± 8.1ab	45.7 ± 4.4ab
	Mahanarva sp.	76.7 ± 7.4ab	76.0 ± 7.8ab	65.7 ± 11.5a	40.9 ± 6.4a	45.9 ± `4.5a	53.6 ± 6.2a
	Z. carbonaria	100.0 ± 0a	86.0 ± 6.7a	70.0 ± 8.4a	28.3 ± 6.1a	31.0 ± 2.9b	30.7 ± 3.9bc
	Z. pubescens	51.5 ± 8.4c	75.0 ± 8.0ab	78.6 ± 7.4a	30.0 ± 7.9a	38.3 ± 4.5ab	41.6 ±3.2ab
CIAT 6294	*A. varia*	16.7 ± 7.4d	24.0 ± 10.5c	31.4 ± 10.8b	6.0 ± 2.1b	8.0 ± 3.8c	19.0 ± 4.6cd
	Mahanarva sp.	0d	0d	0c	4.2 ± 2.3b	6.3 ± 2.7c	11.0 ± 3.0de
	Z. carbonaria	83.3 ± 7.4ab	62.0 ± 9.2bc	61.0 ± 7.1a	7.9 ± 2.8b	15.4 ± 3.1c	14.1 ± 3.7de
	Z. pubescens	66.7 ± 8.6bc	72.0 ± 6.1ab	75.7 ± 7.0a	10.6 ± 4.3b	6.2 ± 3.7c	10.6 ± 5.2e

[a] Nymphs per plant

Means ± SEM of two trials, 10 replications per level of infestation per trial. Means within a column followed by the same letter are not significantly different by LSD. Each level of infestation analyzed separately.

Table 53. Functional plant loss indexes (F.P.L.I.)[a] for susceptible (CIAT 0654) and resistant (CIAT 36062, CIAT 6294) *Brachiaria* genotypes exposed to five levels of infestation (nymphs per plant) with four species of spittlebug.

Levels	*Aeneolamia varia*			*Mahanarva* sp.			*Zulia carbonaria*			*Zulia pubescens*		
	CIAT 0654	CIAT 36062	CIAT 6294	CIAT 0654	CIAT 36062	CIAT 6294	CIAT 0654	CIAT 36062	CIAT 6294	CIAT 0654	CIAT 36062	CIAT 6294
2	73.6	24.9	35.4	69.3	21.6	28.0	70.7	47.4	46.0	67.0	37.9	25.6
3	80.3	30.5	26.0	70.4	22.6	26.0	65.5	48.1	48.6	65.3	40.1	36.0
5	84.2	25.2	35.4	72.5	18.5	26.0	76.2	48.2	64.8	76.2	43.3	38.4
7	84.3	19.2	43.9	76.8	17.7	37.4	77.5	49.5	50.0	74.8	49.2	40.5
10	91.6	15.0	43.0	74.6	17.1	31.8	80.5	56.8	62.9	72.7	41.2	37.0

[a] F.P.L.I. = 1 - [Dry weight of infested plants / Dry weight of uninfested plants] x [1 - damage score / 5] x 100

Activity 2.5 Genetic control and molecular markers for spittlebug and reproductive mode in *Brachiaria*

Highlights

- A new set of *Brachiaria* hybrids with known mode of reproduction is now available.

Progress towards achieving milestones

- **Apomictic *Brachiaria* hybrid selections identified on the basis of progeny testing**
  Using visual assessment of relative uniformity/heterogeneity among open pollinated siblings we selected 18 new apomictic *Brachiaria* hybrids which will now be evaluated in regional trials.

2.5.1 Reproductive mode of new *Brachiaria* hybrids (SX x AP)

Contributors: J. W. Miles (CIAT)

A set of 121 "pre-selected" SX x AP hybrids was identified among over 1,700 hybrids evaluated in field trials during 2000. Reproductive mode of these hybrids is expected to be approximately 50% sexuals and 50% apomicts. In late 2000 and early 2001, open pollinated seed of 56 of these selections was harvested on the spaced plants in the field nursery established at CIAT-Quilichao in 2000. Open pollinated seed of the remainder of the set of 121 was subsequently obtained from pot-grown, vegetative propagules at CIAT-headquarters.

Open pollinated seed of 56 pre-selected hybrid clones was germinated and a maximum of 20 seedlings (where available) established. Seedlings were transplanted to the field at CIAT-headquarters in first semester, 2001, in 20-plant progeny rows. A vegetative propagule of the maternal genotype was established at the head of each progeny row. Reproductive mode of the maternal clone was assessed visually by the relative uniformity/heterogeneity among the OP siblings. A final assessment was made on 18 September (on approx. 3-mo-old plants). Of the 56 progenies, 13 were classified as sexual, 18 as apomictic, with 3 as facultative apomicts (progeny generally uniform with one or more off-types), and the rest more or less ambiguous. (Herbicide damage early in this trial lead to significant loss of plants as well as non-genetic heterogeneity among plants, which confused the results in some progenies.)

2.5.2 Construction of a molecular genetic map of Brachiaria and QTL analysis of spittlebug resistance

Contributors: O. X. Giraldo, J. Vargas, E. Gaitán, M.C. Duque, J. Miles, C. Cardona, and J. Tohme (CIAT)

Rationale

The genus *Brachiaria* Griseb. belongs to the tribe Paniceae, comprises aproximately 100 species, mostly of African origin. Some of these have found commercial use as forage in tropical America, with approximately four-five million hectares of *Brachiaria* pastures in Brazil alone (Valle and Miles 1992). The commercial species of B. *brizantha* and B. *decumbens* are tetraploid apomitic (Valle 1986), The Construction of a Brachiaria molecular map was initiated (BRU annual report pp 123-127, 2000), using a population of 215 F1 individuals derived from a cross between an autotetraploid spittlebug susceptible individual *B. ruziziensis* and a tetraploid spittlebug resistant individual *B. brizantha*. The objective of the study is to increase the saturation of the map using SCARs and SSRs developed at CIAT, AFLPs, RFLPs

probes from other grases species and tag the quantitative trait loci (QTLs) controlling spittlebug resistance in Brachiaria.

Materials and Methods

Plant Material: A sexual tetraploid *B. ruziziensis* (Swenne and Durjardin, 1981), susceptible to spittlebug (accession CIAT 44-3), was used as a female parent in a cross with natural and apomitic tetraploid genotype *B. brizantha* resistant to spittlebug (accession CIAT-6294).

DNA Extraction: DNA was extracted using the protocol described by Carlos Colombo (personal communication) with some modifications. 1g of tissue was dried at 48 °C for 20 hours and ground to fine power; 15 ml of extraction buffer (0.1M Tris-Hcl pH8.0, 0.05M EDTA pH8.0, 0.7 M NaCl, 4% CTAB and 1% ßMe) was added and incubated at 65 °C for 10 min; 15 ml of chloroform:isoamyl alcohol (24:1) was added and centrifuged at 3000 RPM for 30 min. The aqueous phase was transferred to a new tube and 8 ml of chloroform:isoamyl alcohol was added and centrifuged at 3000 RPM for 30 min, repeated twice. A volume of cold isopropanol was added to the supernatant and incubated over night at - 20 °C. The isopropanol mixture was centrifuged at 3000 RPM for 30 min at 4 °C. The DNA pellet was washed with cold 75% ethanol and dried at room temperature, and then resuspended in 300 ul of TE. Pancreatic RNAse was added to a final concentraction of 20 ug/ml. DNA was quantified on a DYNA QUANT 200 fluorometer (Hoffer Scientific Instruments, San Farancisco CA).

Microsatellites: The isolartion of the microsatellites and the methodology for PCR amplification and evaluation of polymorphism have been described previously in (BRU annual report pp 123-127, 2000). An additional set of 26 new SSRs were evaluated this year.

AFLP, RFLP RAPD: All 215 individuals were evaluated using the combination (E-ACG/M-CTA), The screening methodology was described in (BRU annual report pp 123-127, 2000). Protocols for RFLP and RAPD, markers in *Brachiaria* were described previously (BRU Annual report pp 105-110 1997).

Linkage Analysis: Segregation of markers as single dose restriction fragment (SDRF) markers according to the genetic model was determined by departure from the hypothesized 1:1 ratio by the Chi-square test. The data matrixes obtained for presence or absence of bands were analyzed with MAPMAKER v 3.0b for PC (Lander et al. 1987), using LOD score of 6.0 and recombination fraction 0.3. Recombination was translated to genetic distances using the Kosambi map function.

Results and Discussion

Phenotypic screening: The Tropical Forage Entomology section screened the population of the average damage of individual hybrid plant (C. Cardona et al., 1999). The results indicate that approximately 74.5% of the population can be classified as resistant on susceptible individuals to the spittlebug damage. The average damage values cover a continuous range from 1 to 5 suggesting a quantitative trait. Three different ranges were derived allowing the classification of the population as resistant, intermediate or susceptible individuals.

The Genetic Linkage Map: Sixty-eight SSRs, 5 combinations AFLPs (116 markers), and 35 RFLPs segregating in the male parent (CIAT-6294), were tested for linkage using MAPMAKER V.3.0b. Polymorphisms were scored for presence (H), and absence (A), and analyzed for dosage among F1 progeny using Chi-square tests ($P<0,01$). 45 SSRs, 67 AFLPs and 16 RFLPs markers, were found to define 22 linkage groups spanning 1079.031 cM, with an average marker density of 1 marker every 8.43 cM (Figure 18), map distance in centimorgans was calculated using the Kosambi mapping function. Linkage groups were organized according the number of markers presents in each group.

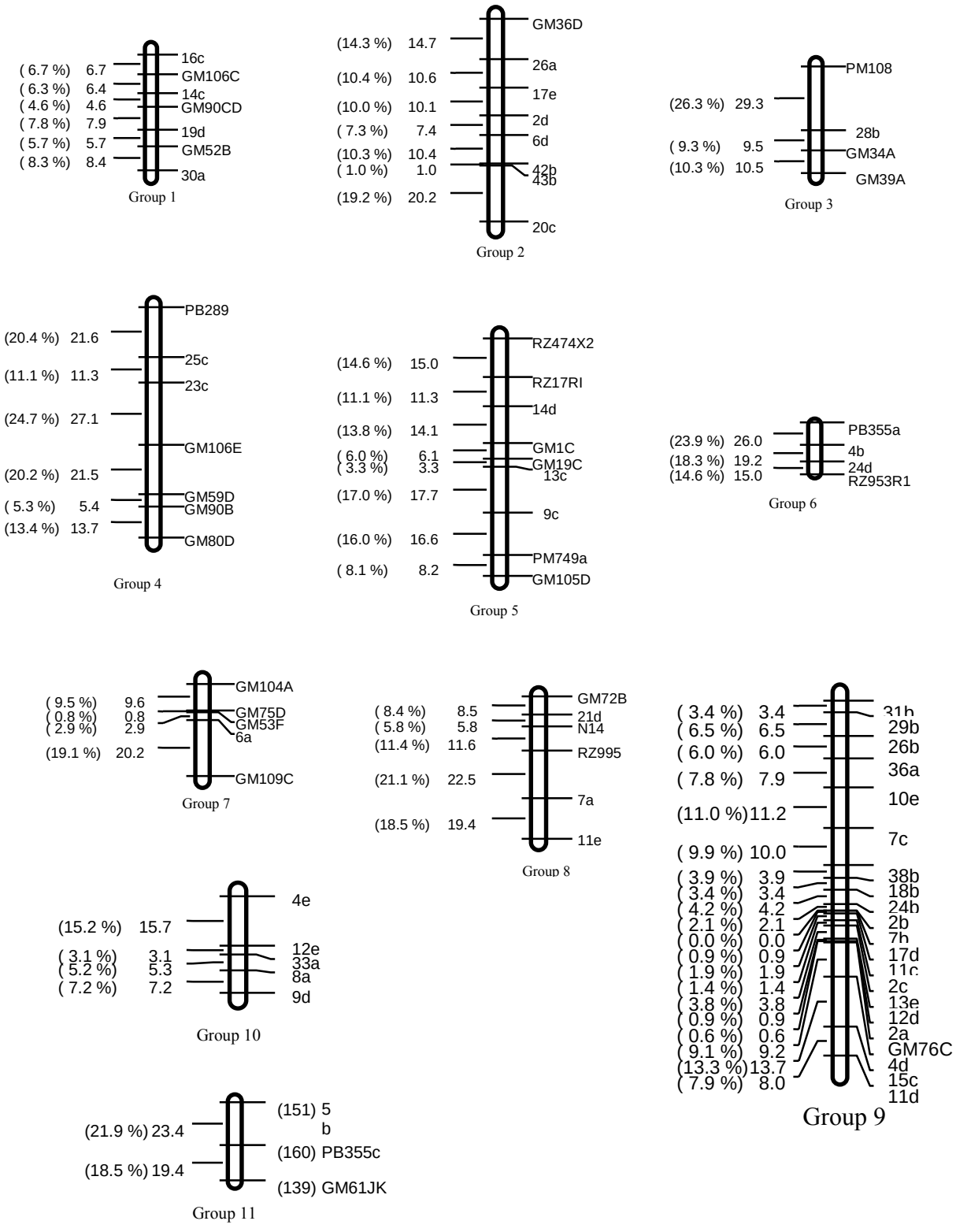

Figure 18. Preliminary *Brachiaria* framework map (LOD:6 Tetha: 0.3) (continues…..)

