

# TROPICAL FORAGE PATHOLOGY

## Activity 1. Characterization of *Xanthomona campestris* pv. *graminis* isolates.

### Introduction

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

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

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

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

### Materials and Methods

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

**DNA isolations:** Bacterial cells were grown overnight in Luria broth medium in a shaker (200 rpm) at 28°C. Cells were collected in microcentrifuge tubes by centrifugation (8000 rpm for 10 min) and discarding the supernatant. The cells were re-suspended in 600-µl TE (50mM Tris, pH

8 and 50mM de EDTA) and stored at  $-20^{\circ}\text{C}$  for 10 min, and subsequently thawed at room temperature. Sixty- $\mu\text{l}$  of a freshly prepared lysozyme (10 mg/mL in 25 mM Tris pH 8) and a 6- $\mu\text{l}$  RNase (stock concentration 10mg/ml) were added to the cell suspension and incubated 15 min at room temperature and transferred to ice for another 15 min. A 120- $\mu\text{l}$  STEP solution (0.5% SDS, 50mM Tris pH 7.5 and 280- $\mu\text{g}$  of proteinase) was added and incubated at  $37^{\circ}\text{C}$  for an hour. Subsequently, 216- $\mu\text{l}$  of ammonium acetate solution (7.5 M concentration) was added and mixed well. The solution was precipitated with phenol: chloroform: isoamyl alcohol (25:24:1) The supernatant was treated twice with equal volume of chloroform: isoamyl alcohol (24:1). The DNA was then precipitated with isopropanol and centrifuged. The DNA pellet was washed with 70% ethanol, air-dried and re-suspended in 100- $\mu\text{l}$  sterile distilled water.

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

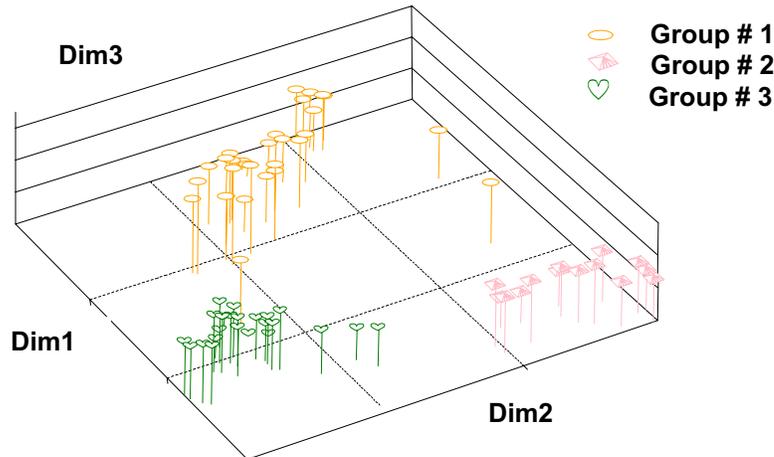
Each 25- $\mu$  reaction mixture contained 30 ng template DNA, 3mM  $\text{MgCl}_2$ , 1.2 (for BOX) and 2 (for ERIC and REP) pmol each primer, 200- $\mu\text{M}$  each of the four dNTPs, 1 U of Taq-DNA polymerase, 50 mM KCl, 10 mM Tris-HCl (pH 8.8), 0.1% Tritón X-100 and 10% (v/v) DMSO (dimethyl sulfoxide).

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

**RAPD-PCR:** Amplifications were carried out with 7 primers from Operon Technologies, Inc. with codes and sequences as follows OPA-01 (5'- CAGGCCCTTC -3'), OPA-02 (5'- TGCCGAGCTG -3'), OPA-03 (5'-AGTCAGCCAC-3'), OPA-04 (5'- AATCGGGCTG-3'), OPAJ-11 (5'-GAACGCTGCC-3'), OPC-02 (5'-GTGAGGCGTC-3'), OPD- 03 (5'- GTCGCCGTC-3'). The reaction had a total volume of 20- $\mu\text{l}$  with 30 ng DNA, 3-mM  $\text{MgCl}_2$ , 0.5- $\mu\text{M}$  primer, 0.26-mM of mixture of dNTPs, 50-mM KCl, 10-mM Tris-HCl (pH 8.8), 0.1% Tritón X-100 and 1 U Taq-DNA polymerase. Amplifications were performed in an automated thermocycler (MJ Research Inc, MA) with an initial denaturation (2 min at  $94^{\circ}\text{C}$ ), annealing (5 min at  $28^{\circ}\text{C}$ ), denaturation (1 min at  $94^{\circ}\text{C}$ ) followed by 45 cycles of denaturation (20 s at  $92^{\circ}\text{C}$ ), annealing (1 min at  $35^{\circ}\text{C}$ ), and extension (1 min at  $72^{\circ}\text{C}$ ), with a final extension (7 min at  $72^{\circ}\text{C}$ ).

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

**Results and Discussion:** Multiple correspondence analysis of the combined data matrix generated using REP-PCR, ERIC-PCR and BOX-PCR resulted in 3 groups with an average similarity index of 78% (**Figure 1**). Isolates *Xc* 44 and *Xc* 45 that were collected in Carimagua appear to be clonal. The same was true with isolates *Xc* 49 and *Xc* 50 that were collected in Palmira.

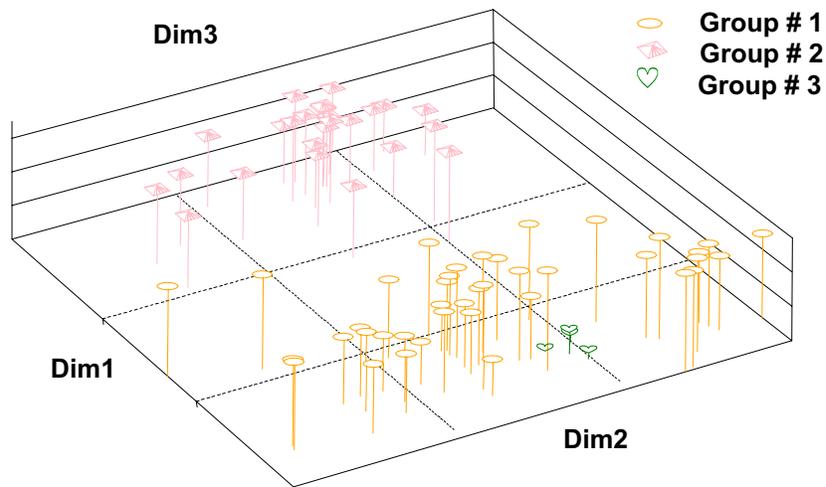


**Figure 1. Correspondence Analysis (MCS) by combining the rep-PCR fingerprints of 69 isolates of *Xanthomonas* obtained with each of the two primers REP and ERIC, and BOX primer.**

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

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

Both RAPD and rep-PCR generated multiple bands. However, correlation between these two techniques was low ( $r = 0.36$ ). RAPD data did not differentiate the two isolates of *X. axonopodis* pv. *manihotis*. The same was the case with ERIC-PCR. However, with BOX primers, as well as the combined data of BOX and REP-PCR separated the two distinct isolates from those of *X. campestris* pv. *graminis*. These results hint that rep-PCR can be used to develop rapid diagnostic tools for *Xanthomonas* pathovars. In addition, both RAPD and rep-PCR revealed genetic diversity among isolates of *X. campestris* pv. *graminis*. In light of this, caution has to be taken in germplasm movement in order not to transfer new isolates of this seed-borne pathogen from one location to another.



**Figure 2. Multiple Correspondence Analysis (MCS) of RAPD-PCR data conducted with 69 *Xanthomonas* isolates.**

**Contributors:** M. Rodriguez, S. Kelemu.

## Activity 2. Resistance to *Xanthomonas campestris* pv. *graminis* (Xcg) in Brachiaria.

### Introduction

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

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

### Materials and Methods

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

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

**Results and Discussion:** Thirteen *Brachiaria* genotypes, BRO-02-193, BRO-02-415, BRO-02-445, BRO-02-465, BRO-02-968, BRO-02-1045, BRO-02-1405, BRO-02-1474, CIAT 16322, CIAT 26110, CIAT 26990, CIAT 36061, 36062, were tested for their reaction to *X. campestris* pv. *graminis*. Seventeen isolates of the pathogen were used to inoculate each of the genotypes. Three genotypes, CIAT #16322, 26110 and 26990, showed no disease symptoms after inoculations with each of the 17 isolates. CIAT 36062 was the most susceptible of the genotypes evaluated, being infected with 16 of the isolates.

