TROPICAL FORAGE PATHOLOGY

Activity 1. Antifungal proteins in tropical forages.

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

Rationale

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

Seeds use strategies to germinate and survive in soils that are inhabited by a wide range of microfauna and microflora. Various antifungal and/or antibacterial proteins such as chitinases, β-glucanases, thionins, ribosome-inactivating proteins and permatins have been detected in seeds. Antimicrobial proteins and peptides have been isolated and characterized from seeds of maize (*Zea mays* L.), radish (*Raphanus sativus* L.) and various other plants. They are believed to play a role in plant defense because of their strong antimicrobial activity *in vitro*. This belief is further supported by their ability to confer resistance (to pathogens) in transgenic plants containing genes that encode them. The list of antifungal proteins from various organisms is long, with new ones continuously being discovered.

Other plant-derived proteins have insecticidal properties that can, for example, protect seeds from attack by larvae of various bruchids and inhibit the growth and development of *Helicoverpa punctigera* (Wallengren) larvae. Of particular interest are plant-derived proteins called cyclotides (circular proteins in which the N and C termini are linked via a peptide bond), which have antimicrobial and insecticidal properties. Ocatin, a protein isolated from the Andean tuber crop oca (*Oxalis tuberosa* Mol.), is reported to have antibacterial and antifungal effects.

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

Materials and Methods

Protein extractions: Large quantities of seeds *Clitoria ternatea* CIAT 20692 were produced on field plots at CIAT headquarters in Palmira, Colombia, for protein extractions. Seeds (100 g) of *C. ternatea* CIAT 20692 were surface-sterilized in 3.25% NaOCl solution for 10 min, then in 70% ethanol (3 min), and rinsed 6 times with sterile distilled water. The seeds were left in sterile distilled water overnight to facilitate maceration. The imbibed seeds were then macerated in 1,000 mL of sterile distilled water with a sterilized mortar and pestle. The macerated solution

was filtered through several layers of cheesecloth to get rid of the seed debris. The filtrate was then centrifuged at 4 ^{o}C in tubes (50 mL) at 13 000 × g for an hour. To remove any potential microbe associated with the filtrate, the supernatant was filtered through 0.22-µm-pore-size cellulose acetate membranes. Aliquots (7 mL) of the filtrate were distributed in 15-mL tubes and lyophilized for 7 hours. The lyophilized samples were stored at - 20 ^{o}C for further use.

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

Inoculum: A highly virulent isolate (PG8 HND) of the pathogen *Phaeoisariopsis griseola*, causal agent of angular leaf spot, was grown on V8 agar at 24°C for 12 days. Conidia were collected and suspended in sterile distilled water at a concentration of 2 x 10^4 conidia per mL. This inoculum was used on *Phaseolus vulgaris* variety Sprite bean plants. This variety is one of the most susceptible varieties to *P. griseola*.

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

Plant inoculations and treatment applications**:** Seeds of a highly susceptible bean variety (Sprite) were planted in pots in the greenhouse at CIAT headquarters. Seventeen-day old bean plants (15 plants per treatment) were first sprayed with, either the fungicide benlate (500 µg/ml), crude antifungal protein preparation, or sterile water. Two hours later all the plants were inoculated with *P. griseola* conidia at a concentration of 2 x 10^4 conidia per mL. The inoculated plants were placed in a humidity chamber for 4 days. They were then transferred to the greenhouse for development and symptom expression. Treatments with crude antifungal protein, benlate or sterile water continued every 2 days. Disease evaluations were conducted 7, 10, 12, 14 and 17 days after inoculation.

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

Treatment of P. griseola conidia with the protein Finotin**:** A conidial suspension of 2 x 10^4 conidia per mL was diluted 10^{-2} - 10^{-5} and examined under a microscope in order to determine the right concentration with evenly distributed and separated conidia. The dilution 10^{-4} was chosen for treatment with the antifungal protein Finotin and further examination. Twenty-µl of this conidial suspension was placed on a slide and subsequently covered with a thin layer of potato dextrose agar medium. A 200-µl crude protein preparation (the same concentration that was used to spray onto bean plants) was applied on the agar. Control slides had water instead of the antifungal protein. These were placed in Petri dishes containing wet filter paper and incubated at room temperature. Pictures of conidia were taken under the microscope at 0, 2, 7, 24, 32 and 96 hours to observe the development of individual conidia.

Results and Discussion

Effect of antifungal protein Finotin on bean angular leaf spot: The crude protein extract from seeds of *C. ternatea* CIAT 20692 showed antifungal activity in vitro on the pathogen *P. griseola* (data not shown). Plants treated with the crude antifungal protein preparation consistently developed fewer angular leaf spot disease lesions than the control plants that were treated with sterile distilled water **[Figure 1; Figure 2]**. Had a purified protein been used to control the disease on bean plants, the level of disease control would perhaps have been even higher. It is interesting to note that even a crude protein extract sprayed directly onto plants provided protection against the disease. Experiments are currently in progress to control tomato diseases under field conditions and natural infections using crude protein preparations. Tomatoes are generally susceptible to a number of diseases. The purpose of these experiments is to develop a simple disease control strategy for small producers using this antifungal protein.