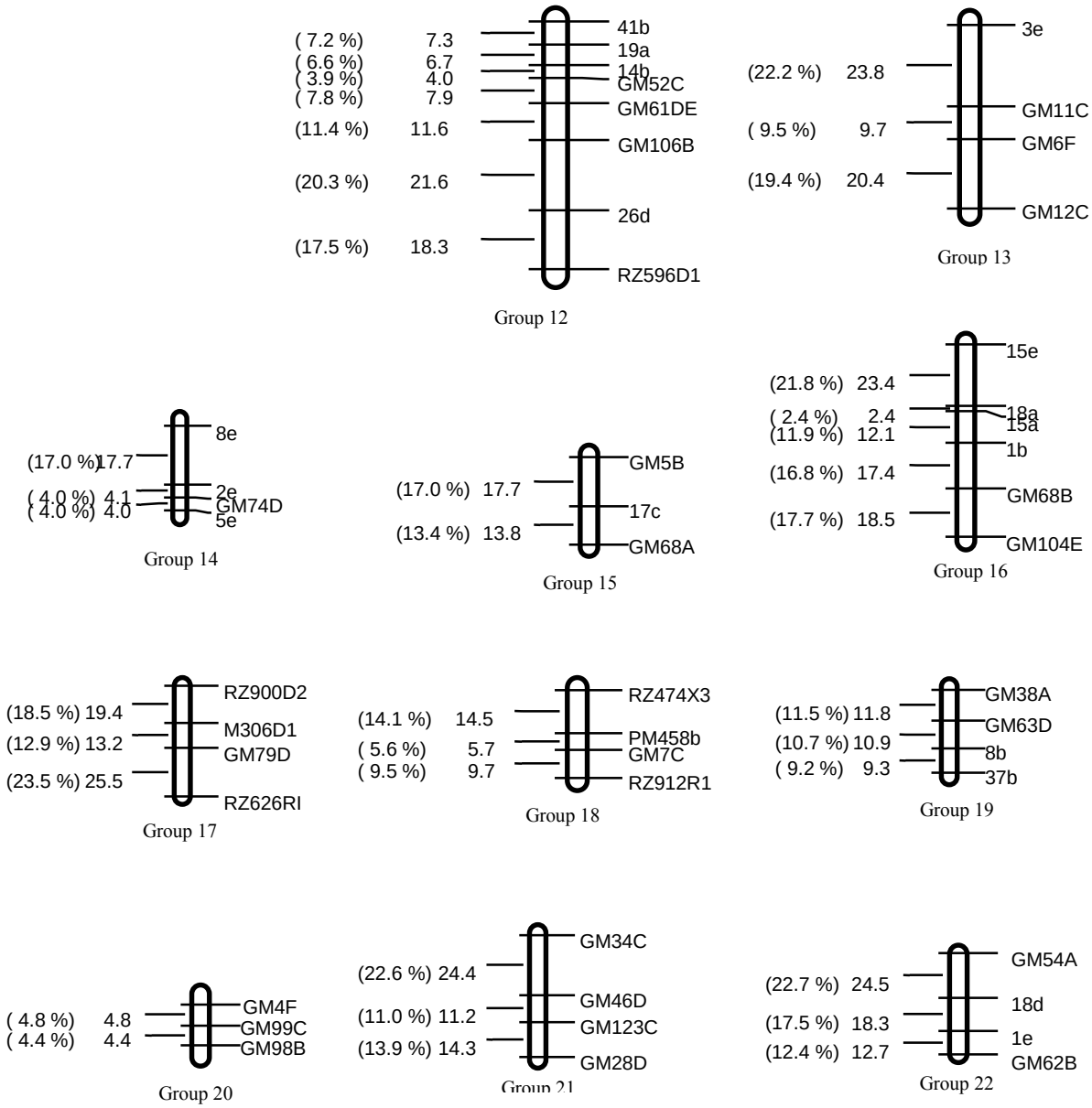

Figure 18. Preliminary *Brachiaria* framework map (LOD:6 Tetha: 0.3) (Continuation….)

The most densely populated linkage group 9 spanned 99.0 cM with 21 markers, follow by the linkage group 5 with 9 markers, spanned 92.306 cM, the groups 2 and 12 with 8 markers spanned 74.404 and 77.275 cM each one, while the least populated group was the linkage group 20 with 3 markers. The markers were grouped using a LOD = 6 and a recombination fraction of 0.3.Only the markers from linkage group 9 were grouped using a LOD=34 and recombination fraction of 0.3 (Figure 18).

Quantitative Trait Loci (QTL) analysis The average damage values from each genotype (F1) were analyzed with QTL Cartographer software using the map generated by Mapmaker. First, a search test was conducted, to find the association of segregant markers and the trait of interest, through lineal regression for each marker in relation to the quantitative trait and using the Composite Interval mapping Method

(CIM). Statistical significance levels of 0.01% were obtained by evaluation of F test in 6 markers of linkage group 2 (GM36D, 42b, 6d, 2d, 17e, 26a), and 3 markers of linkage group 16 (1b, 16a, 15a). These significance levels indicate a strong genetic linkage. Using the Composite Interval Mapping method, two major QTLs were found on linkage groups 2 and 16 with a LOD of 21 and 8 respectively (Figure 19) suggesting a strong evidence for the presence of QTLs for resistance to spittlebug. The most significant QTLs explained up to 37% and 15 % of the variance for QTL 1 and QTL2 respectively.

More makers will be placed on the Brachiaria map as they become available. The QTLs data will be integrated with the work on the isolation of Brachiaria resistance gene analogs (RGAs). We plan to saturate the region of the different QTLs with additional markers using AFLP and Dart to fine map the region of QTL1 and QTL2. Such markers could eventually be used for a marker assisted selection program. However we will need to confirm the QTL1 and QTL2 in a different background.

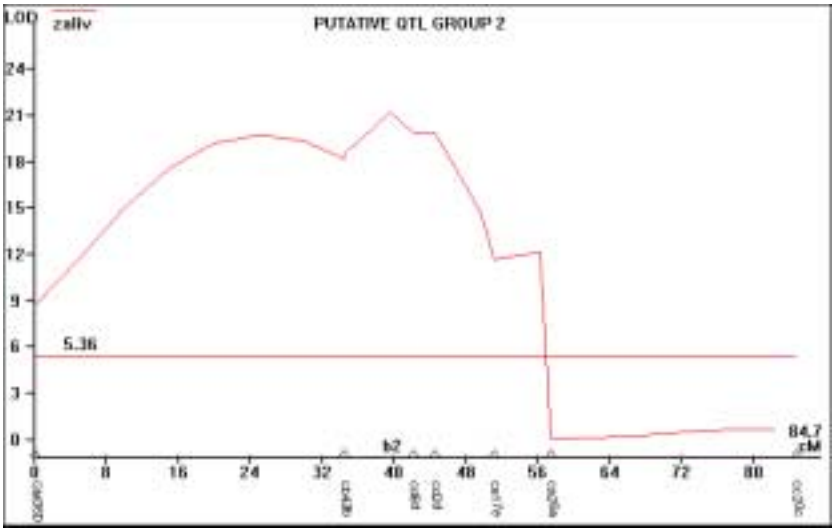

Figure 19. Putative QTLs associate with susceptibility to spittlebug in the linkage group 2.

Activity 2.6 Role of endophytes in tropical grasses

Highlights

- Endophyte isolates were successfully introduced into nine plants of two different accessions.
- DNA fragment common to most of the isolates of *A. implicatum* has been cloned, sequenced, and specific primers synthesized for use in detection of endophytes in species of *Brachiaria*.
- The genetic diversity of isolates of *Brachiaria* endophytes was determined.
- New endophyte isolates have been isolated and characterized.
- The presence of an endophytic fungus in a *Brachiaria brizantha* accession may have a major contribution to its *Rhizoctonia* foliar blight resistance.

Progress towards achieving milestones

- Isolated and characterized new isolates of endophytes
- Synthesized and tested endophyte-specific primer
- Defined effect of a new endophyte isolate on *Rhizoctonia solani*

We have isolated and characterized new isolates of endophyte from some *Brachiaria* hybrids and accessions. One of the accessions (*B. brizantha* CIAT 16320) which contained an endophyte has high levels of resistance to *Rhizoctonia* foliar blight. Elimination of the endophyte from the plant using a fungicide eliminates the antfungal properties of plant extracts. We have cloned and sequenced a RAPD fragment common to most isolates of the endophyte *A. implicatum*. A specific primer was synthesized based on the sequence data. The primer differentiates endophytic fungi from other fungi and can be used to detect endophytes in the plant.

2.6.1 Endophyte seed transmission studies in *Brachiaria*

Artificial introductions of endophytes in *Brachiaria* genotypes

Contributors: X. Bonilla, C. Zuleta, H. Dongyi and S. Kelemu (CIAT)

Rationale

Successful and efficient artificial inoculation methods are essential for studies of effects of endophytes on plant physiological processes. Beneficial endophytes, which have the feature of maternal transmission through the ovule and seeds, can then be introduced into the plant genotype of interest via artificial inoculations so that the grass-endophyte association becomes self-replicating as long as the infected seeds are stored under conditions which keep the fungus alive.

Materials and Methods

Seeds of *Brachiaria brizantha* CIAT 6780 and CIAT 26110 were surface-sterilized as follows: lemas and peleas were carefully removed without damaging embryos. These were washed with 70% ethanol for 2 minutes, in 2.5% NaOCl for 10 minutes, rinsed 5 times with sterile distilled water. The seeds were transferred to sterile filter papers to remove excess moisture, then planted onto magenta vessels (Sigma) containing plant growing medium (MS), 8-10 seeds/vessel, and incubated at room temperature. Seedlings were inoculated with selected isolates of *Acremonium implicatum* 15-20 days after germination. Fungal mycelium was introduced into meristem tissues with the use of an entomological needle under a stereoscope.

The wound area where the fungus was introduced was sealed with sterile vaseline. Inoculated plants were transplanted onto magenta vessels with MS medium and incubated for 10-15 days. Surviving plants were transplanted to pots containing sterile soil. Plants were checked for the presence or absence of the endophyte 2.5 -3 months after inoculations.

Results and Discussion

Of the 70 inoculated and surviving plants of *B. brizantha* CIAT 6780, only 6 plants tested positive for the presence of the endophyte. Forty-nine inoculated *B. brizantha* CIAT 26110 plants survived, of which only 3 tested positive for the endophyte. Examinations for endophyte presence were done 4-12 months after inoculations.

The plants which tested positive for endophytes were transplanted into big pots for further tillering and propagation. Half of these tillers were treated with the fungicide Folicur in order to create genetically identical clones of *Brachiaria* with and without the endophyte.

2.6.2 Synthesis of endophyte specific DNA fragment for quick detection of endophytes

PCR analysis and screening of *Brachiaria* genotypes
PCR analysis of other fungi (pathogens or non-pathogens) and new endophytes

Contributors: H. Dongyi, (South China University of Tropical Agriculture, The People's Republic of China), Y. Takayama (Tamagawa University, Tokyo, Japan), and S. Kelemu (CIAT)

Rationale

Both the benefits and harmful effects of endophytes make it important to examine their presence in forage grass *Brachiaria*. Microcopy and culture isolation methods had been established earlier. But these methods are time and resource consuming. PCR detection methods have been used to detect fungi in plants. A RAPD fragment common to six endophyte isolates (generated with primer OPAK10) had been cloned and sequenced (Figure 20). Specific primers were synthesized based on this sequence data. Two sets of primer pairs were tested for specific detection of endophytes.

These primer pairs were: P1 (5'-TTCGAATGATAAGGCAGATC-3') and P4 (5'-ACGCATCCACTGTATGCTAC-3'); P4 and P5 (5'-TGAGAAGACCTCTTGTTATG-3').

CAAGCGTCACGGAGTACATAGTTTCACCCGGTGTTCGAATGATAAGGCAGATCCGAG
GAGCACTTTGCGGATGAGAAGACCTCTTGTTATGCCGGAGGGGGGTTGTTGGAGTTA
CGCTGATCTCTATGAGCCCAGATTATTAGAGGAGGAGACGAGTTGGAAATTTGAGTC
CCGTCGCCCCTTCCCGACTTCTGACCAGACTGTGGTGACCACTTGCGCCTGCCATTGC
CACCTGGCGTCCCGACTGTTGCCAACCCAGTTTTCTTCCATGGCCGGCTATGCTCAGT
CTTGCATTTGTGAGAAAAGAAAGATCCTGCTCTCTATTCGTGCAGCGAAAAATGTTT
GCGGAACCCCCTTTCACCCCTTGGATGCCGATCCATGCGCGACTTCCTCTTGGTTGAT
CTAGCTGGTGTTATNCACATGTCCCGATCTCGCCTTCTTGTACTAGTAGTGACGAGAC
AGCTGGACCGTAGCATACAGTGGATGCGTCCTATACTCCTGTCATGACGGTGACGCT
TG

Figure 20. Sequence data of a RAPD fragment common to six endophyte isolates from species of *Brachiaria*.

This system can be used, without a doubt, to correctly identify endophytes of *Brachiaria in vitro*. However, because of the high sensitivity of the method, and a lack of completely endophyte-free control clonal plants, we have not yet been able to routinely use the method for endophyte detection *in planta*. Further improvements of this PCR method are currently in progress. These 2 sets of primer pairs could specifically amplify the target fragment (about 450-bp), using genomic DNA template of all endophyte isolates or endophyte-containing species of *Brachiaria*. With primer pair P1/P4, the genomic DNA of two major pathogens of *Brachiaria*, *Drechslera* spp. and *Rhizoctonia solani*, a very faint and insignificant band was amplified, whereas a strong specific band was amplified in genomic DNA of all endophyte isolates (Photo 5). Plants with codes of "P + numbers" are plants artificially inoculated with endophytes. P015 and P018 are *B. brizantha* 26110. P56, P63, P100 and P111 are *B. brizantha* 6780. Other codes (H + numbers) are *Brachiaria* hybrids. Endophytes were isolates on culture from hybrid plants H19, H32, H45 and H47, but not from H50. The endophyte used to inoculate the plants was successfully re-isolated from adult plants P015, P056, P63 and P111, but not from plants P018 and P100. These PCR and culture data are compatible indicating that the PCR detection method can be highly effective.

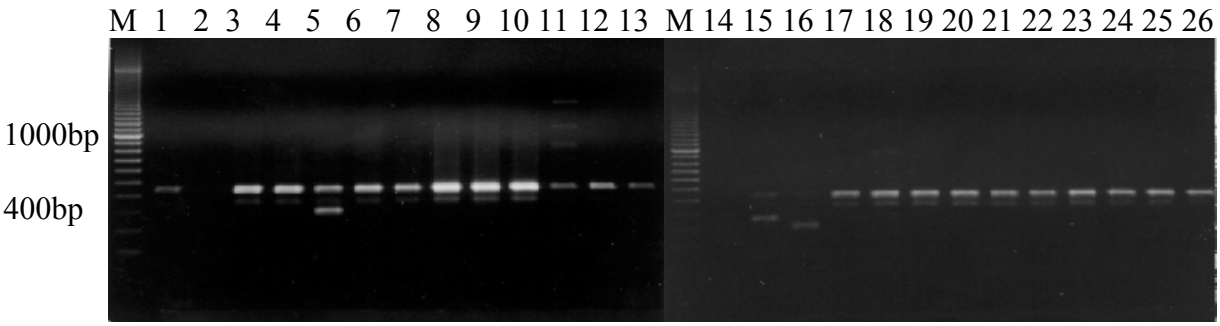

Photo 5. PCR amplified products with specific primer pairs of P1/P4. M, 00-bp marker.
Lanes 1-13 are amplifications of genomic DNA from *Drechslera* spp., *Rhizoctonia solani*, endophyte isolates EH45, EH19, EH47, EH32a, EH32b, EB606, EB6780 (P-201), EB16845 (P-904), EB6780 (P-501), EB16845 (P-909) and Epinedo, respectively. All of the fungi were isolated from species of *Brachiaria*.
Lanes 14-26 are amplifications of plant genomic DNA from P018, H50, P100, H32F', H32F, H19, H32, H45, H47, P015, P056, P063 and P111, respectively.