Isolates of *X. campestris* pv. *graminis* that infect *Brachiaria* exhibit a wide range of genetic diversity. Pathogenic variation reveal that a wide range of pathotypes exist within the pathogen population. It is encouraging to note that high levels of resistance exist in *Brachiaria*, and it is possible to combine the available resistance in a breeding program.

**Contributors:** M. Rodriguez and S. Kelemu.

### **Activity 3. Elucidate the role of endophytes in tropical grasses.**

#### **Endophyte seed transmission studies in *Brachiaria***

##### **Introduction**

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

DNA from isolates of *A. implicatum* was amplified using 10-base random primers. Primer OPAK 10 (Operon Technology Inc.) amplified bands including a 500-bp product common to all of the isolates tested. This fragment has been cloned and sequenced. Based on this sequence data, several primers were designed and synthesized. A primer pair designated P1 (5'-TTCGAATGATAAGGCAGATC-3' and P4 (5'-ACGCATCCACTGTATGCTAC-3') amplified a 500-bp product with template DNA from isolates of *A. implicatum* in pure cultures and in tissues of *Brachiaria* infected with *A. implicatum*. No amplification product was detected in plants free from *A. implicatum* or using DNA of non-endophytic fungi or the bacterium *Xanthomonas campestris* pv. *graminis*, a pathogen of species of *Brachiaria* (Kelemu et al., 2003. Molecular Plant Pathology 4: 115-118).

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

##### **Materials and Methods**

**Endophyte elimination:** The fungicide Folicur® was used to generate endophyte-free *Brachiaria* clones. Twenty or more plantlets were propagated from a mother plant naturally or artificially infected with the endophyte. Half of these plantlets were soaked in a solution of 0.6 mL/L of Folicur® (250 g a.i./L) for 6 h to eliminate the endophyte, and the other half were left untreated to serve as controls. All plantlets were individually planted in small pots and placed in the greenhouse. Plants were examined 4-6 weeks after treatment for the presence or absence of *A. implicatum*.

**DNA isolations:** Fresh mycelia of endophyte isolates cultured on PDA plates, endophyte-infected or endophyte-free plant leaves, or seeds were collected and macerated in liquid nitrogen for genomic DNA isolation. Genomic DNA was extracted using the DNeasy™ Plant Mini Kits (QIAGEN, Valencia, CA) according to the manufacturer's instructions.

**PCR Amplifications:** Specific primers P1 (5'-TTCGAATGATAAGGCAGATC-3') and P4 (5'-ACGCATCCACTGTATGCTAC-3') were used in the PCR reactions. Amplifications were carried out in a Programmable Thermal Controller (MJ Research, Inc.), programmed with 44 cycles for genomic DNA of endophyte pure cultures or plant leaves, and 54 cycles for DNA

from *Brachiaria* seeds, of a 30 sec denaturation step at 94°C (3 min for the first cycle), followed by 1 min at 65°C, and primer extension for 1 min (10 min in the final cycle) at 72°C. The amplification products were separated by electrophoresis in a 1.0% agarose gel (Bio-Rad), stained with ethidium bromide and photographed under UV lighting.

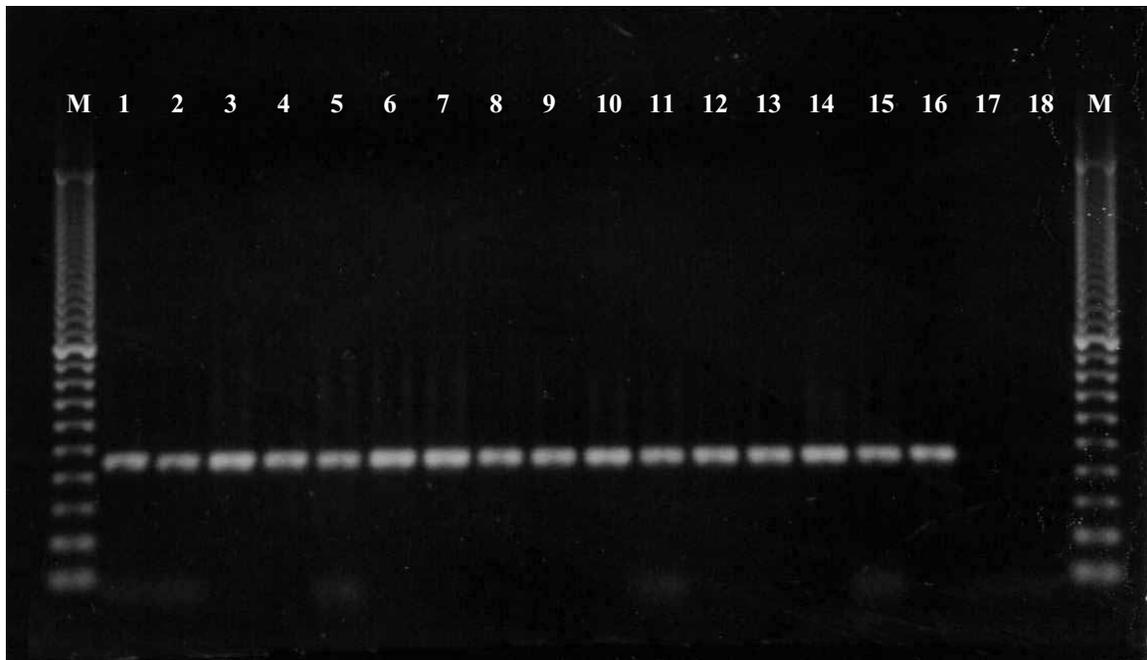
Seed samples were collected from plants confirmed to be endophyte-infected or endophyte-free using the PCR tests with template DNA isolated from plant tissues, and fungal endophyte isolation on culture media.

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

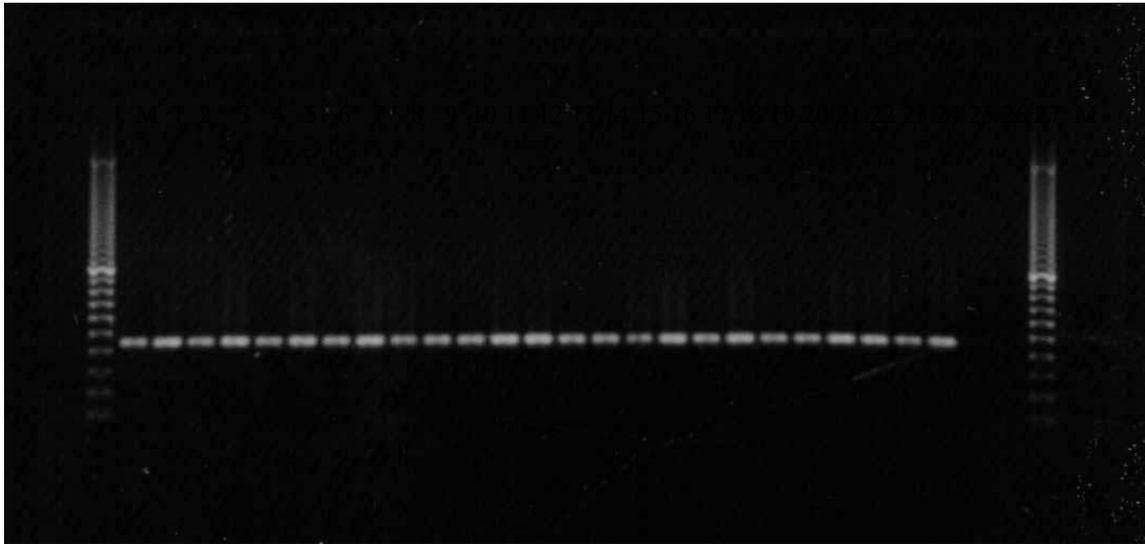
The primer pair, P1/P4, allows the precise and rapid detection of *A. implicatum* in *Brachiaria* plants and permits a differentiation between endophytic and non-endophytic fungi (Kelemu et al., 2003. *Molecular Plant Pathology* 4: 115-118).

A single band of about 500-bp in all examined isolates of *A. implicatum* was amplified. Endophyte-containing and endophyte-free plants were also consistently differentiated using this primer combination (data not shown). Seeds were collected from plants whose tissue samples were used as well as other plants. All seed DNA from endophyte-containing plants had a 500-bp amplified product. No amplification product was detected with seed DNA from endophyte-free plants (**Figure 1**).

Seedlings generated from seed samples of endophyte-containing and endophyte-free plants had consistently tested positive or negative, respectively, for the diagnostic 500-bp amplified product (**Figure 2**). From these results, we concluded that *A. implicatum* maintains its symbiotic association with species of *Brachiaria* through seed transmission.