2.6.3 Genetic diversity of isolates of endophytic fungi from *Brachiaria*, and search for new endophytes in hybrids of *Brachiaria*

Contributors: H. Dongyi (South China University of Tropical Agriculture, The People's Republic of China), and S. Kelemu (CIAT)

Rationale

The search for new endophytes continued this year in new hybrids of *Brachiaria* species. We believe that the search for new isolates of endophytes may enable us find endophytes with various beneficial properties such as disease and insect control without other deleterious effects. Fungal endophytes were shown to be widely distributed in tropical forage *Brachiaria* through tissue staining and microscopy detection. Eleven endophyte isolates were isolated from different accession or hybrids of *Brachiaria*. Table 54 shows the list of isolates and their plant origin. Molecular methods such as RFLP (restriction fragment length polymorphism), SSR (simple sequence repeat, microsatellite), RAPD (random amplified polymorphic DNA) and AFLP (amplified fragment length polymorphisms) were successfully used to show the genetic variation among species or biotypes. In this study, we used RAPD and AFLP to measure diversity in the 11 endophyte isolates listed in Table 54. Both methods were used successfully to determine genetic diversity and relations among endophyte isolates from *Brachiaria*.

Table 54. List of endophytic isolates and their original plant hosts.

Endophyte isolate codes	Host grass of endophyte isolates	
EH45	*Brachiaria* hybrid # 45	
EH19	*Brachiaria* hybrid # 19	
EH47	*Brachiaria* hybrid # 47	
EH32a	*Brachiaria* hybrid # 32a	
EH32b	*Brachiaria* hybrid # 32b	
EB606	*Brachiaria* 606	
EB6780.201	*Brachiaria* 6780	(201)
EB16845.904	*Brachiaria*16845	(904)
EB6780.501	*Brachiaria* 6780	(501)
EB16845.909	*Brachiaria*16845	(909)
EPinedo	*Brachiaria* seeds (Santander de Quilichao)	

Materials and Methods

Endophyte isolates were cultured on potato dextrose agar for a period of 2-3 weeks to produce fresh mycelia for DNA extractions. Genomic DNA extractions were done accoring the manufacturer's instructions using Dneasy™ plant mini Kit (Qiagen).

RAPD analysis. PCR was carried out in 20 µl of reaction mix containing 2.0 µl of 10 x PCR buffer, 1.2 µl of 25 mM $MgCl_2$, 2.6 µl of 2 mM dNTPs, 1.0 µl of 10 µM Primer, 0.2 ul of 5 U/µl Taq DNA polymerase, 10.0 µl of dd H_2O and 3.0 µl of 10 ng/µl Template DNA (DNA of endophyte isolate), placed in a PTC-100™ Programmable Thermal Controller (MJ Research Inc.) programmed for initial denaturation at 94°C for 2 min, annealing at 28°C for 5min, 45 cycles of 94°C for 1 min, 92°C for 20sec, 35°C for 1 min, 72°C for 1 min, following a final extension at 72°C for 10 min. PCR products were resolved by running a 1.2% agarose gel, stained with ethidium bromide, and photographed under UV lighting.

After preliminary testing of 3 isolates with 30 arbitrary10-base oligonucleotide primers (Operon), 11 primers were selected and used to evaluate all 11 fungal endophyte isolates. About 20 different bands were amplified with every primer. Eleven primers generated a total of 220 bands. Comparisons of each banding profile for each primer were conducted on the basis of presence or absence (1/0) of RAPD products of the same size (Photo 6). Matrices were generated and analyzed using NTSYSpc, Dice coefficient and UPGMA clustering method to produce a similarity dendrogram.

Results of RAPD data. There were some differences in similarity analysis results of data matrix generated from amplified products by different primers. However, the trend was very similar. The similarity clustering diagram tree of UPGMA using the entire RAPDs data showed that EH45, EH19 and EH47 are identical (Figure 21).

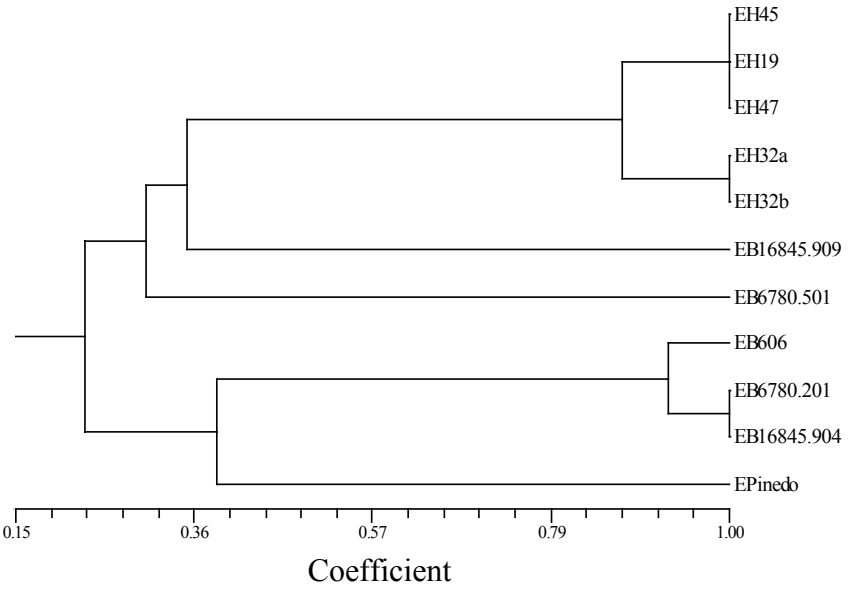

Figure 21. Similarity dendrogram of 11 endophyte isolates isolated from species and hybrids of *Brachiaria*, based on RAPDs data.

EH32a and EH32b are also identical. The two groups' similarity coefficient is above 0.8. EB6780 (201) and EB16845 (904) have same identity and very similar to EB606 with coefficient of over 0.9. EB6780 (501), EB16845 (909) and Epinedo are very different from each other and the former group, with less than 40% similarity. A single primer data analysis produced similar results and conclusions (eg. Figure 22).

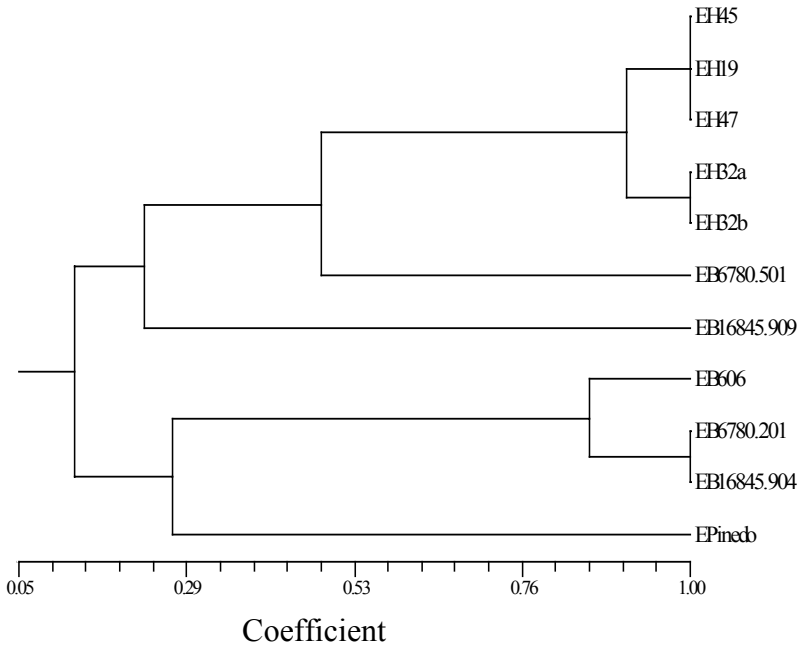

Figure 22. Similarity dendrogram of 11 endophyte isolates isolated from species and hybrids of *Brachiaria*, based on RAPDs data using primer AJ12.

AFLP analysis. In order to possibly differentiate those isolates that showed high similarity using the RAPD data, a further study was conducted using the AFLP method.

AFLP assays were performed with AFLP Analysis System for Microorganisms (Gibco) according to the manufacturer's instructions. Genomic DNA of each isolate was digested with the restriction enzymes *Eco*RI and *Mse*I, and ligated to their specific adapters. The restriction fragments were then amplified by PCR. Fifteen combinations of *Eco*RI and *Mse*I selective primers were first used to perform PCR of 2 very similar isolates (based on RAPDs data) in order to determine which primer pairs could give best results.

The primer pairs E-A/M-C, E-C/M-C, E-C/M-G and E-G/M-T were the most appropriate and were used to conduct PCR for all the 11 isolates. Amplification products were separated by electrophoresis on a 6% denaturing polyacrylamide gel. The gel was stained with silver nitrate after electrophoresis and developed on APC film. Methods of band reading, matrix preparation, and PCR conditions were the same as those used in RAPD analysis. Correlation of the matrices of RAPDs and AFLPs was conducted using MxComp program of NTSYSpc.

Results of AFLP analysis. Four hundred sixty-six bands of sizes between 100- and 330-bp were used in AFLPs similarity analysis. The results of AFLPs clustering analysis were very consistent with those of RAPDs (Figure 23). The correlation coefficient of matrices based on AFLPs and RAPDs was as high as 0.98. Each matrix from different selective primer pairs data also had high consistence, with a correlation coefficient value between 0.97 and 0.99. Both methods of RAPDs or AFLPs, and even one set of selective primer pairs in AFLP (eg. Figure 24), could reveal the relationship of these endophyte isolates.

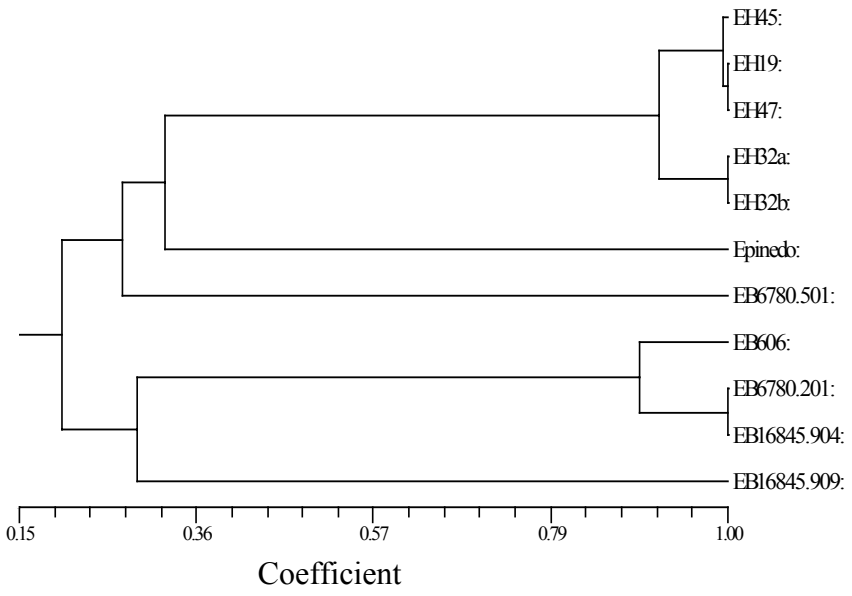

Figure 23. Similarity dendrogram of 11 endophyte isolates from species and hybrids of *Brachiaria* based on AFLP data.