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



**Figure 2.** Specific detection of *Acremonium implicatum* in seedlings generated from seeds of endophyte-infected and endophyte-free *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. Lanes 1-7, seedlings from seeds harvested from naturally endophyte-infected plants SX99/3488 (8), BRN99NO/4132 (22), BRN99NO/4132 (39), *B. decumbens* accession CIAT 606 (42), BRUZ4X/4402 (44), FM9201/1873 (48), SX99/073 (52), respectively; lanes 8-17, seedlings generated from seeds of ten artificially infected *B. brizantha* CIAT 26110 (15) plants; lanes 18-25, seedlings generated from seeds of eight naturally infected *B. brizantha* CIAT 16320 (32) plants; lanes 26 & 27, seedlings generated from seeds of two endophyte-free *B. brizantha* CIAT 16320 (32-25) plants; lanes M, 100-bp ladders.

**Contributors:** H. Dongyi and S. Kelemu.

#### **Activity 4. Endophyte distribution in *Brachiaria* plants, and PCR analysis and screening of *Brachiaria* genotypes for endophytes.**

##### **Introduction**

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

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

##### **Materials and Methods**

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

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

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

**Results and Discussions:** A diagnostic 500-bp amplification product was observed in all examined leaf sheaths, leaf blades, stems, and roots of *Brachiaria* plants infected with *A. implicatum* (**Figures 1-4**). The amplification product was also detected in whole seeds, embryo, and endosperm of seeds (data not shown). These results indicate that *A. implicatum* is distributed in the plant parts described above. Johnson et al. (1985, *Plant Disease* 69:200-202) described the concentration and distribution of *Epichloë typhina* in tall fescue individual plants with decreasing order in leaf sheaths, seeds, crowns, stems, leaf blades, and roots. Because amplifications with template DNA from leaf sheaths, leaf blades and fresh seeds generate the diagnostic 500-bp product with just 45 cycles, as opposed to 55 cycles with DNA from roots and stems, it is likely

that mycelial concentrations and distributions in *A. implicatum*/*Brachiaria* associations have a similar trend as those reported in *Epichloë typhina*/ tall fescue. There was no obvious difference in sensitivity with genomic DNA from leaf blades and sheaths, although isolations on culture media is more routinely and successfully done from leaf sheaths than leaf blades.

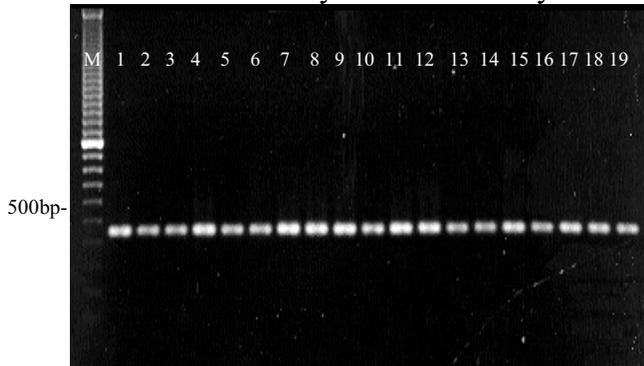


Figure 1.

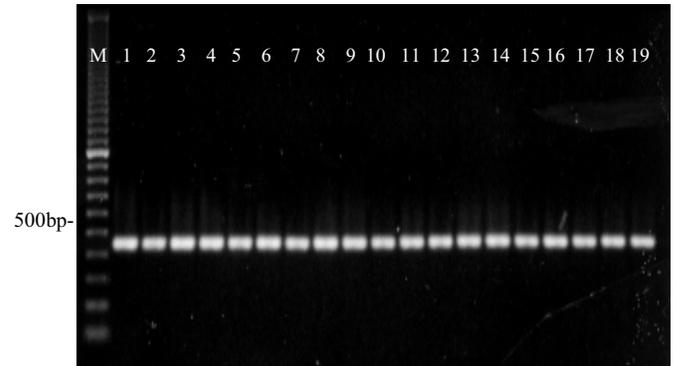


Figure 2.

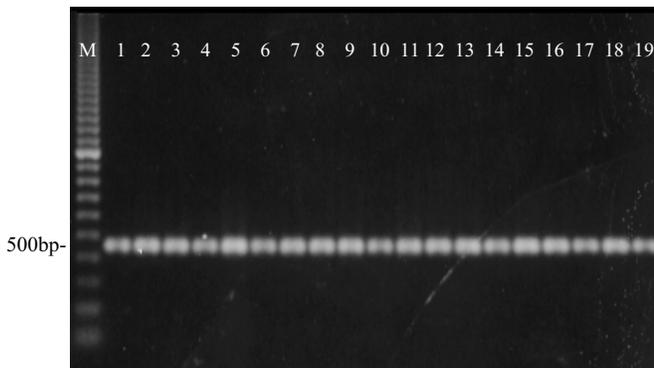


Figure 3.

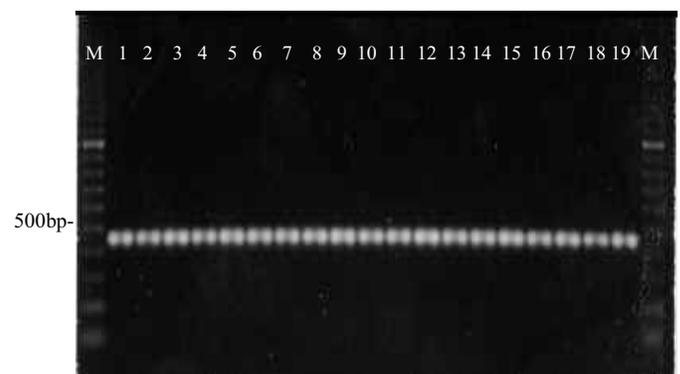


Figure 4.

Figures 1-4. Specific detection of *Acremonium implicatum* in leaf blades (Figure 3), leaf sheaths (Figure 4), stems (Figure 5), and roots (Figure 6) collected from endophyte-infected *Brachiaria* plants using polymerase chain reaction (PCR) with primer pair P1/P4. Lanes 1~19: *Brachiaria* hybrid plants SX99/3488 (8), SX99/0275(14), BR99NO/4132 (22), SX99/1513 (23), FM9201/1873 (29), BR99NO/4015 (37), BR99NO/4132 (39); *B. decumbens* accession CIAT 606 (42); *B. hybrids* BRUZ4X/4402 (44), FM9201/1873 (48), SX99/0731(52), FM9503/S046/024 (19); *B. brizantha* accession CIAT 16320 (32a); *Brachiaria* hybrid FM9503/S046/024 (45), SX99/2341(47); *B. brizantha* accession CIAT 26110 (15), *B. brizantha* 6780 (56), *B. brizantha* 6780 (63), *B. brizantha* 6780 (111), respectively. *Brachiaria* hybrid FM9503/S046/024 (19), *B. brizantha* CIAT 16320 (32a), *Brachiaria* hybrid FM9503/S046/024 (45), *Brachiaria* hybrid SX99/2341(47) were naturally infected with isolates we have previously characterized. Plant *B. brizantha* CIAT 26110 (15), *B. brizantha* CIAT 6780 (56), *B. brizantha* CIAT 6780 (63), and *B. brizantha* CIAT 6780 (111) were artificially infected with an isolate (EB 6780(201) of *A. implicatum*. All remaining plants were naturally infected with yet to be isolated and characterized strains.

Contributors: H. Dongyi, T. Sakai, S. Kelemu.

## Activity 5. Effect of fungal endophytes on pathogens *in planta*.

### Introduction

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

Although many endophyte isolates show antifungal activities *in vitro*, there are only a few reports on resistance to pathogens conferred by endophytes *in planta*. Reduction of tall fescue seedling density due to *Rhizoctonia zeae* was inversely correlated with endophyte (*N. coenophialum*) infestation level of the seed lot (Gwinn and Gavin, 1992, *Plant Disease* 76:911-914). Plant protection by *E. typhina* against *Cladosporium phlei*, the causing pathogen of purple leaf spot of timothy grass, was reported (Greulich et al. 1999, *Ann. Phytopathol. Soc. Jpn.* 65:454-459).

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

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

### Materials and Methods

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

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

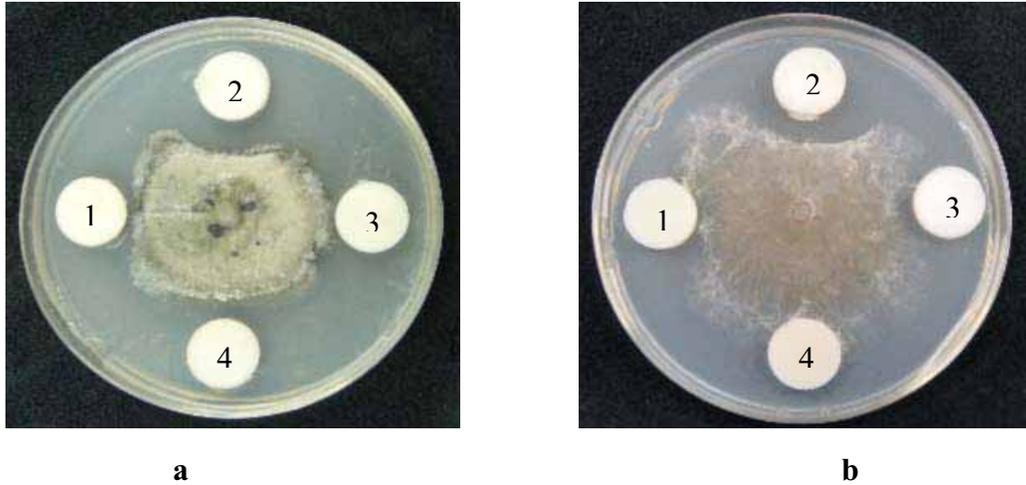
**Antifungal activity tests:** Filter paper discs were soaked with 400- $\mu$ l endophyte mycelial/conidial extract prepared as described above. These were placed on PDA-containing petri dishes individually inoculated with *Drechslera* sp. and *R. solani* as shown in **Figures 1a and b**. These were incubated at 28 °C in the dark for 3–5 days.

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

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

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

Extracts from *A. implicatum* strain EH32 showed strong inhibition to *Drechslera* sp and *R. solani* (**Figures 1a and 1b**). Extracts treated with heat or pronase lost their antifungal activity. Further extensive studies are needed to determine the nature of the antifungal activity in *A. implicatum*.



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

**Contributors:** H. Dongyi, S. Kelemu.

## Activity 6. Search for nitrogen-fixing bacteria associated with species of *Brachiaria*.

### Introduction

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

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

### Materials and Methods

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

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

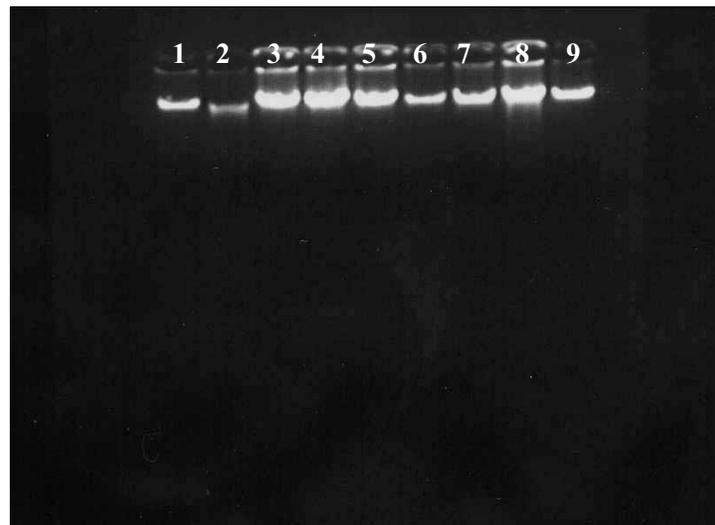
**DNA isolations from soil samples:** A protocol described by Porteous et al. (1997. An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. Technical note. *Molecular Ecology*. 6: 787-791) was used to isolate DNA from soil. The method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

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

**Nested PCR Amplification:** Three primers were used, which were originally designed by Zehr and McReynolds (1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. *Appl. Environ. Microbiol.* 55: 2522-2526) and Ueda, et al. (1995. Remarkable N<sub>2</sub>-fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. *J. Bacteriol.* 177: 1414-1417), to amplify fragments of *nifH* genes. Amplification steps described by Widmer et al (1999. Analysis of *nifH* gene pool complexity in soil and litter at a douglas fir forest site in the Oregon cascade mountain range. *Applied and Environmental Microbiology* 65:374-380) were adopted.

## Results and Discussion

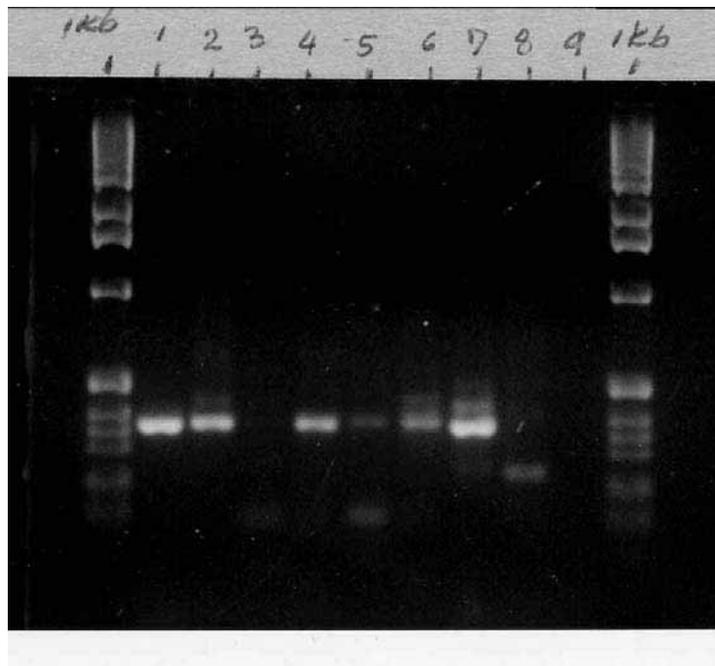
**DNA extraction from bacterial cells:** DNA extracted from bacterial cells is shown in Figure 1.



**Figure 1.** DNA isolated from: 1) *Bradyrhizobium* 3101, 2) *Bradyrhizobium* 2469, 3) unidentified bacterium BR97-155 CBT, 4) unidentified bacterium 16445 CBT, 5) unidentified bacterium 16497 CBH, 6) unidentified bacterium FM97-383 CACT, 7) *Rhizobium* 668, unidentified bacterium. BR97-1371, 9) *Xanthomonas campestris* pv. *graminis* 1015.

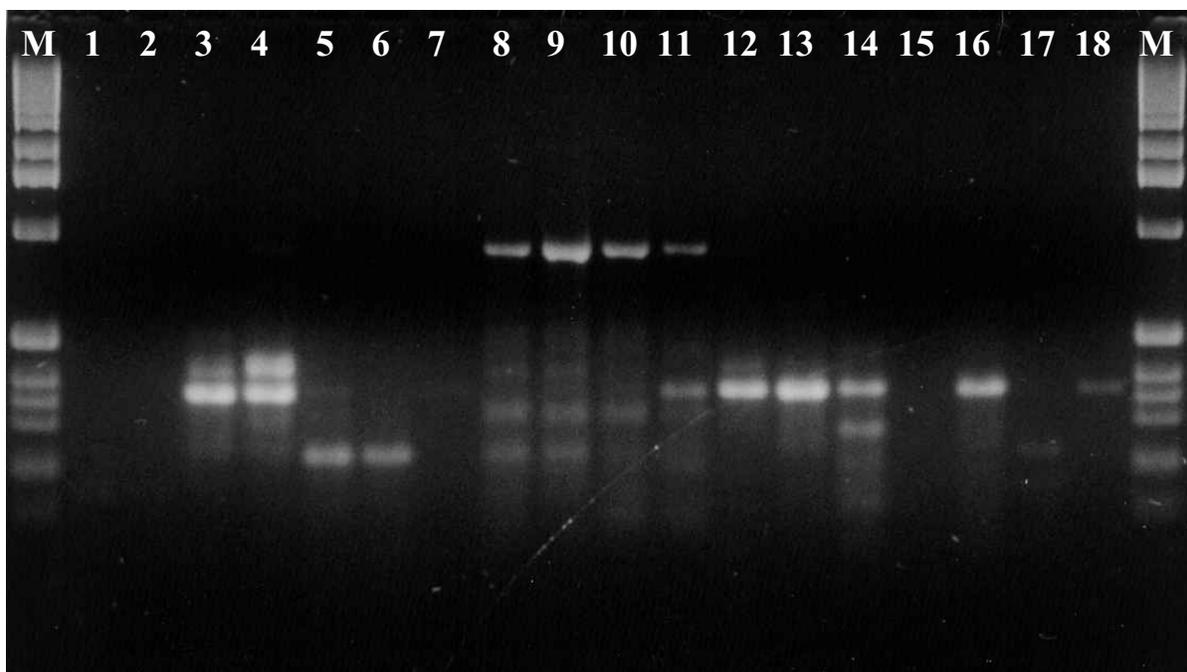
**Nested PCR amplifications:** Amplified products of approximately 370-bp size were amplified with template DNA from nitrogen-fixing bacteria *Rhizobium* and *Bradyrhizobium*, as well as from those randomly picked bacterial colonies isolated from *Brachiaria* CIAT 16445, *Brachiaria* CIAT 16497, and *Brachiaria* FM97-383 (**Figure 2**). Template DNA from a randomly picked

bacterial colony from *Brachiaria* CIAT 36062 amplified a product with approximately 210-bp size (**Figure 2**). No amplification products were observed with DNA from the pathogen *X. campestris* pv. *graminis* (**Figure 2; lane 9**) and with that of a bacterium isolated and selected from *Brachiaria* BR97-155 (**Figure 2; lane 3**).