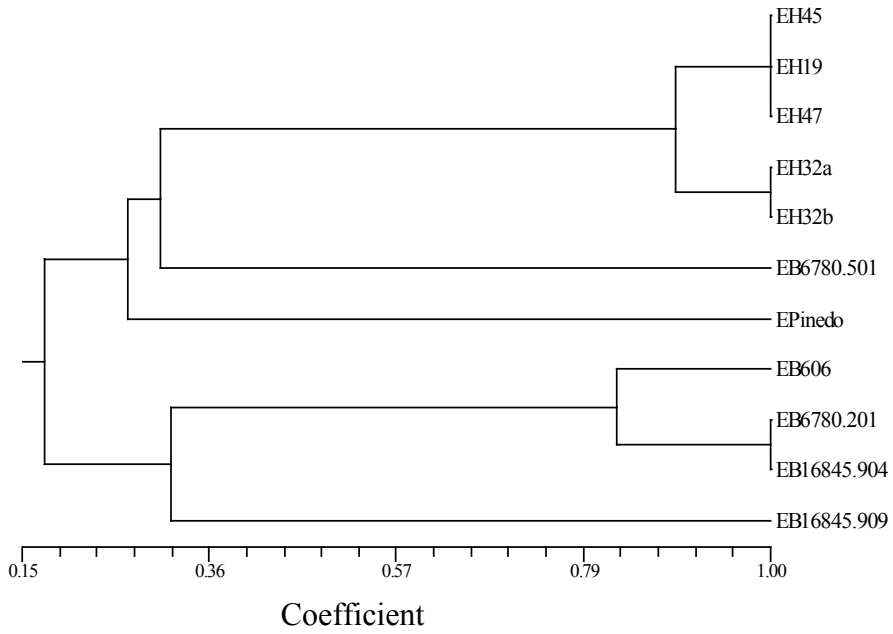

Figure 24. Similarity dendrogram of 11 endophyte isolates from *Brachiaria* based on AFLP data using primer pairs E-A/M-C

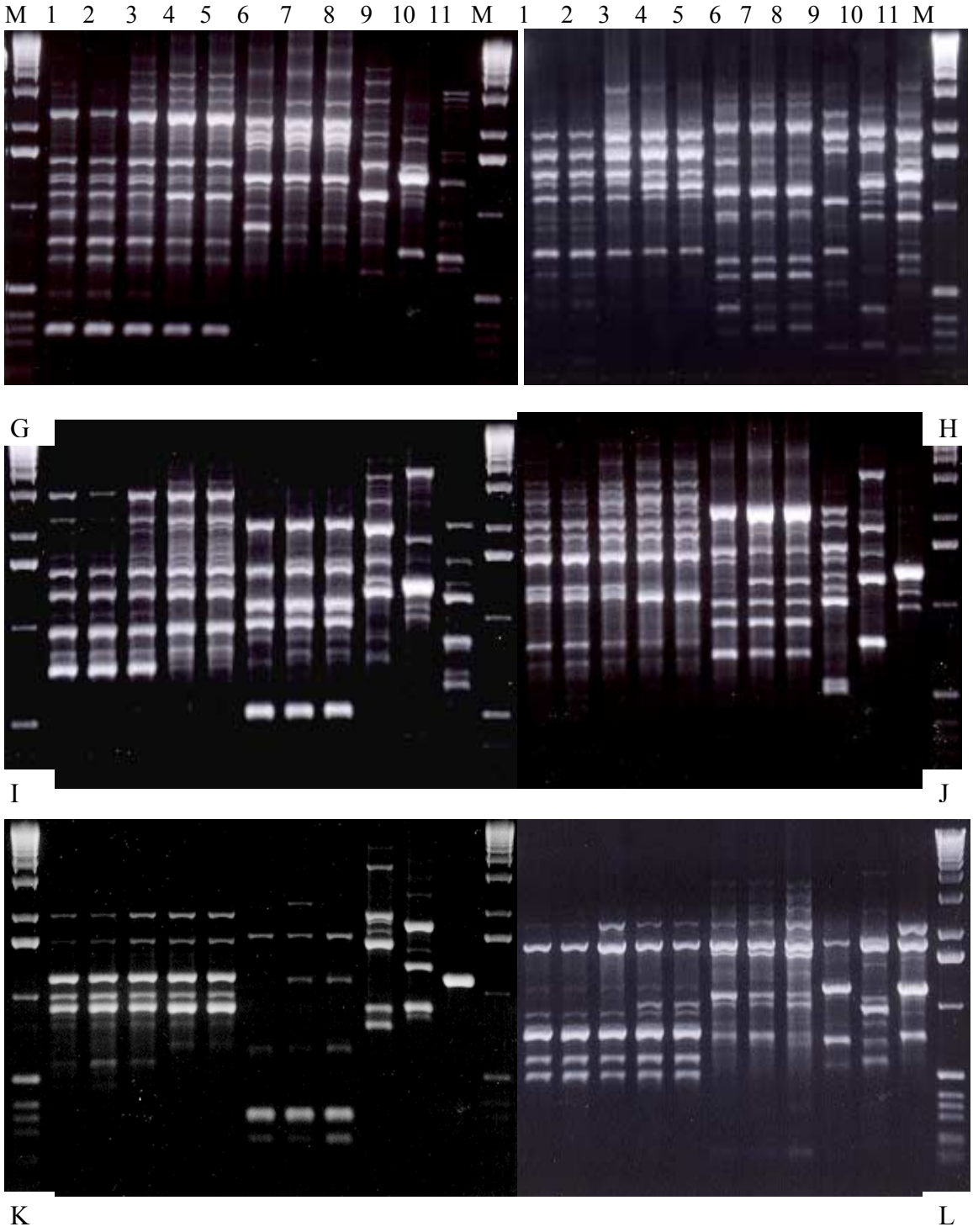

Photo 6. Electrophoresis gels of RAPD products of isolates of endophytic fungi. Gels G, H, I, J, K, L, with primers OPAK12 (5'-AGTGTAGCCC-3'), OPAK19 (5'-TCGCAGCGAG-3'), OPAJ01 (5'-ACGGGTCAGA-3'), OPAJ04 (5'-GAATGCGACC-3'), OPAJ12 (5'-CAGTTCCCGT-3'), and OPAJ13 (5'-CAGCCGTTCC-3'), respectively. Lanes 1-11 are endophyte isolates from *Brachiaria* hybrids #45, 19, 47, 32a, 32b, *Brachiaria* accessions 606, 6780 (P-201), 16845 (P-904), 6780 (P-501), 16845 (P-909), and endophyte Pinedo (isolated from seeds), respectively.

2.6.4 Effect of endophyte on *Rhizoctonia solani* (*in vivo*)

Contributors: H. Dongyi (South China University of Tropical Agriculture, The People's Republic of China) and S. Kelemu (CIAT)

Rationale

Previous results had shown that *B. brizantha* CIAT 16320 had high levels of resistance to Rhizoctonia foiar blight. It is interesting to note that 2 endophyte isolates (EH32a and EH32b) were isolated from CIAT 16320. We have demonstrated that endophytes of *Brachiaria* inhibit some fungal pathogens *in vitro* (IP-5 Annual Report, 1999, 2000) and *in vivo* (Kelemu et al., 2001). In this study, we examined the antifungal activities of clonal H32 plants with and without the endophyte.

Materials and Methods

Brachiaria material used in this study are: (1) CIAT 16320 *Brachiaria* plant naturally infected with an endophytic fungus; resistant to *Rhizoctonia* foliar blight, maintained in greenhouse. (2) Hybrid 30 *Brachiaria* plant, no endophyte detected, susceptible to *Rhizoctonia* foliar blight, maintained in greenhouse. (3) CIAT 16320F, which are tillers treated with 0.1 ml/L Folicur to eliminate endophytes (2 months old after treatment), maintained in greenhouse.

Pieces of surface sterilized leaf blades and sheaths were macerated in sterile distilled water (3-gm plant tissue in1-ml water). Plant debris was removed by centrifugation at 10,000 rpm for 5 minutes. The supernatant was filter sterilized and used to test its anti-fungal properties. Sterile filter paper discs were each soaked with 200 - 400 µl plant extract (supernatant) and placed at ends of plates containing potato dextrose agar with appropriate controls. A fresh sclerotium of *R. solani* was placed in the center of each plate. The plate was incubated at 28 C till measurements of mycelial coverage were taken Table 55. .

Table 55. Inhibition zone distance (mm) between filter paper disc treatments with extracts of *Brachiaria* hybrids and mycelial growth of *Rhizoctonia solani* on potato dextrose agar.

Treatment	CIAT 16320 (+E)*	CIAT 16320F (-E)	Hybrid 30 (ND-E)
200 µl water	0.00	0.00	0.00
200 µl extract	2.00	0.00	1.00
400 µl extract	6.33	0.00	1.00

* + E = naturally infected with an endophyte; -E = the endophyte eliminated using the fungicide Folicur; ND-E = endophyte not detected.

Sclerotia of *R. solani* were produced, and plants were inoculated as described earlier (Kelemu et al. 1995, Tropical Grasslands 29:257-262) to assess the level of resistance to the pathogen.

Results and Discussion

Three *Brachiaria* genotypes (codes FM9503/S046/024 [plants # 19 and 45], CIAT 16320 [plant # 32a, 32b], and SX99/2341 tested positive for endophytes. Of these, *B. brizantha* CIAT 16320 was highly resistant to *R. solani*. In order to determine whether the presence of an endophytic fungus was responsible for the resistance to *R. solani*, genetically identical lines of CAT16320 were created by treating half of the tillers derived from a single mother plant containing an endophyte with the fungicide Folicur while the remaining half were left untreated. Plant extracts from endophyte-infected and endophyte-free plants had differences in their growth inhibitory properties against *R. solani*. Results showed that extracts of CIAT 16320 produced inhibition zones while H30 (a hybrid with no detectable endophyte) and CIAT 16320F

(genetically identical clone to hybrid H32, but the endophyte was removed by treatment with the fungicide Folicur) did not (Photos 7, 8).

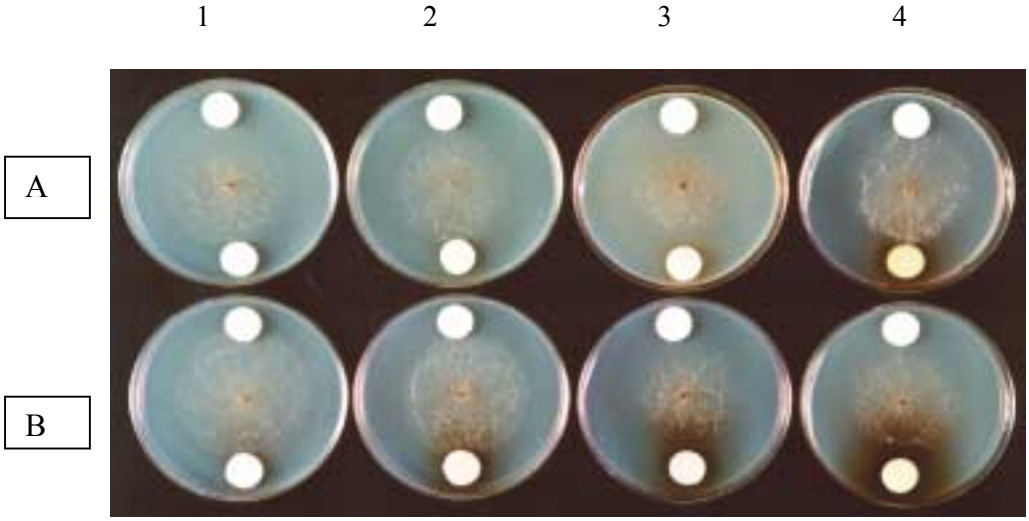

Photo 7. *Rhizoctonia solani* growth inhibition test by extracts from *Brachiaria brizantha* CIAT 16320 H32 and Hybrid 30. CIAT 16320 is naturally infected with an endophyte and is highly resistant to *R. solani*. H30 had no detectable endophyte and is susceptible to *Rhizoctonia*.
The filter disc in the upper part of each plate was treated with sterile water as control, whereas the disc in the lower part of each plate was treated with plant extracts.
Filter paper discs in plates A1 (200 µl), A3 (400 µl), B1 (200 µl) and B3 (400 µl) were treated with extracts from leaf sheaths, those in plates A2 (200 µl), A4 (400 µl), B2 (200 µl) and B4 (400 µl) were with extracts from leaf blades
Plates A1-A4 and B1-B4 had extracts from Hybrid 30 and CIAT 16320, respectively.

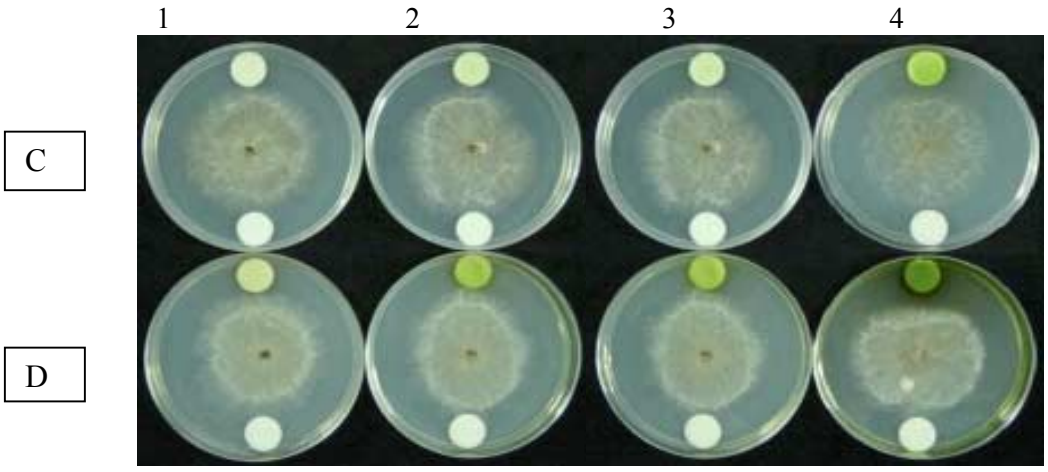

Photo 8. Growth inhibition test of *Rhizoctonia solani* by leaf and leaf sheath extracts from *Brachiaria brizantha* CIAT 16320 and CIAT 16320F. Extracts in plates C1, C2, D1 and D2 were from CIAT 16320F (tillers of CIAT 16320 treated with Folicur to eliminate the endophyte).
Extracts in plates C3, C4, D3 and D4 were from CIAT 16320 naturally infected with an endophyte. The filter disc in the lower part of each plate was treated with sterile water as control, whereas the disc in the upper part of each plate was treated with plant extracts. Extracts in plates C1 (200 µl), D1 (400 µl), C3 (200 µl) and D3 (400 µl) were from leaf sheaths. Extracts in plates C2 (200 µl), D2 (400 µl), C4 (200 µl) and D4 (400 µl) were from leaf blades

Since CIAT 16320 contained endophyte and had resistance to *Rhizoctonia*, and because elimination of the endophyte using a fungicide also eliminated the antifungal activities of the plant extracts, we concluded

that the fungal inhibition (and possibly the resistance) was from the endophye/plant interactions. Studies are in progress to assess the reactions of live plants (CIAT 16320 and CIAT 16320F) to *R. solani*. The compound (s) involved in the resistance will also be studied in the future.

2.7 Define interactions between host and pathogen in *Brachiaria, Arachis* and *Stylosanthes*

Highlights

- More than 200 isolates of *Colletotrichum gloeosporioides* infecting *Stylosanthes*
- *guianensis* have been characterized.
- A rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease in transgenic *S. guianensis*. Segregation of resistance in the selfed progeny of a transgenic plant (118 resistant: 38 susceptible) suggest that resistance behaves as a single, dominant Mendelian factor.
- Sources of resistance to *Rhizoctonia* foliar blight have been identified in *Brachiaria*. It appears that the presence of endophytes contributes to this resistance.
- Sources of resistance to *Xanthomonas campestris* pv. *graminis* have been identified in *Brachiaria*.

Progress towards achieving milestones

- Determined *Colletotrichum gloeosporioides* diversity at a field in Quilichao using RAPD and AFLP
- Defined the inheritance of a rice chitinase gene in *Stylosanthes guianensis*
- Defined resistance of transgenic *S. guianensis* to *Rhizoctonia* foliar blight
- Identified sources of resistance to *Rhizoctonia* foliar blight in *Brachiaria* hybrids

We have determined the genetic diversity of more than 200 isolates of *C. gloeosporioides* collected at a field site in Quilichao, Colombia. The inheritance of a rice chitinase gene introduced into *S. guianensis* via *Agrobacterium*-mediated transformation was shown to be of Mendelian fashion. Transgenic *S. guianensis* plants were resistant to *Rhizoctonia* foliar blight. We have evaluated and identified some sources of resistance to *Rhizoctonia* foliar blight disease of *Brachiaria*.

2.7.1 Biodiversity studies on the anthracnose pathogen of *Stylosanthes*

Contributors: M. Rodriguez, G. Segura, X. Bonilla and S. Kelemu (CIAT)

Rationale

Anthracnose disease continues to be the most important limitation to *Stylosanthes* production. The pathogen population is diverse and complex. Knowledge on the pathogen population structure is crucial to effective disease management. The studies were conducted to advance understanding of anthracnose epidemiology and the population genetics of *Colletotrichum gloeosporioides* for improved disease management.

Materials and Methods

Fungal cultures, PCR analysis, plant inoculations, and pathogenicity evaluations, statistical data analysis were all as described before in various publications (Kelemu, et al., 1996, 1997 and 1999).

For evaluations of pathogenicity, we compared the commonly used visual evaluation method described by Horsfall-Barratt, with 4 control isolates and 12 differentials.

To construct dendrograms of RAPD and pathogenicity data, the presence and absence of RAPD bands was coded in a binary matrix with 1/0, respectively. The pathogenicity data was equally coded as 1 for disease evaluation values of 3-9 (Horsfall-Barratt scale) and 0 for values ≤ 3. Dendrograms were generated using SAHN of NTYS.

To determine the genetic and pathogenic variability, 217 isolates of *Colletotrichum gloeosporioides* were analyzed. DNA isolations, PCR reactions, plant inoculations and race determinations are as described earlier (Kelemu, et al. 1999. Genetic diversity in South American *Colletotrichum gloeosporioides* isolates from *Stylosanthes guianensis*, a tropical forage legume. European Journal of Plant Pathology 105:261-272). RAPD primers and their sequences used in the study are listed in Table 56.

Table 56. RAPD primers and their sequences used in this study.

Code	Sequence (5'……3')
OPA- 2	TGCCGAGCTG
OPA- 3	AGTCAGCCAC
OPA- 4	AATCGGGCTG
AJ- 4	GAATGCGACC
AJ- 6	GTCGGAGTGG
AJ- 8	GTGCTCCCTC
AJ- 9	ACGGCACGCA
AK- 4	AGGGTCGGTC
AK- 19	TCGCAGCGAG
D- 3	GTCGCCGTCA

AFLP analysis was initiated to complement the information obtained with the RAPD analysis. AFLP technology is used to visualize genetic polymorphisms among isolates, generating fingerprints that can be used to assess the relatedness between isolates. The strength of this methodology is that it combines two technologies, the digestion of the DNA from the classical RFLP analysis (restriction fragment length polymorphism), followed by the amplification of the fragments from the PCR (polymerase chain reaction) technique, and the visualization of the polymorphism from RFLP.

The "AFLP® Analysis System for Microorganisms" kit from Gibco BRL (USA) was used according to the manufacturer's instructions. Genomic DNA of each isolate was digested with the restriction enzymes *Eco*RI and *Mse*I. Double stranded DNA specific adapters were ligated to the *Eco*RI and *Mse*I ends of the fragments. On the next step, the fragments were amplified. Seven primers for the *Eco*RI and five primers for the *Mse*I ends were used for the amplification. The primers contained either zero, one or two selective nucleotides. To determine which primer combinations would give the most information on the genetic diversity, a preliminary evaluation was performed. All possible primer combinations were evaluated on four randomly picked isolates. The amplification was done with a MJ-Research PTC-100 thermal cycler. The amplification conditions were as described by the manufacturer of the kit (Gibco BRL). Amplification products were separated on a 6% denaturing polyacrylamide sequencing gel. At the end of the electrophoresis period, gels were stained with silver nitrate. The resulting banding pattern was analyzed manually.

For analysis of genetic similarity, each band was codified either as 1 or 0, whether it was present or not, in any particular isolate. The genetic similarity between two isolates was calculated based on Dice's coefficient with NTSYS-pc Version 1.8 (Exeter Software, NY). For each coefficient , the similarity matrix was used to construct dendrograms with the help of the unweighted pair grouping by mathematical averaging (UPGMA) methods using the SAHN and Tree programs in NTSYS. Multiple correspondence analysis was also employed to assign isolates to separate clusters.

Results and Discussion

AFLP. Four primers were used combined as follows: *Eco*RI primer E-AC with *Mse* I primer M-A, *Eco*RI primer E-AC with *Mse* I primer M-C, *Eco*RI primer E-AC with *Mse* I primer M-G, *Eco*RI primer E-T with *Mse* I primer M-A. In order to determine whether the information generated using these four primer combinations were complimentary or redundant, a correlation index between the similarity matrix generated using the four primers and that using only three of the primer combinations. The results showed a 97% correlation between the two matrices, which indicated that the information generated using three primer combinations (*Eco*RI primer E-AC with *Mse* I primer M-A, *Eco*RI primer E-AC with *Mse* I primer M-C, *Eco*RI primer E-AC with *Mse* I primer M-G) was sufficient to differentiate the isolates. A total of 371 bands were generated using these three combinations.

The analysis generated 9 groups of isolates (A-I). Group A is a heterogeneous group which contained 34 isolates from various geographic origins. This group has a 27% similarity with the rest of the groups.

Group B is the largest group with 153 isolates. There were three well-differentiated sub-groups within this large group. The first sub-group contained 75 isolates. Internal divisions within this sub-group showed a tendency for individual isolates to group based on their geographic origins. In general, the isolates from Quilichao and Caquetá formed consistent groups. The AFLP data showed that three isolates from Caquetá (isolate # 16116, 16120, 16160) were identical. This was interesting as the three isolates were collected from the same accession (*S. guianensis* CIAT 184) on the same date and at the same field. The remaining two sub-groups contained isolates collected in Quilichao from various host accessions between December 1999 and October 2000.

Group C had only four isolates from Quilichao collected from different accessions of *S. guianensis* within a month period. The similarity range within this group is between 59-82%, with an average of 68% similarity. The isolates 16203 (Carimagua), 16611 and 16296 (Quilichao) formed the AFLP groups D, H, and I, respectively. Interestingly, these three isolates were collected from three different resistant *S. guianensis* bred genotypes. The RAPD data did not differentiate these three.

Group E consisted of 13 isolates (12 of them from Quilichao). Interestingly, seven of these isolates isolated from a mixture of resistant hybrids 11833/11844 formed a sub-group. The second sub-group contained 5 isolates which originated from three resistant genotypes. The isolates in this group are most likely emerging pathogen genotypes.

Group F consisted of 8 isolates from Quilichao and Brazil.

Group G is a minor group with only two isolates collected in Carimagua, Colombia.

RAPDs. Analysis of the 217 isolates with the 10 random primers generated a total of 226 bands. A dendrogram constructed using this data generated 9 groups. As with the AFLP data, sub-groups of isolates were formed with a tendency based on their geographic origins. The second of these 9 major groups (group B) contained 69% of the isolates.

A closer look at AFLP and RAPDs groupings of the isolates and their original hosts revealed something interesting. Resistant host genotypes (eg. CIAT 2340, FM9405, CIAT 11833, CIAT 11844) provided isolates which belonged to more groups than very susceptible ones such as CIAT 2312 and Endeavour. For example, isolates collected from *S. guianensis* CIAT 2340 over time belonged to AFLP groups A, B, E and H; those from FM9405 clustered in AFLP groups A, B, C, E and F; whereas the highly susceptible genotypes like CIAT 2312 and Endeavour provided isolates which belonged to the predominant groups A

or B. These observations indicate that isolate sample collections for population studies should include as many diverse host genotypes as possible including those with high level of resistance to the pasthogen.

Pathogenicity studies. The isolates CIAT 16066 (Carimagua) and CIAT 1609 (Caquetá) were the most pathogenic infecting 11 of the 12 differentials. Eleven of the isolates did not cause any symptoms on any of the differentials used. Three of the differentials (CIAT 2312, Endeavour and 1875) were susceptible to more than 100 of the isolates. None of the differentials was susceptible to all the isolates tested. Two of the differentials (CIAT 2340, 1507) were infected by less than 30 of the isolates. When a matrix of 0 (resistant) and 1 (susceptible) was generated and a similarity dendrogram constructed, the groups formed did not show a clear association with location or collection date. The analysis showed no direct correlation between pathogenic groups and the genetic variability groups of AFLP and RAPDs data. However, the variability observed within the pathogen population in the three tests (AFLP, RAPDs and pathogenicity) was high.

Multiple Correspondence Analysis. Analysis of AFLP data using Multiple Correspondence Analysis (MCA) in three dimensions generated 7 groups with an average similarity of 58% (Figures 25, 26 different orientations of the same graph). The first dimension explains 47% of the variation within the isolates studied. This clearly separates group 1 and group 3. The second dimension differentiates groups 1, 2, 3, 6 and 7. It also separates groups 4 and 5 from the others. The third dimension divides the population in two clusters: the first forming the groups 3, 6 and 7; the second with groups 1, 2, 4 and 5.

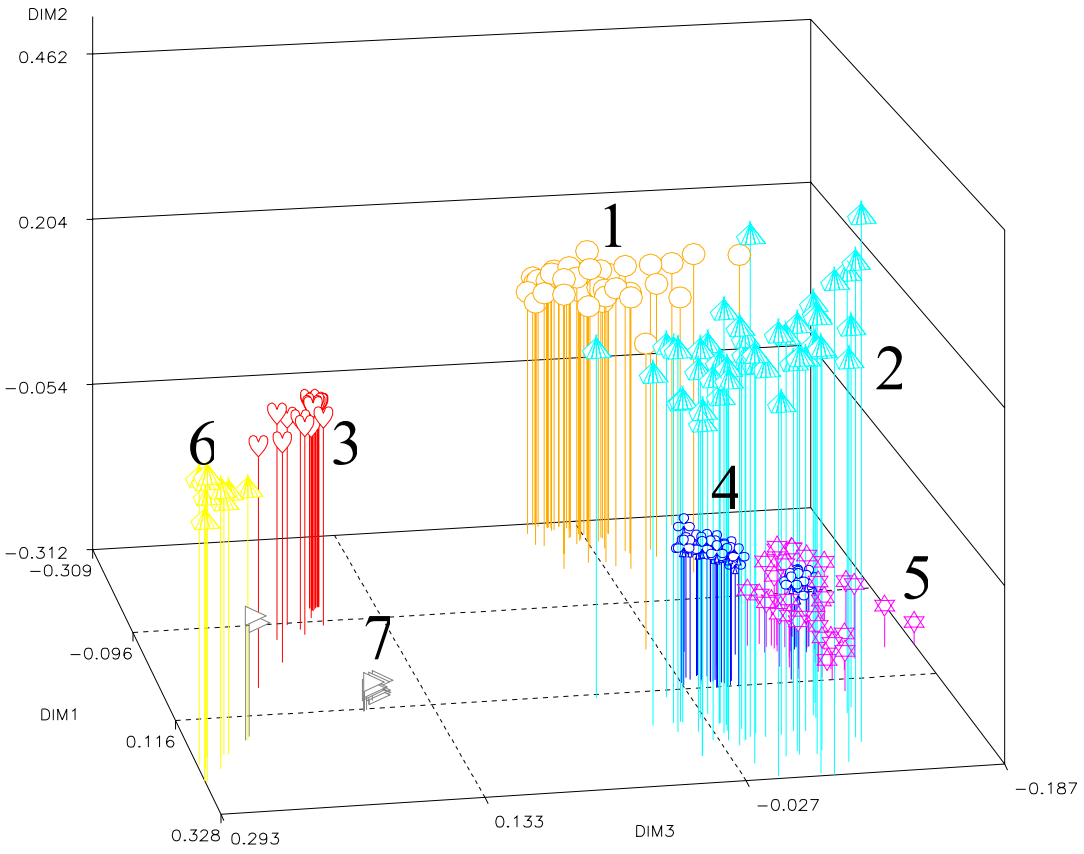

Figure 25. Cluster analysis of AFLP data on isolates of *Colletotrichum gloeosporioides*.

Group 1 consisted of 54 isolates with an average similarity of 35%. The isolates in this group together with those of group 3 formed one group (B1) using UPGMA analysis.

Group 2 contained 56 isolates. With the exception of some isolates, this group combines the groups A, E, F, H, and I formed using UPGMA. In general, this group had an average similarity index of 73%. This group is related to groups 4 and 5 in the third dimension with an average similarity of 56%.

Group 3 had 20 isolates with an average similarity of 52 %. As it appeared in group 1, two of the isolates were separated from the rest of the group.

Group 4 contained 39 isolates with an average similarity of 56%. Within this group, a sub-group of 12 isolates was formed in the third dimension which appeared more related to group 5 isolates. This same sub-group was formed in the UPGMA dendrogram.

Thirty-four isolates belonged to group 5. The UPGMA grouped 24 of these isolates in group B3, 4 in B2, 4 in C and 2 en group F. Similarity within this group is 76%. Groups 4 and 5 were all collected from Quilichao and are related to each other.