**Figure 2.** Nested PCR amplification products with three primers of sequences of *nifH* gene. Lanes 1-9, *Bradyrhizobium* 3101, *Bradyrhizobium* 2469, BR97-155 CBT, 16445 CBT, 16497 CBH, FM97-383 CACT, *Rhizobium* 668, BR97-1371, *Xanthomonas campestris* pv. *graminis* 1015 (negative control), respectively. Size markers 1kb ladder.

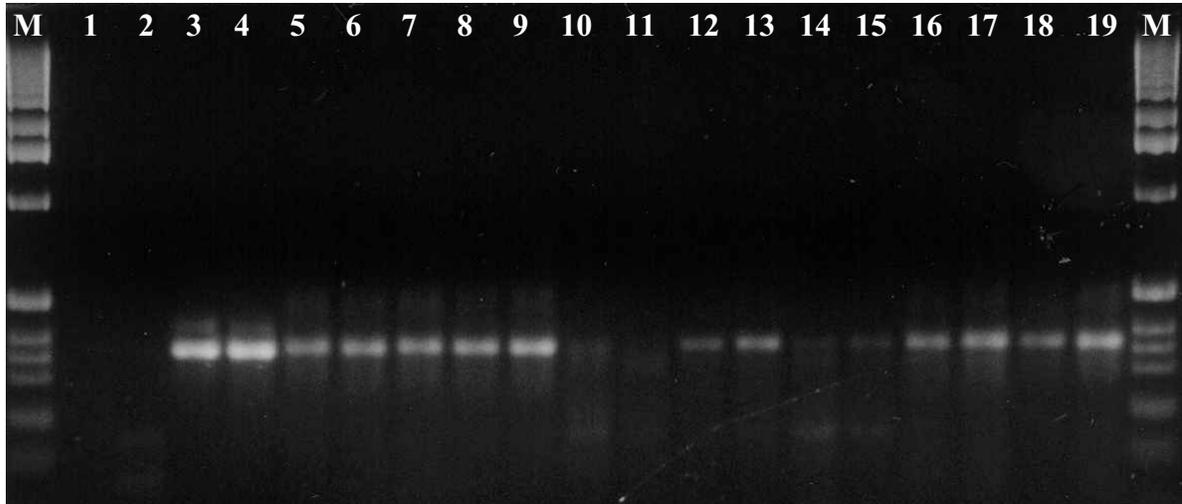
*Brachiaria* hybrid CIAT 36062 (BR97-1371) is of particular interest because of its maintenance of green color in the absence of nitrogen input. We have plants of this hybrid in pots in the glasshouse for the last 4 years with no application of nitrogen fertilizer, but are still green. We, therefore, concentrated on this hybrid and isolated independent bacterial colonies from roots, leaves, stems, and soil around the plant roots. Roots were sectioned into three parts: superficial (next to stems), middle and bottom parts. Pieces plant tissues were surface sterilized, macerated in sterile distilled water and plated on nutrient agar for bacterial isolations. Cells from individual bacterial colonies (random colony selection was based on colony color and morphology) were transferred to fresh nutrient agar for further increment. Nested PCR amplification with DNA of these colonies resulted in various size products ranging between 200-1000 bp sizes (**Figure 3**). Two colonies (**Figure 3; lanes 7, 15**) gave no amplification products. Some bacterial colonies isolated from the bottom part of the root, the leaf and stem generated strong amplification products with the same size as that produced by nitrogen-fixing bacteria used as positive controls (**Figure 3**).



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

DNA isolated from soil samples taken from the surface, middle and bottom part of the potted plants all generated strong amplification products with the same size as those produced by nitrogen-fixing bacteria used as controls (**Figure 4**).

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



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

**Contributors:** C. Zuleta, R. Sedano, S. Kelemu.

## Activity 7. Bacterial endophytes isolated from *Brachiaria*.

### Introduction

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

Soil bacteria of the genera *Allorhizobium*, *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium*, and *Sinorhizobium* are of great agricultural importance, because of their ability to fix atmospheric nitrogen in a symbiosis association with legumes. Populations of rhizobia can survive in the soil as saprophytes in the absence of legumes. In recent years, the natural habitat of rhizobia was extended to the roots of gramineous plants. *Rhizobium leguminosarum* bv. *trifolii* was reported to exist inside the roots of rice plants grown in rotation with clover in Egypt (Yanni et al. 1997. Plant Soil 194:99-114), without forming root nodules or nodule-like structures. Various other N<sub>2</sub>-fixing endophytic bacteria, known as plant growth-promoting rhizobacteria (PGPR), such as *Acetobacter diazotrophicus* and *Herbaspirillum seropedicae* in sugarcane, *Azoarcus* spp. in Kallar grass (*Leptochloa fusca*), and *Azospirillum* spp. in maize and rice have been reported. *Herbaspirillum seropedicae* has also been found in association with maize, sorghum and other gramineous plants. Sugarcane plants inoculated with a wild type strain of *A. diazotrophicus* had a higher nitrogen content those inoculated with a *nif* mutant strain or uninoculated controls in nitrogen-deficient conditions.

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

### Materials and Methods

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

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

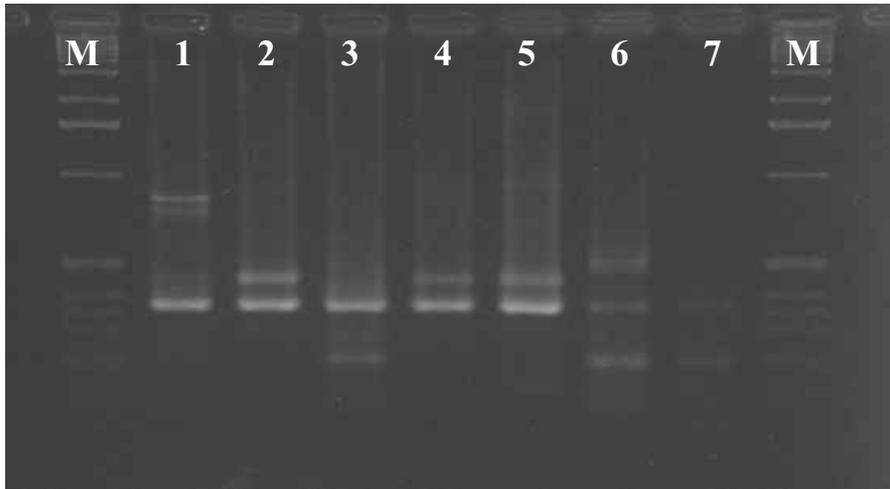
cleaning with phenol-chloroform and chloroform/isoamyl alcohol and precipitation with ethanol. The quality of DNA was checked on 1 % agarose gel.

**Nested PCR Amplification:** Three primers were used, which were originally designed by Zehr and McReynolds (1989. Use of degenerate oligonucleotides for amplification of the *nifH* gene from the marine cyanobacterium *Trichodesmium thiebautii*. Appl. Environ. Microbiol. 55: 2522-2526) and Ueda, et al. (1995. Remarkable N<sub>2</sub>- fixing bacterial diversity detected in rice roots by molecular evolutionary analysis of *nifH* gene sequences. J. Bacteriol. 177: 1414-1417), to amplify fragments of *nifH* genes. Amplification steps described by Widmer et al (1999. Analysis of *nifH* gene pool complexity in soil and litter at a douglas fir forest site in the Oregon cascade mountain range. Applied and Environmental Microbiology 65:374-380) were adopted.

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

**Results and Discussion:** The three bacterial isolates 36062-R2, 36062-H4, and 36062-V2 consistently isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively, tested positive in nested PCR amplifications (**Figure 1**). The fatty acid analysis matched the bacterium coded 03-36062-V2 with *Flavimonas oryzihabitans* at 0.887 similarity index. *F. oryzihabitans* has been described as a plant growth promoting rhizobacterium in graminicolous plants (Luz, W.C., <http://www.ag.auburn.edu/argentina/pdfmanuscripts/luz.pdf>, accessed on 05 August, 2003). The analysis matched isolate 02-36062-H4 with *Agrobacterium rubi* at 0.845 similarity index. The name *A. rubi* is synonymous to *Rhizobium rubi* (Young et al. 2001. Int. J. Syst. Evol. Microbiol. 51:89-103). The match using fatty acid data of the isolate 01-36062-R2, however, was not conclusive, matching it with *Leclercia adecarboxylata*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, at 0.879, 0.841, and 0.820 similarity index, respectively. Of these, *E. cloacae* has been described as one of the dominant endophytic bacteria isolated from citrus plants (Araújo et al.2002. Applied and Environmental Microbiology 68:4906-4914).

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



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

**Contributors:** C. Zuleta, R. Sedano, S. Kelemu.

## Activity 8. Antifungal protein isolated from seeds of tropical forage legume.

### Introduction

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

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

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

### Materials and Methods

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

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

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

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

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

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

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

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

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

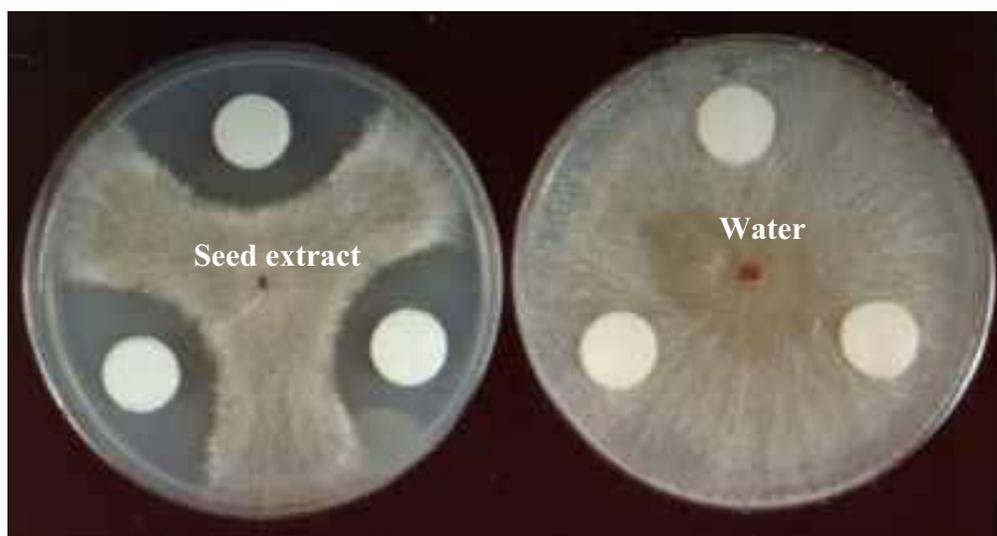
The purified protein was mixed with flour of 'ICA Pijao' at different concentrations (0, 0.0625, 0.125, 0.25, 0.5, 1.0, 2.0, and 5% w/w). Infestation procedures were as follows: for *Z. subfasciatus*, seeds were infested with at least eight pairs of bruchids per seed. After five days, seeds were examined under a dissecting microscope and 5-6 eggs were left per seed by destroying the excess with a needle. For *A. obtectus*, seeds for each protein concentration were infested with 5-6 neonate larvae per seed. Larval penetration was subsequently checked to guarantee correct mortality counts. All artificial seeds were individually evaluated in glass vials.

Percent adult emergence and days to adult emergence until the last insect emerged were the parameters recorded, although insect survival has been expressed in terms of percent mortality. At the end of the trial, when no more adult emergence occurred, the instar of dead larvae within the seed was determined by measuring the width of the head capsule after dissection of the seeds.

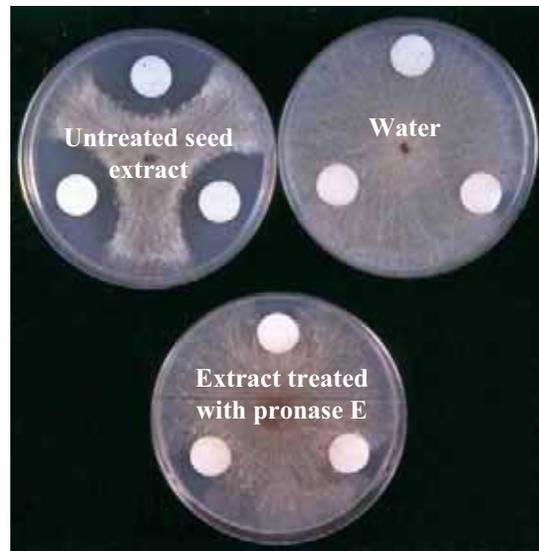
**Statistical analysis:** Concentration-mortality responses were estimated by means of probit regression analysis (SAS Institute 1989). The Statistix package (Analytical Software 2000) was used for analysis of variance performed with data on days to adult emergence

## Results and Discussion

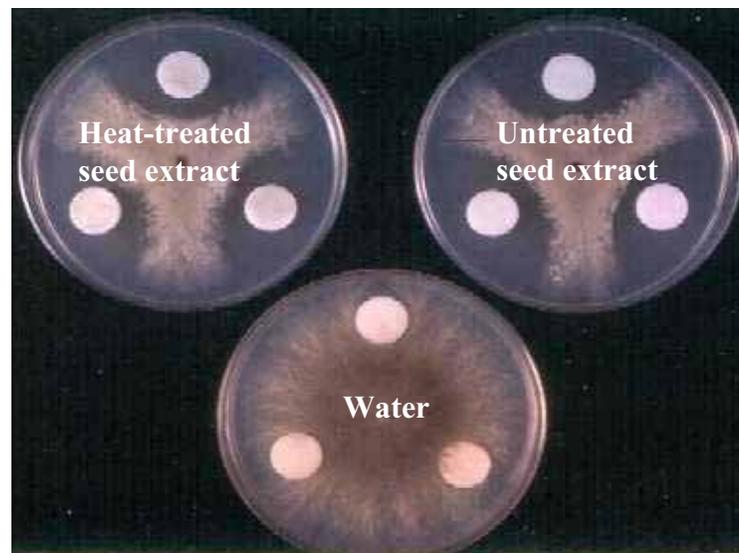
**Antifungal activity:** The crude extract from seeds of *C. ternatea* CIAT 20692 showed strong antifungal activity on the test fungus *R. solani* (Figure 1). This activity could be eliminated by treatment with Pronase E (Figure 2), indicating that the active compound is a protein. The activity was heat stable (Figure 3). Seeds release this heat stable proteinaceous antifungal compound after mechanical disruption of their seed coat (Figure 4) or after germination (data not shown).



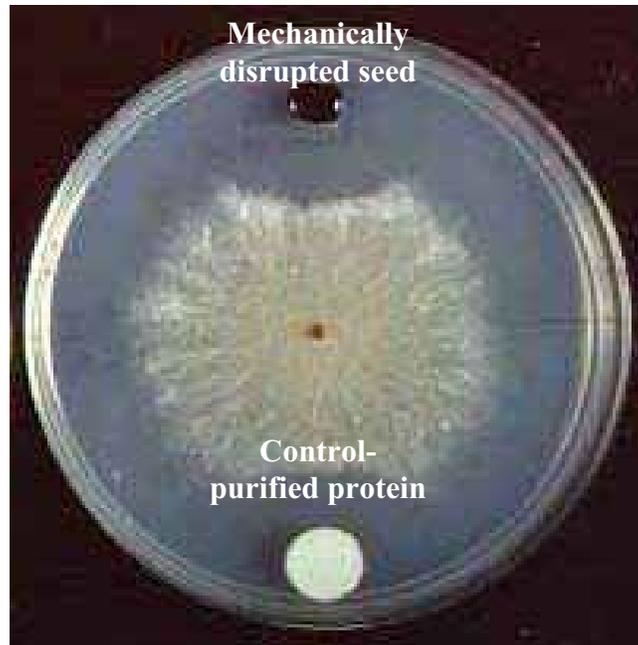
**Figure 1.** Growth inhibition of *Rhizoctonia solani* by seed extract from *Clitoria ternatea* CIAT 20692 on potato dextrose agar plates incubated at 28 °C for 2 days. Seed extract filtrate (300- $\mu$ l) was applied on each of the three filter paper discs, whereas the control plate had discs with equal volumes of sterile distilled water. A single sclerotium of *R. solani* was placed on the center each plate.