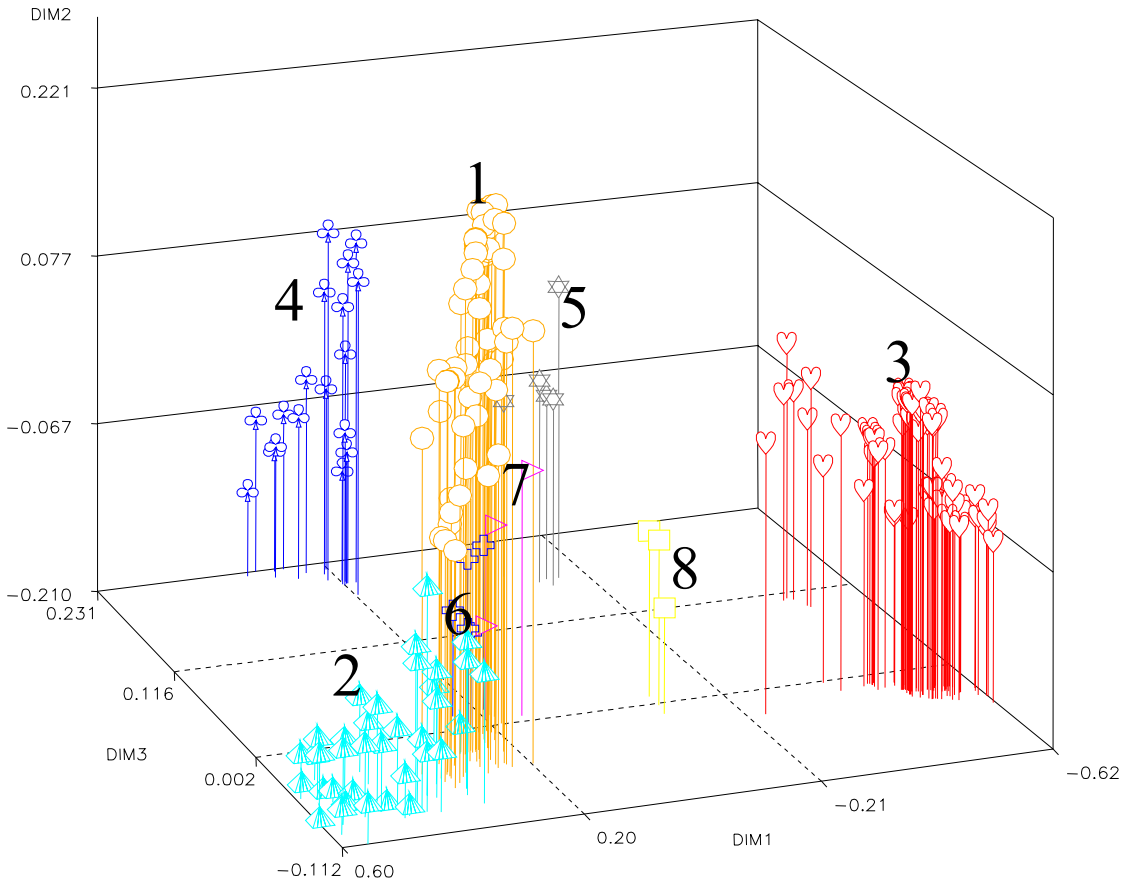

Figure 26. Cluster analysis of RAPDs data on isolates of *Colletotrichum gloeosporioides*.

The isolates of group 6 (9 isolates) had an average similarity of 88%. These isolates were dispersed in different groups using NTSYS. This group is related to groups 3 and 7. Isolates which formed group 7 are

very closely related to each other (average similarity of 91%) and were all collected from Quilichao. With the exception of few of the member isolates of this group, all were collected on the same date. This group of isolates was grouped together with RAPDs data and other statistical analysis.

The RAPDs data produced 8 groups of isolates with an average similarity of 54% (Figure 26). Group 1 had 60 isolates with an average of 45% similarity. The group is related to group 2 (76% similarity), and group 6 (55% similarity). This group of isolates is analogous to groups 1 and 3 formed in the AFLP data (Figure 25). Group 2 contained 36 isolates with an average similarity of 77%. Group is 3 has 87 isolates (all from Quilichao, years 1999, 2000 from various host genotypes) and is the most differentiated from the rest of the groups (Figure 26). Group 4 had 18 isolates with an internal average similarity of 83%. Groups 5 and 6 had 5 isolates each. With an average similarity of 70% and 52 %, respectively. Groups 7 and 8 had 3 isolates each. In general there was a good correlation between groups formed using UPGMA and those with multiple correspondence analysis. The results showed that isolates collected from Caquetá had less variability than those from Quilichao.

2.7.2 Epidemiology studies on the anthracnose pathogen of *Stylosanthes*

Contributors: Gustavo Segura, M. Rodriguez, and S. Kelemu (CIAT)

Thirteen accessions and advanced hybrids/populations of *S. guianensis* and two accessions of *S. scabra* were planted at the CIAT Experiment Station in Quilichao, Department of Cauca (3^0 6' N, 76^0 31' W; altitude 1000 m; mean annual rainfall 1,700 mm with normally a bimodal distribution of March-June and September-December; mean annual temperature 24^0 C; ultisols) on 1999-03-30. There was an establishment period of six months before the first disease evaluation (see field establishment problems on page 5). Disease evaluation started on 1999-10-21. Five branches per plot were marked and each one rated on a scale of 0 to 9, according to the Horsfall-Barratt scale. Evaluation has been done on a monthly basis up until May of this year, and will continue for at least 12 more months. Weather data were obtained from an automatic weather station (Monitor Sensors, Australia). Continuous data was obtained for foliage temperature, relative humidity of the air and precipitation. Because of the inconsistency of the weather data, no statistical analysis could be performed on the relationship of the epidemic progress with weather. On average disease severity increased steadily over time although, there was a clear reduction during January and May. There were marked differences on epidemic development depending on the accession.

2.7.3 Characterization of transgenic *Stylosanthes* plants with chitinase gene

Contributors: J. Changshun (CATAS, China), H. Guixi (CATAS, China), G. Segura and S. Kelemu (CIAT)

Rationale

Foliar blight, caused by *Rhizoctonia solani* Kühn (teleomorph *Thanatephorus cucumeris* Donk), affects species of *Stylosanthes* in many parts of tropical America where the annual rainfall is more than 1500 mm (Lenné and Calderon, 1984, In: Stace, H. M. and Edye, L. A. (eds) Biology and Agronomy of *Stylosanthes*. Academic Press, North Ryde, Australia, pp. 279-194). The disease has also been reported in many parts of the world (O'Brien and Pont, 1977, Queensland Agricultural Journal 103:126-128; Lenné, 1990, Phytopathology Papers No. 31, CAB International, Wallingford, UK). The disease initially appears as water-soaked spots on infected leaves. Under favorable conditions such as prolonged humidity, these progress into rotting and extensive foliar death. The pathogen has an extensive host range and it can produce a substantial foliar damage on susceptible genotypes (Baker, 1970, In: Parameter, J. R. Jr (ed.) *Rhizoctonia solani*: Biology and Pathology. Pp.125-148. University of California Press: Berkeley), including various tropical and subtropical forage legumes and crops (Hepperly et al., 1982, Plant Disease

66: 256-257; Galindo et al., 1983, Plant Disease 67:1016-1021; Yang et al., 1990, Plant Disease 74: 501-504).

Sclerotia of the fungus can survive in the soil or on plant debris for long periods. Sclerotia first appear as white masses on infected plant tissues, and as they mature, they turn brown and loosely attached which then easily shed forming the primary source of inoculum.

Chitinases have been reported from a number of plants and various microbes (Punja and Zhang, 1993, J. Nematol 4:526-540; Ohme-Takagi et al., 1998, Mol Gen Genet 259:511-515). Both biotic and abiotic stresses can induce chitinase expression at relatively high level although many plant chitinases are often expressed constitutively at low levels. Chitinase catalyzes the hydrolysis of the β-1,4 linkages of the N-acetyl-D-glucosamine polymer chitin, a structural component in a number of organisms. However, no chitin-like substrates are present in plants (Boller et al., 1983, Plant Mol. Cell Biol. 5:145-174). In contrast, chitin constitutes 3-60% of the cell wall in fungi (Bartnicki-Garcia, 1968, Annu Rev Microbiol 22:87-108). Purified chitinases obtained from: (1) bean were effective against cell walls of *Fusarium solani* (Boller et al., 1983) and *Trichoderma viride* (Schlumbaum et al., 1986, Nature 324:365-367); (2) tomato (Young and Pegg, 1982, Physiol Plant Pathol 21:411-423), tobacco (Mauch et al., 1988, Plant Physiol 88:936-942), and pea (Sela-Buurlage et al., 1993, Plant Physiol 101:857-863) could inhibit fungal growth by lysis of fungal tips; and (3) *S. guianensis* leaves could kill *C. gloeosporioides* hyphae (Brown and Davis, 1992, J. Phytopathol 136:247-256). Chitinases have also been reported as pathogenesis-related proteins in cucumber (Metraux et al., 1988, Physiol Mol Plant Pathol 33: 1-9). These and other findings support the hypothesis that plant chitinases have antibiotic functions and thus probably constitute a defense mechanism in plants against pathogens.

Recombinant DNA techniques have allowed the isolation of specific genes and their introduction into plants which otherwise is not possible with conventional breeding. Complimentary DNA clones and genomic clones have been isolated, and the amino acid sequences deduced for several chitinases obtained from a number of plants (Van Damme et al., 1993, Physiol Plant 87: 177-186; Samac et al., 1990, Plant Physiol 93:907-914; Swegle et al., 1989, Plant Mol Biol 12:403-412; Broglie et al., 1986, Proc Natl Acad Sci USA 83:6820-6824; Metraux et al., 1989, Proc Natl Acad Sci USA 86:896-900; Huynh et al., 1992, J Biol Chem 267:6635-6640; Zhu and Lamb, 1991, Mol Gen Genet 226:289-296; Huang et al., 1991, Plant Mol Biol 16:479-480).

Some transgenic plants with chitinase-encoding genes such as canola (Benhamou et al., 1993, The Plant Journal 4:295-305), cucumber (Raharjo et al., 1996, Plant Cell Rep. 15:591-596; Tabei et al., 1998, Plant Cell Reports 17:159-164), rice (Lin et al., 1995, Bio/Technology 13:686-691), rose (Marchant et al., 1998, Mol Breed 4:187-194), tobacco (Broglie et al., 1991, Science 254:1194-1197), and tomato (Hironaka et al., 1993, In: Proc. Conference on "Molecular genetics of plant-microbe interaction", Rutgers, NJ. (Abstract no. 46) have also been generated to control fungal pathogens. A rice-chitinase gene, controlled by the CaMV 35S promoter and introduced into *Indica* rice, was shown to enhance resistance to *Rhizoctonia solani* (Lin et al., 1995). A rice-chitinase gene in a transgenic rose plant reduced the development of blackspot disease caused by the fungus *Diplocarpon rosae* Wolf (Marchant et al., 1998). Tabei et al. (1998) had shown that transgenic cucumber plants containing a rice chitinase gene had enhanced resistance to gray mold disease caused by *Botrytis cinerea*. Increased chitinase activity has also been reported to enhance anthracnose resistance in *S. guianensis* (Brown and Davis, 1992).

In this study, we introduced a rice-chitinase gene (Huang et al., 1991) into the widely grown *S. guianensis* CIAT 184 accession in order to enhance foliar blight resistance. This accession has broad adaptation to the humid tropics and has been released as a cultivar in various countries including Peru and southern China. It has also performed well in parts of Africa.

Materials and Methods

DNA manipulation. A 1.5-kb DNA fragment, containing a 1.1-kb rice chitinase gene and the CaMV 35S promoter, was recovered from the *Hind*III digested plasmid pBSKS-G11 (R) with the chitinase gene (provided by Dr. S. Muthukrishnan, Department of Biochemistry, Kansas State University), using a DNA recovery kit (BIO-RAD) according to the manufacturer's instructions. This DNA fragment was ligated to the *Hind*III site of pCAMBIA2301, a vector generously provided by the Center for the Application of Molecular Biology to International Agriculture (CAMBIA), Australia. The ligated DNA product was used to transform *Escherichia coli* DH5α. Two constructs, designated pCIATCH1 and pCIATCH2, with opposite orientations of the insert were selected. All recombinant DNA techniques were carried out with standard procedures (Sambrook et al., 1989).

Bacterial transformation. Bacterial strains and plasmids used are listed in Table 1. Competent cells of *E. coli* DH5α were prepared and subsequent transformations with plasmid DNA carried out according to the protocol described by Inoue (1990, Gene 96:23-28). Transformed cells were cultured on Luria agar medium (bacto-tryptone, 10 g; bacto-yeast-extract, 5 g; NaCl, 10 g; and agar, 15 g; per L of distilled water) with appropriate antibiotics (100 μg/mL ampicillin for pBSKS-G11(R), and 50 μg/mL kanamycin for pCAMBIA2301 and its derivatives) and incubated overnight at 37 °C. For α-complementation screening, 40 μL/plate X-gal and 4 μL/plate IPTG were used.

pCIATCH2 DNA was directly transformed into *Agrobacterium tumefaciens* strain LBA4404 (Jefferson et al., 1987, EMBO J. 6:3901-3907). Transformed cells were selected by plating them on Luria agar medium with 25 μg/mL, and 50 μg/mL kanamycin. Recombinant DNA was isolated from transformant *A. tumefaciens* cells and digested with *Hind*III for verification. All transformants were maintained at –80 °C in 20% glycerol.

Plant transformation and regeneration. Seeds of *S. guianensis* CIAT 184 were surface-sterilized with 3% NaOCl solution for 15 min and rinsed with sterile distilled water, then treated for 5 min with 70% ethanol and rinsed three times with sterile distilled water. Seeds were then germinated on a basal MS medium (Murashige and Skoog, 1962, Physiol Plant 15:473-479). Cultures were maintained under fluorescent light at 55 $\mu E/m^2$ per second at 24 °C, with a 12-h photoperiod. Segments were excised from leaves for transformation.

A. tumefaciens LBA4404, containing plasmid pCIATCH2, was incubated overnight at 28 °C, with shaking at 200 rpm, in 10 mL of Luria broth (LB) containing 100 μM acetosyringone, 25 μg/mL streptomycin, and 50 μg/mL kanamycin. For co-cultivation, cells were collected from the overnight cultures by centrifugation and re-suspended in fresh LB liquid medium.

Leaf-segment explants were inoculated by swirling for 2–5 min in the bacterial suspension, blotted dry on sterile filter paper, plated onto regeneration medium (basal medium with 1.0 μg/mL α-NAA and 4.0 μg/mL BAP) (Sarria et al., 1994), and incubated at 28 °C in the dark for 2 days. The explants were then washed in sterilized distilled water, blotted dry on sterile filter paper, and cultured on the regeneration medium, containing 15 μg/mL kanamycin and 250 μg/mL carbenicillin.