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

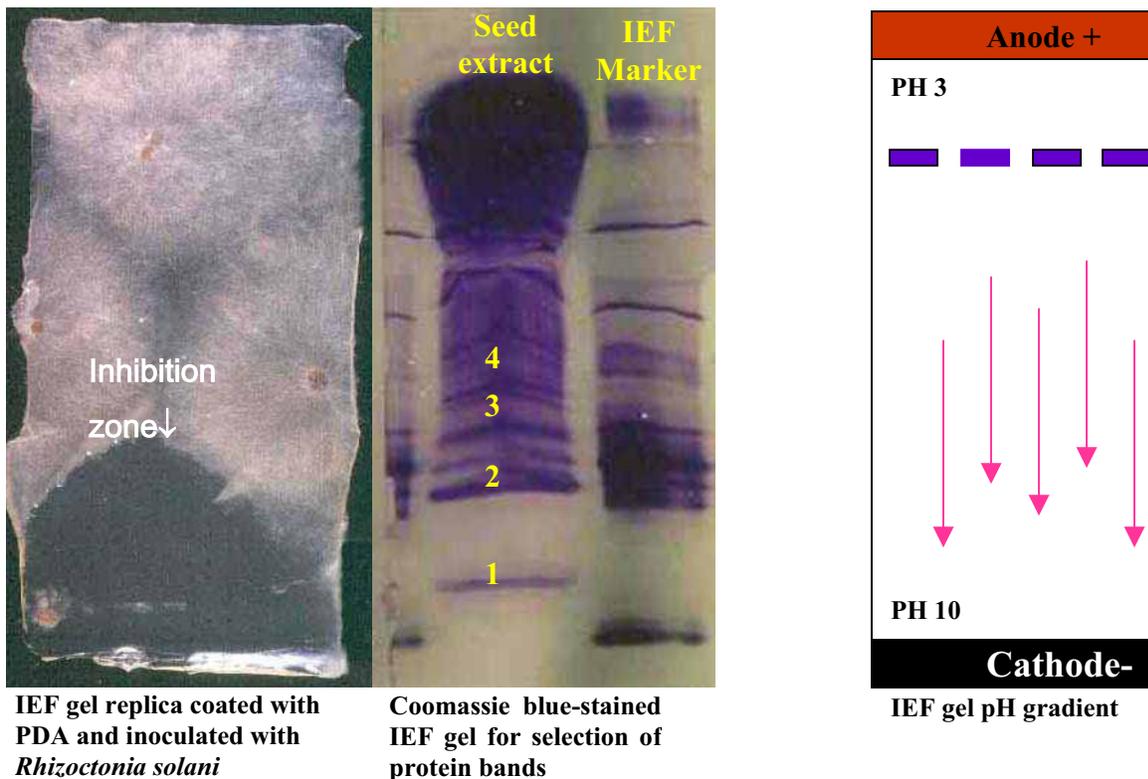


**Figure 3.** Heat stability of antifungal activity of extracts from *Clitoria ternatea* CIAT 20692 seeds after boiling for 5 min. A single sclerotium of *R. solani* was placed on the center each plate containing potato dextrose agar and incubated at 28 °C for 2 days. Seed extract filtrate (300- $\mu$ l), either boiled or untreated, was applied on each of the three filter paper discs, whereas the control plate had discs with equal volumes of sterile distilled water.



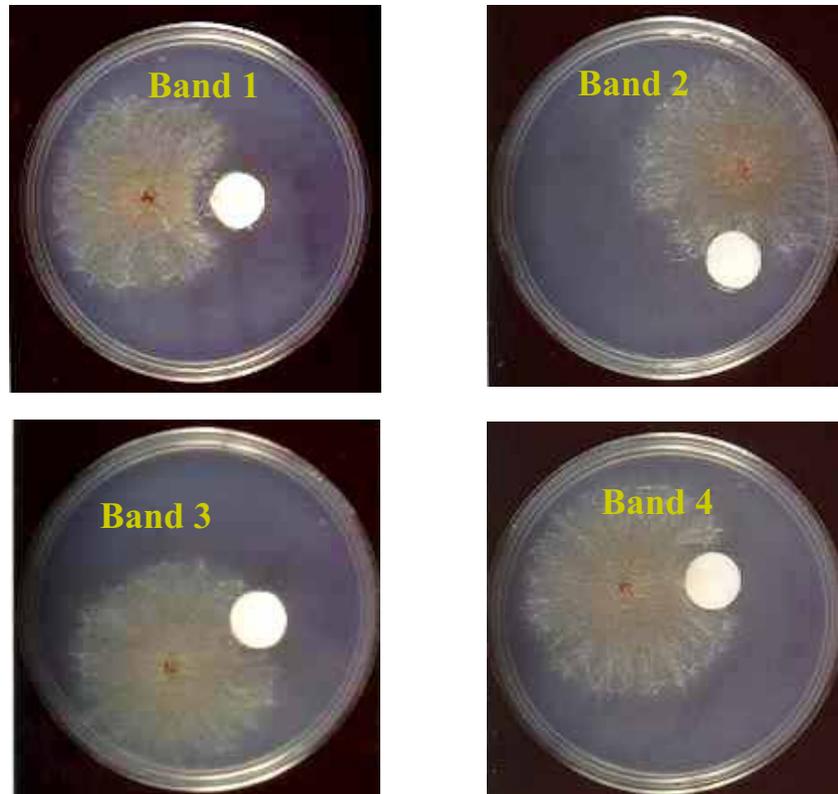
**Figure 4.** Seeds release an antifungal compound after mechanical disruption of their seed coat. Note that a single seed can release a compound with substantial antifungal activity against the test fungus *Rhizoctonia solani*.

**Identification of antifungal protein and purification:** Resolving the seed extract by isoelectric focusing gel revealed a number of proteins. Thus, identifying the specific protein (s) responsible for the antifungal activity seemed a daunting task. To reduce the complexity of that task and to facilitate the identification of the specific protein of interest, we created a new protocol which involves: 1) resolving the seed extract by an IEF gel, 2) neutralizing the gel to eliminate the pH gradient, 3) lightly and uniformly coating the gel with warm PDA, 4) inoculating the gel/PDA composition with *R. solani* sclerotia, 5) wrapping it with Saran wrap to avoid loss of moisture and incubating it at 28 °C for 2 days. This protocol, in fact, had greatly facilitated our task. *R. solani* grew uninhibited in the large portion of the gel/PDA composition, but was inhibited in the area where proteins with alkaline pI run. The specific antifungal protein was identified by cutting out ultra-thin-layer polyacrylamide gel areas corresponding to individual protein bands in a duplicate Coomassie-stained gel. The sliced gels were each macerated in 100- $\mu$ l sterile distilled water in Eppendorf tubes and used for antifungal activity. The results of these tests show that a highly basic protein (**numbered 1 in Figure 5**) was responsible for the antifungal activity (**Figure 6**).

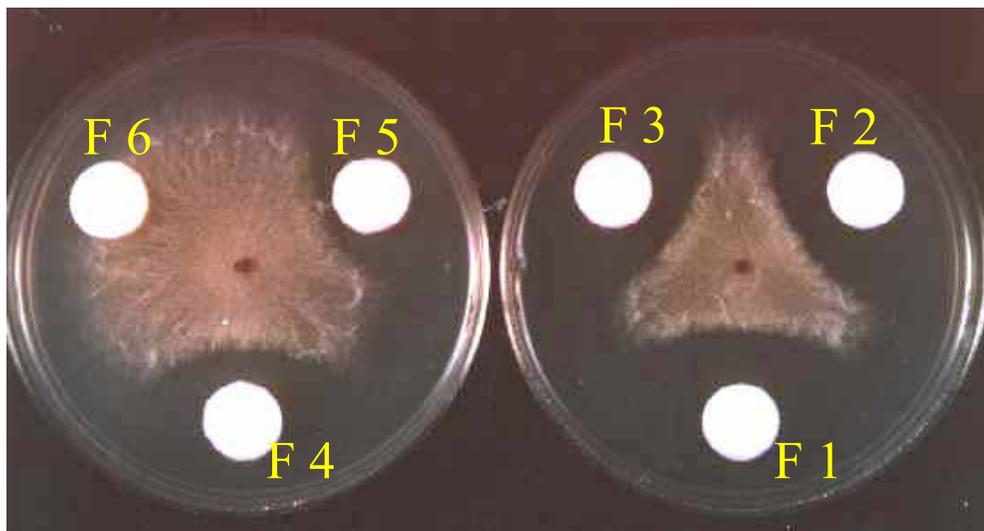


**Figure 5.** Extracts of *Clitoria ternatea* seeds resolved by isoelectric focusing (IEF) gel. *Rhizoctonia solani* growth inhibition zone indicated that the protein (s) responsible for antifungal activity were in the alkaline part of the gel. A triplicate IEF gel was superimposed on an identical Coomassie-stained IEF gel. Gel areas corresponding to the stained bands 1-4 were cut out for identification of the antifungal protein.

The peptide was well separated from the other proteins on IEF gels, making the purification procedure using the preparative granulated bed isoelectric focusing a relatively easy task. Five of the fractions showed activity with decreasing intensity starting from the highly alkaline pI (isoelectric point) [Figure 7]. The active protein which was mostly recovered in fraction 1 was named Finotin. Both SDS-PAGE (Figure 8) and IEF gels (data not shown) showed that fraction 1 is pure and free of other proteins.



**Figure 6.** Identification of specific antifungal protein band. The protein band with the most alkaline pI (isoelectric point) numbered 1 in Figure Finotin 4 demonstrated growth inhibition of the test fungus *Rhizoctonia solani*.



**Figure 7.** Purification of an antifungal protein from seeds of *Clitoria ternatea* using preparative granulated bed isoelectric focusing. Five fractions scooped from the gel starting from the alkaline part of the gel as # F1 demonstrated antifungal activities, with F5 having the least growth inhibitory activity against *Rhizoctonia solani*.



**Table 1. Toxicological responses of bean bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus* to a purified protein (Finotin) isolated from seeds of *Clitoria ternatea*.**

No. Tested	LC <sub>50</sub> (95% FL) <sup>a</sup>	LC <sub>95</sub> (95% FL)	Slope ± SEM	χ <sup>2</sup>
<i>Zabrotes subfasciatus</i>				
147	1.21 (0.99 - 1.47)	2.88 (2.17 - 5.21)	4.3 ± 0.88	2.78
<i>Acanthoscelides obtectus</i>				
155	0.36 (0.28 - 0.43)	0.77 (0.61 - 1.28)	4.9 ± 0.96	0.84

<sup>a</sup> FL, fiducial limits.

**Table 2. Effect of increasing concentrations of a purified protein (Finotin) isolated from seeds of *Clitoria ternatea* on the biology (days to adult emergence) of the bean bruchids *Zabrotes subfasciatus* and *Acanthoscelides obtectus***

Protein Concentration (% w/w)	Days to Adult Emergence	
	<i>Zabrotes subfasciatus</i>	<i>Acanthoscelides obtectus</i>
0.0 <sup>1</sup>	43.1e	34.4c
0.0625	45.0e	33.8c
0.125	51.5d	35.2c
0.25	55.6c	49.4b
0.5	57.3c	63.4a
1.0	72.7b	N.E.
2.0	80.0a	N.E.
5.0	N.E. <sup>2</sup>	N.E.

Means within a column followed by the same letter are not significantly different according to Fisher's protected LSD. ANOVA on data testing for differences among dosages (protein concentrations). For *Z. subfasciatus*:  $F = 106.9$ ;  $df = 6,21$ ;  $P < 0.001$ ; for *A. obtectus*:  $F = 184.9$ ;  $df = 4,16$ ;  $P < 0.001$ .

<sup>1</sup> Background flour prepared with 'ICA Pijao'

<sup>2</sup> N.E., no adult emergence, 100% larval mortality.

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

**Contributors:** G. Segura, C. Cardona, S. Kelemu.

## Publications

### Refereed Journal Articles

- Kelemu, S., Dongyi, H., Guixiu, H. and Takayama, Y. 2003. Detecting and differentiating *Acremonium implicatum*: developing a PCR-based method for an endophytic fungus associated with the genus *Brachiaria*. *Molecular Plant Pathology* 4(2): 115-118.
- Kelemu, S. and Dongyi, H. 2003. Endophytic fungus *Acremonium implicatum* is seed transmitted in *Brachiaria* spp. (Abstract). *Phytopathology* 93:S43.
- Kelemu, S. and Mahuku, G. 2003. Harnessing Biotechnology to Improve Food and Forage Legumes: The Case of Plant Disease Resistance. Paper for the Second National Workshop on Food and Forage Legumes, 22-27 September 2003, Addis Ababa, Ethiopia.
- Weeds, P.L., Chakraborty, S., Fernandes, C.D., Charchar, M. J. d'A., Ramesh, C.R., Kexian, Y. and **Kelemu, S.** 2003. Genetic Diversity in *Colletotrichum gloeosporioides* from *Stylosanthes* spp. at centers of origin and utilization. *Phytopathology* 93:176-185.

### Submitted

- Chakraborty, S., Ghosh, R., Mukherjee, M., Fernandes, C. D., Charchar, M. J. and **Kelemu, S.** 2003. A comparison of artificial neural network and multiple regression analysis for weather-based prediction of anthracnose.
- Kelemu, S., Mahuku, G., Fregene, M., Pachico, P., Johnson, N., Calvert, L., Rao, I., Buruchara, R., Amede, T., Kimani, P., Kirkby, R., Kaaria, S., and Ampofo, K. 2003. Harmonizing the agricultural biotechnology debate for the benefit of African farmers. *African Biotechnology Journal*.

### Conference Papers

- Kelemu, S. and Mahuku, G. 2003. Harnessing Biotechnology to Improve Food and Forage Legumes: The Case of Plant Disease Resistance. Paper for the Second National Workshop on Food and Forage Legumes, 22-27 September 2003, Addis Ababa, Ethiopia.

### Book Chapters

- Chakraborty, S., Ghosh, R., Ghosh, M., Maji, A. K., White, N., Fernandes, C. D., Charchar, M. J., Ramesh, C. R. and **Kelemu, S.** 2003. Weather dependency of anthracnose and risk mapping. In: S.Chakraborty (editor) High yielding anthracnose resistant *Stylosanthes* for Agricultural systems'
- Kelemu, S., Miles, J. W. and Rao, I. M. 2003. Biotic and abiotic constraints to *Stylosanthes* production. In: S.Chakraborty (editor) High yielding anthracnose resistant *Stylosanthes* for Agricultural systems'

### Other Publications

- T Amede, E Amézquita, J Ashby, M Ayarza, E Barrios, A Bationo, S Beebe, A Bellotti, M Blair, R Delve, S Fujisaka, R Howeler, N Johnson, S Kaaria, **S Kelemu**, P Kerridge, R Kirkby, C Lascano, R Lefroy, G Mahuku, H Murwira, T Oberthur, D Pachico, M Peters, J Ramisch, I Rao, M Rondon, P Sanginga, M Swift and B Vanlauwe. 2003. BNF: A key input for integrated soil fertility management in the tropics. (A working group paper).

**Staff:** Raul Sedano, B.Sc, Research Assistant II. Martin Rodriguez, B.Sc., Research Assistant III, Ximena Bonilla, B.Sc., Research Assistant III (part time), Carolina Zuleta, Research Assistant III, Tomoko Sakai, Visiting Scientist, Japan Overseas Cooperation Volunteers (JOCV), JICA, Japan, Gustavo Segura, Technician II, Alvaro Baena, Technician II, Dario Viveros, Technician III.

**Donors:** The Ministry of Foreign Affairs, Japan.

**Collaborators:** Japan International Cooperation Agency (JICA). Dr. C. Schardl, University of Kentucky, USA.

<b>TROPICAL FORAGE PATHOLOGY.....</b>	<b>146</b>
<i>Activity 1. Characterization of Xanthomona. campestris pv. graminis isolates.....</i>	<i>146</i>
<i>Activity 2. Resistance to Xanthomonas campestris pv. graminis (Xcg) in Brachiaria.....</i>	<i>150</i>
<i>Activity 3. Elucidate the role of endophytes in tropical grasses.....</i>	<i>151</i>
<i>Activity 4. Endophyte distribution in Brachiaria plants, and PCR analysis and screening of Brachiaria genotypes for endophytes.....</i>	<i>155</i>
<i>Activity 5. Effect of fungal endophytes on pathogens in planta.....</i>	<i>157</i>
<i>Activity 6. Search for nitrogen-fixing bacteria associated with species of Brachiaria.....</i>	<i>160</i>
<i>Activity 7. Bacterial endophytes isolated from Brachiaria.....</i>	<i>165</i>
<i>Activity 8. Antifungal protein isolated from seeds of tropical forage legume.....</i>	<i>168</i>