After 2 weeks, all growing calli were transferred to a fresh regeneration medium for further selection. The selected green calli were then transferred to basal medium, containing 0.01 μg/mL α-NAA and 4.0 μg/mL BAP for shoot induction. After shoots appeared, the regenerated plantlets were transferred to basal medium, containing 0.1 μg/mL α-NAA and 0.4 μg/mL BAP for elongation. Shoots were excised and cultured on basal medium with quarter-strength salt and 0.1 μg/mL α-NAA for rooting. Kanamycin and

carbenicillin were used in all regeneration steps. Regenerated plantlets were transferred to pots containing autoclaved soil and placed in a glasshouse.

Plasmid isolations. Medium-scale plasmid DNA isolations were done using the protocol described by Marko (1982, Annu Biochem 121:382). Mini-preparations of plasmids were made according to the protocol described by Birnboim and Doly (1979, Nucleic Acids Res 7:1513-1523). Highly purified plasmids were extracted with the QIAGEN plasmid kit (QIAGEN, USA).

DNA isolations. Genomic DNA was isolated from fresh leaves of *S. guianensis* using DNeasy Plant Mini Kit (QIAGEN). DNA concentration was estimated using a Hoefer® DyNA Quant® 200 Fluorometer (Amersham Pharmacia Biotech, USA).

Southern and dot blot hybridizations. For Southern blot analysis, 10 µg DNA sample was digested to completion with Hind III. For dot blot analysis, 5 µg DNA was used. A 1.5 HindIII DNA fragment containing the chitinase gene from pCIATCH2 was used as a probe. The probe was labeled using the DIG High Prime DNA Labeling and Detection Kit (Roche Molecular Biochemicals, Germany) according to the manufacturer's instructions. All hybridizations and detections were carried out according to the manufacturer's instructions.

Screening of transformed plants by PCR analysis. PCR amplifications were carried out in 25µl reaction mixtures containing 0.25mM dNTPs, 3.0 mM $MgCl_2$, 0.6µM primers (for Gus gene primers; NPT II gene primers using 0.12 µM), 1 unit of Taq DNA polymerase, 1 X PCR buffer and 250 ng of template DNA. The reaction was performed with pre-denaturation at 94 °C for 1 min, and then 35 cycles of denaturation at 94 °C for 30 sec., annealing at 56 °C for 30 sec., and extension at 72 °C for 1.5 min, followed by a final extension at 72 °C for 7 min.

Sequences of the primers used in this study are 5'-CTGCGACGCTCACACCGATACC-3', 5'-TCACCGAAGTTCATGCCAGTCCAG -3' (Gus gene primers) and 5' - ATCGGGAGCG-GCGATACCCTA-3', 5'- GAGGCTATTCGGCTATGACTG-3' (NPT II gene primers). The primers were synthesized by Operon Technologies, USA.

Inoculum production. Sclerotia of *R. solani* AG-1 were produced in a peptone sucrose yeast broth (PSY; 20 g peptone, 20 g sucrose, 5 g yeast and 1 L deionized water). Mycelial discs (4 mm in diameter) were taken from a 4 to 5-day-old culture of *R. solani* AG-1 grown on potato dextrose agar (Difco). One disc was added to each of several 250-ml Erlenmeyer flasks, each containing 30 ml PSY. The flasks were wrapped with aluminum foil and incubated as still cultures at room temperature (about 25 C) for 10 days. Sclerotia were harvested with sterile forceps that separated them from the mycelial mats. They were then air-dried overnight in a laminar flow hood. Dry sclerotia were placed in sterile glass tubes and stored at 4 C till use.

Plant inoculations and disease evaluations. Seedlings of both transformed and non-transformed *S. guianensis* plants were handled as described in Kelemu et al. (1999, European Journal of Plant Pathology 105:261-272). Ten weeks old plants were artificially inoculated with sclerotia by placing one sclerotium on the soil surface in contact with each plant's stem. Humidity was maintained at 100% by placing the inoculated plants in a plastic box with one side made of cheesecloth, and immersing the entire box in a tray of water until disease symptoms developed. Plants were evaluated for their reactions to R. solani starting six days after inoculation. The number of sclerotia produced per plant, diseased and defoliated leaves per plant, and disease upward progression per plant were measured.

Inheritance of resistance attributed to chitinase. Seeds were collected from selfed, transformed, and non-transformed plants and germinated in Petri dishes containing moist filter papers, and subsequently transplanted in potted soil in the glasshouse as described in Kelemu et al (1999). The transgenic plant (designated as plant No. 22) chosen for subsequent inheritance study in the progenies, showed high levels of foliar blight resistance, GUS activity, strong dot blot signal and positive PCR results with NPTII gene primer and GUS gene primer. One hundred fifty six selfed transgenic progeny seedlings and 20 selfed control progeny were transplanted each to a separate pot. Inoculations and disease evaluations were conducted as described above. The probability values for goodness of fit to expected ratios were calculated using the Chi-square analysis.

Results

Plant transformation and regeneration. Calli were induced from leaf segments infected by *A. tumefaciens* on selective medium, containing 15 mg/L kanamycin. Shoots were induced from kanamycin-resistant calli cultured on regeneration medium with half-strength salt. These shoots grown from kanamycin-resistant calli were elongated on regeneration medium, containing 20 mg/L kanamycin. Rooting occurred from shoots excised from calli. Fifty putatively transformed kanamycin-resistant plants were generated. Some of these showed strong GUS activity.

Southern and dot blot analysis. DNA isolated from the putative plant transformants were tested using dot blot analysis. DNA of those which showed positive signals were further analyzed using Southern blot analysis. Digestion of genomic DNA to completion with *Hind*III resulted in a band which hybridized to the probe. No hybridizing band was detected in DNA from untransformed control plants.

PCR analysis: DNA from randomly picked progenies of transgenic plants were quickly tested using NPTII or GUS gene primers in PCR reactions. These progenies segregated either generating an amplification DNA product or none (Photo 9). DNA from progenies of the control plant showed no amplification product.

Reactions of transgenic and control plants to *R. solani*. Transgenic *Stylosanthes* plants constitutively expressing the rice chitinase gene showed no visible differences in growth and vigor when compared with control plants. Results showed that transgenic plants had a higher level of resistance to *R. solani* than control plants (Photo 10). A larger number of sclerotia were produced on the infected adult control plant (137 sclerotia) than on the transgenic plant No# 22 (11 sclerotia). In addition, more leaves were infected and defoliated from control plants than from transgenic ones (data not shown).

These results have practical significance. Sclerotia produced on infected plants shed to the soil upon maturation. These sclerotia can survive in the soil for long periods of time and form sources of inoculum for the next disease cycle. Therefore, transgenic plants not only confer resistance to *Rhizoctonia* foliar blight disease, but also help drastically reduce the production of sclerotia and thus sources of inoculum and maintain healthier soil.

Inheritance of resistance to R. solani in selfed progeny. In order to determine the inheritance of resistance to *R. solani*, seeds were collected from selfed transgenic mother (T_0) plants. Foliar blight, seedling blight, root rot and brown-girdling are among the disease symptoms caused by *R. solani*, a chitinous soil-borne pathogen with a wide host range. The number of diseased and defoliated leaves, number of sclerotia produced on diseased tissues of each plant, and disease upward progression have been determined in each plant. There were 118 plants rated as resistant (showing either no symptoms or limited symptoms) and 38 plants rated as susceptible (heavily infected as control plants) .

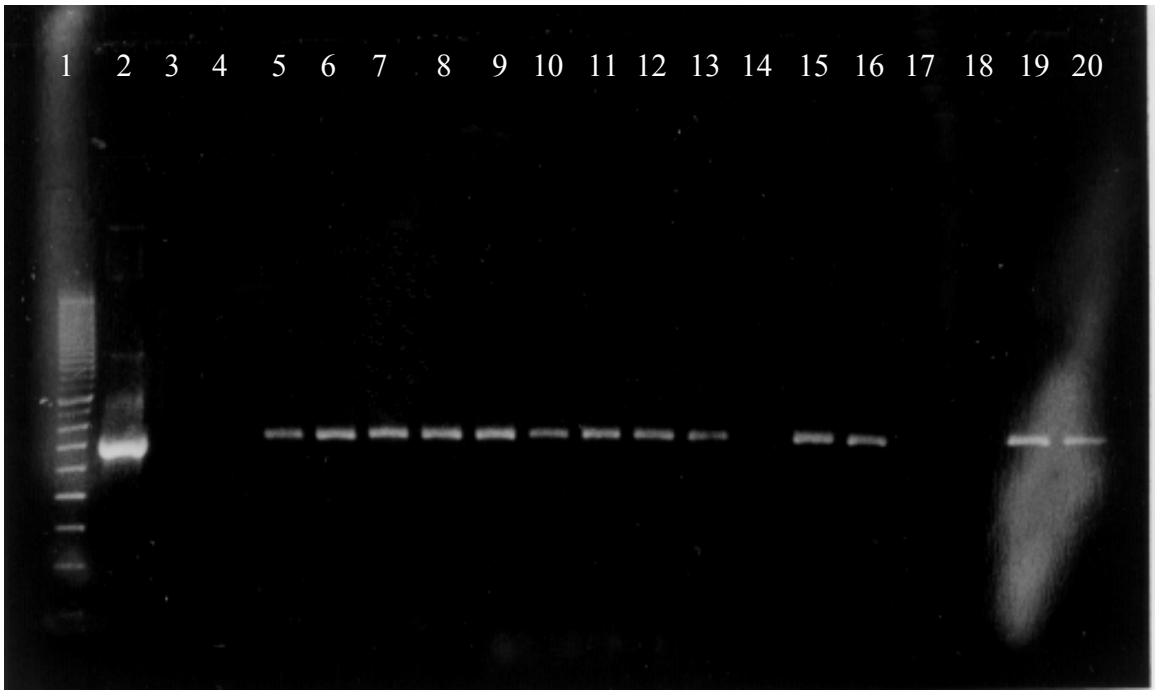

Photo 9. PCR amplifications of genomic DNA isolated from randomly picked selfed progenies and untransformed control plant with *npt*II gene primers. Lane 1 = marker 100-bp ladder; lane 2 = positive control; lane 3 = negative control; lane 4 = untransformed control plant; lanes 5-20 = selfed progenies of transformed plants. *Note:* All progenies lacking the amplified band were susceptible to *Rhizoctonia solani*.

Photo 10. Reactions of transgenic (left) and control (right) *Stylosanthes guianensis* accession CIAT 184 plants to *Rhizoctonia solani* AG-1 eight days after inoculations. Note the blighted leaves and stems on the lower part of the control plants.

This segregation in the progenies was in agreement with the expected Mendelian ratio of 3 resistant:1 susceptible, when analyzed by Chi-square test (Table 57). The number of infected and defoliated leaves per resistant progeny is significantly lower than that in each of the susceptible ones (Figure 27). Susceptible plants sustained more sclerotia per plant than the resistant ones (Figure 27). In addition, the

upward disease progression was significantly higher in susceptible segregants than the resistant ones (Figure 27).

The average root length and root dry weight were significantly higher in resistant segregants than those of susceptible ones. The average root length in each of the resistant progenies was 25 cm, and 21 cm in susceptible ones. The average root dry weight of resistant segregants was 0.228 gm. vs 0.156 gm of susceptible segregants. Disease development and progression over time on each of the resistant progenies was faster and greater than on susceptible ones (Figure 28).

These results confirm that the introduced rice chitinase gene in the transgenic *S. guianensis* plant was inherited in the progenies along with resistance to *Rhizoctonia* foliar blight disease.

Table 57. Inheritance of resistance to *Rhizoctonia solani* AG-1 conferred by a rice chitinase gene in progenies from a selfed transgenic *Stylosanthes guianensis* plant.

Plant Description	Observed Resistant	Observed Susceptible	Expected Resistant	Expected Susceptible	Chi-square value	P (%)
Selfed transgenic	118*	38♦	117	39	0.03418	85
Selfed control (accession CIAT No. 184)	-	20	-	20	-	-

*64 plants with no macroscopic symptoms of *Rhizoctonia* foliar blight, and 54 plants with some visible symptoms with upward disease progression of less than 6 cm.

♦38 progenies were as susceptible as the selfed control plants.

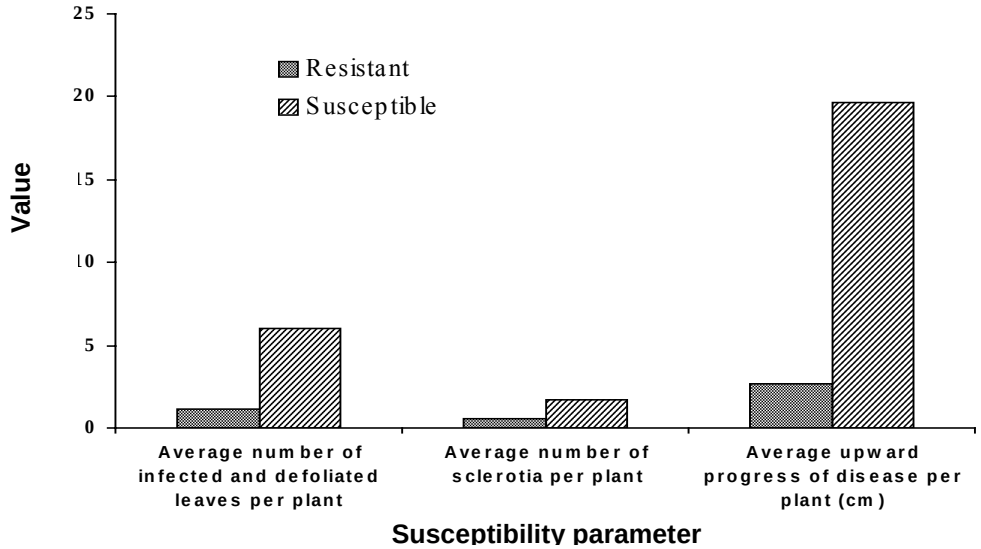

Figure 27. *Rhizoctonia* foliar blight disease development in selfed progenies of transgenic plant No. 22 according to the number of defoliated and infected leaves, number of sclerotia formed in infected plant tissues and disease progress.

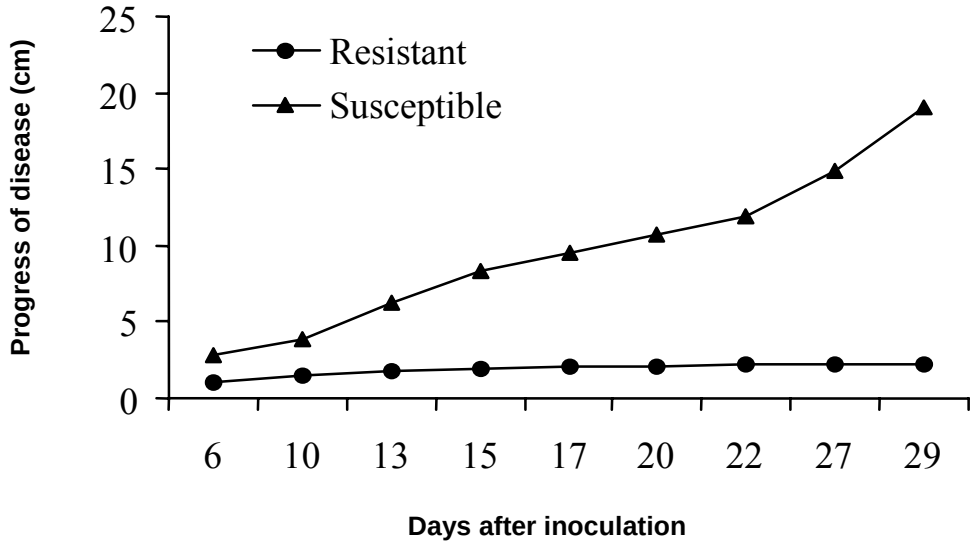

Figure 28. Progress of *Rhizoctonia* foliar blight disease in selfed progenies of transgenic plant No. 22 over time.

Discussion

Chitinases have no known function and have no known endogenous substrate in higher plants, but because its substrate, chitin, is a major component of the cell walls of many filamentous fungi with the exception of Oomycetes, it has been speculated to have a plant protection function. Plants naturally respond to microbial invasion by activating a number of defenses such as antibiotic synthesis, stimulation of enzymes, reinforcement of their cell walls and so on (Dixon and Lamb, 1990, Ann. Rev. Plant Physiol. Plant Mol. Biol. 41:339-367). Chitinases are among a group of proteins called pathogenesis-related proteins that are induced in response to attack by plant pathogens and some abiotic stresses (Boller, 1988, Plant Mol. Cell Biol. 5:145-174).

In this report, we have shown that the presence of a rice chitinase gene under the control of CaMV 35S-promoter in transgenic *S. guianensis* accession CIAT No. 184 confers resistance to foliar blight disease caused by *R. solani*. This resistance is manifested by a significant reduction in the upward progression of the disease, lower number of infected and defoliated leaves, greater root biomass and length, and fewer number of fungal sclerotia produced in transgenic plants than control plants. *Stylosanthes* is a perennial forage legume and the production of significantly fewer fungal sclerotia on transgenic plants than on control plants has important implications on curtailing the disease cycle through reductions of sources of inoculum. Constitutive production of chitinase in transformed tobacco plants has been shown to reduce plant mortality in soil infested with *R. solani* (Broglie et al., 1991; Vierheilig et al., 1993).

The segregation of selfed progeny fitting a ratio of 3:1 (resistant: susceptible) is consistent with a single locus of an active chitinase gene. The level of resistance expressed in most of the segregating resistant progeny (T_1) is comparable or similar to that observed in the transgenic mother plant, although some expressed a much higher resistance. Some of the progeny showing higher levels of resistance than the mother plant have been selected for further seed productions and evaluations of T_2 and T_3 generations.

Several studies have been reported on transgenic plants containing chitinase genes. For example, tobacco and canola transgenic plants expressing bean chitinase gene conferred resistance to *R. solani* (Broglie et al., 1991); rice plants transformed with a rice chitinase gene under the 35S promoter were shown to have resistance to the sheath blight disease caused by *R. solani* (Lin et al., 1995); transgenic cucumber plants containing a rice chitinase gene expressed an enhanced resistance to gray mold disease caused by *Botrytis cinerea* (Tabei et al., 1998); rose plants transformed with a rice chitinase gene had a reduced blackspot disease, caused by the fungus *Diplocarpon rosae*, severity (Marchant et al., 1998). However, our work is the first report on transgenic *S. guanensis* containing a rice chitinase gene for control of *Rhizoctonia* foliar blight disease.

This study indicates that there are benefits associated with the introduction of naturally existing defense related genes, which are automatically triggered in plants upon attack by pathogens or injury by abiotic stresses. The transfer of a gene from rice to a legume forage plant *Stylosanthes* is not possible using traditional breeding methods. Recombinant DNA techniques made this possible thus generating a genetic diversity for disease resistance. As more cloned plant defense genes and effective promoters become available, it is likely that more disease resistant, environmental friendly plants would be created. The challenge ahead would be to educate the general public and the media on the ever-growing debate of transgenics, their potential benefits and possible unknown draw-backs.

2.7.4 Bacterial blight of *Brachiaria* caused by *Xanthomonas campestris* pv. *graminis*: Isolate collection, pathogenicity evaluation and seed transmission

Contributors: C. Zuleta and S. Kelemu (CIAT)

Rationale

A bacterial wilt disease of *Brachiaria*, its casual agent, artificial inoculation methods and bacterial population dynamics in resistant and susceptible genotypes of *Brachiaria* have been described (IP-5 Annual Report, 2000; Zuleta et al. 2001). *Xanthomonas campestris* pv. *graminis* infects a number of cultivated forage grasses. Some of the first symptoms are chlorotic/necrotic stripes along the leaves. As the disease advances, the whole leaf may die. Under severe conditions, the whole plant may turn yellow and die. Another typical symptom is wilting and curling of leaves without any discoloration or lesions, which result in quick plant death. Isolates have been collected from three sites in Colombia to determine pathogenicity and distribution.

Materials and Methods

Infected tissues were collected from various accessions and hybrids of *Brachiaria*. After surface sterilization, these tissues were macerated in sterile distilled water and plated on nutrient agar. Single bacterial colonies were isolated 48 hours later. Bacterial cells originating from individual colonies were used for pathogenicity tests. Inoculation methods were as described by Zuleta et al.(2001). For seed transmission tests, seeds collected from diseased susceptible plants in the field were surface sterilized (in 1% NaOCl solution for 5 minutes, in 70% ethanol for 1 minute, and rinsed three times with sterile distilled water). The samples were transferred to sterile filter papers to remove excess moisture. These seed samples were then divided into two: 1) planted onto magenta vessels (Sigma) containing plant growing medium (MS), 9 seeds/vessel. These were maintained in a growth camber with 14 hours light period at 28 C; 2) plated on nutrient agar and incubated at 28°C.

Results and Discussion

Eighty seven percent of the isolates collected from Popayán and Santander de Quilichao were pathogenic on the most susceptible *Brachiaria* CIAT 36062. Disease symptoms typically appeared 15 days after inoculations. The disease was more prevalent than the previous years. Approximately 10 % of the seeds germinated, all of which developed bacterial wilt-like symptoms. Of these, we were able to isolate the wilt-causing causing bacterium from only 4% of the plants. These results indicate that the bacterium is seed transmitted. Approximately 7% of the seeds plated on nutrient agar produced colonies of the casual agent (Photos 11, 12) which were pathogenic on the susceptible *Brachiaria* CIAT 36062. In addition to seed transmission, the disease is transmitted via vegetative propagation and tools used for cutting.

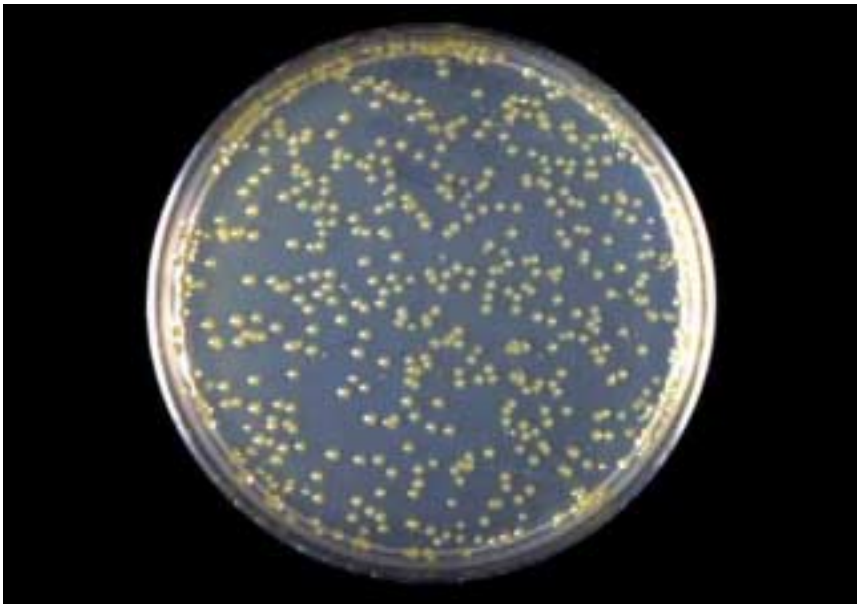

Photo 11. Bacterial colonies on nutrient agar

Photo 12. Symptoms of bacterial wilt disease at its initial (left) and later (right) stages after inoculations

2. 8 Antifungal compounds isolated from seeds of tropical forage legumes

2.8.1 Screening of tropical forage legume collection for antifungal properties

Contributors: W. Kunxin (South China University of Tropical Agriculture, The People's Republic of China), G. Segura and S. Kelemu (CIAT)

Highlights

- Seeds of several tropical forage legumes contained compounds with anti-fungal properties.

Progress towards achieving milestones

- **Detected antifungal compounds in seeds of tropical forage legumes**
  We have examined seeds of 239 different tropical forage species for anti-fungal properties. It was demonstrated that using the methods we applied, some of these clearly contained compounds with properties which inhibit fungal growth.

Rationale

Plants have developed a variety of defense systems to protect themselves from potential pathogens. Plants produce various anti-microbial compounds. Some are constitutive while others are induced upon pathogen infection. Anti-microbial proteins and peptides have been realized to play important roles in plant defense systems and have been detected in a wide range of plants and plant tissues.

Seed germination is probably one of the most vulnerable periods for pathogen attack. Many anti-microbial proteins or peptides have been identified in seeds, such as plant defensins (Osborn *et al.*, 1995, FEBS Lett.368: 257-262; Terras *et al.*, 1992, J. Biol. Chem. 267:15301-15309), proteinase inhibitors (Joshi *et al.*, 1998, Biochem. Biophys. Res. Comm. 246:382-387), chitin-binding proteins (Broekaert *et al.*, 1992, Biochemistry 31:4308-4314), lipid-transfer proteins, knottin-type peptides, and several 4-cysteine-type peptides (reviewed by Broekaert *et al.*, 1997, Crit. Rev. In Plant Sci. 16:297-323), chitinases (Schumbaum *et al*., 1986, Nature 324: 365-367). These anti-microbial proteins or peptides showed potent anti-microbial activity *in vitro*. The expression of certain anti-microbial peptides is induced both locally and systemically by pathogen attack. The expression of an antifungal protein (defensin) in transgenic potato plants has been shown to provide resistance to the fungal pathogen *Verticillium dahliae* in the greenhouse and in the field (Gao *et al*, 2001, Nature Biotechnology 18:1307-1310).

Materials and Methods

Rhizoctonia solani CIAT 5596 was used as a test fungus. Seeds were kindly provided by the germplasm bank at CIAT.

Intact seeds were surface sterilized as follows: 4 min in 70% ethanol, 15 min in 2.5% NaOCl solution, and 6 washes of 5 min each in sterile distilled water.

An incision was made with a scalpel in the seed coat. The incised seeds were soaked overnight in sterile distilled water (10 ml dH_2O per gram seeds) at 4°C. The seeds/dH_2O were ground with mortar and pestle. The solution was filtered through 4 layers of cheese cloth to remove plant debris. The filtrate was centrifuged at 15,000 rpm for 20 min. The suspension was sterilized with a 0.2-µm pore-size nylon membranes (Sigma). Ten-ml filter-sterilized suspension was concentrated by lyophilization. The dry material was re- dissolved in 1 ml sterilized deionized water and used to test its anti-fungal properties.

R. solani inhibition assays were conducted by placing an agar disc containing fungal mycelium at the center of a potato dextrose agar (Difico) containing plate (diameter 9 cm). The plate was then incubated at 28 °C for 1 day, after which two autoclaved 1.5 cm diameter filter disks were placed at opposite ends of the plate. One of the disks was soaked with 400-µl seed extract while the second one was treated with sterile deionized water as control. The plate was re-incubated at 28 °C for 2 days before measurements of mycelial expansion were taken.

Seed extracts (200 µl) of each plant species which showed anti-fungal activity were digested with pronase E (Sigma, P-6911) in reaction buffer (10 mM NaCL, 10mM Tris HCL, PH 7.5) by incubating at 37 °C for 16 h. The suspension was used to test whether the anti-fungal activity was lost.

Results

About 239 forage species were examined, and Table 58 shows those with some anti-fungal properties. Anti-fungal substances which can inhibit the hyphal growth of *R. solani* were detected, but treatment of samples with pronase E did not destroy the anti-fungal properties.

Table 58. List of tropical forage species seeds with anti-fungal properties.

Species	Measurement of zone of inhibition (mm) (two plates average value)	
	With pronase E	Without pronase E
Acacia glauca	4.6*	5.0
Calliandra calothyrsus	9.0	10.0
Calliandra houstoniana	6.5	7.0
Calliandra magdalenae	5.5	6.0
Antifungal protein (control)	2.0	12.5
Leucaena diversifolia	7.0	7.5
Leucaena diversxleucoc	6.3	6.8
Leucaena esculenta	5.5	6.0
Leucaena lanceolata	7.0	6.8
Leucaena leucocephala	6.6	7.0
Leucaena leucocxpulver	6.4	7.0
Leucaena macrophylla	5.7	6.2
Leucaena pallida	6.5	7.0
Leucaena pulverulenta	5.8	6.2
Leucaena shannonii	7.0	6.5
Leucaena retusa	6.8	6.4
Leucaena trichodes	6.6	7.0
Tephrosia africa	5.5	5.0
Tephrosia cinerea	6.4	7.0
Tephrosia pescador	5.5	6.0
Tephrosia polystachya	7.0	6.0
Tephrosia sinapou	6.0	5.5
Tephrosia vogelii	5	5.4
H_2O	0	0

*Values are the average of data from two plates