Effect of antifungal protein on conidia of P. griseola in vitro**:** Conidia treated with crude protein or sterile water along with a layer of potato dextrose agar as described earlier reacted differently. Conidia failed to germinate in the presence of the antifungal protein Finotin 96 hours after treatment, whereas those treated with sterile water germinated and converted into mycelia **(Figure 3)**. Thus, one of the mechanisms of pathogen control by the protein may be by preventing fungal spore germination. However, a more detailed work on plant tissue as well as on culture is needed to fully establish the mechanism of disease control by this protein.

Effect of antifungal protein Finotin on Brachiaria foliar blight**:** Detached *Brachiaria* CIAT 36061 leaves sprayed with crude protein extract and subsequently inoculated with *R. solani* mycelial discs developed very limited or no foliar blight lesions, whereas control leaves developed severe lesions **(Figure 4)** when evaluated 72 hours after inoculation. Although we don't intend to use the antifungal protein for direct applications in the control of foliar blight disease in *Brachiaria* (it will be impractical to do so), we are exploring the possibilities of transforming some of the endophytic microbes associated with *Brachiaria* with the gene encoding the protein.

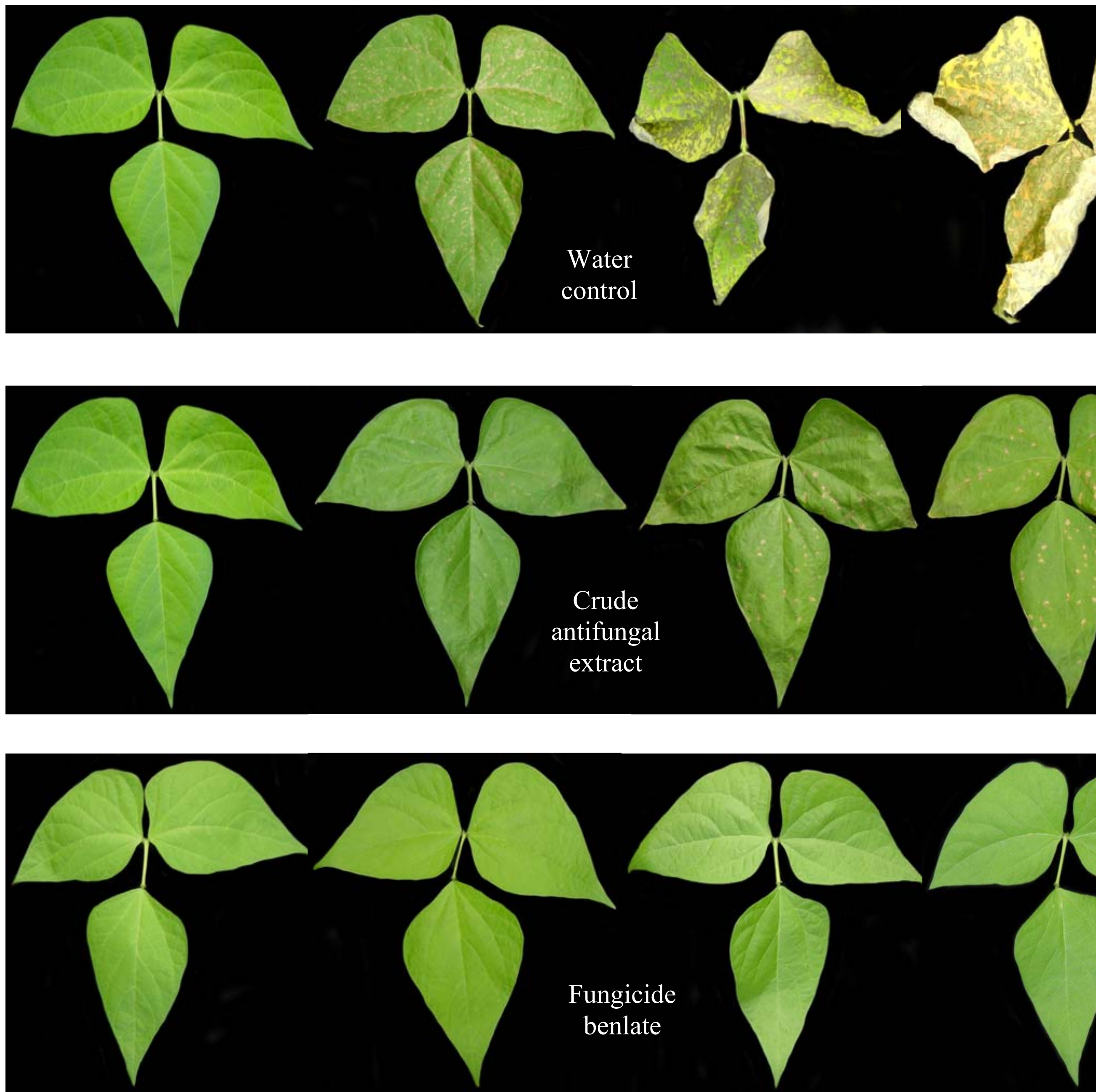

Figure 1. Treatment of bean plants with crude protein extract from seeds of *C. ternatea* CIAT 20692 against the fungal pathogen *P. griseola*, causal agent of angular leaf spot disease. Plants treated with the crude antifungal protein preparation consistently developed fewer angular leaf spot disease lesions than the control plants that were treated with sterile distilled water.

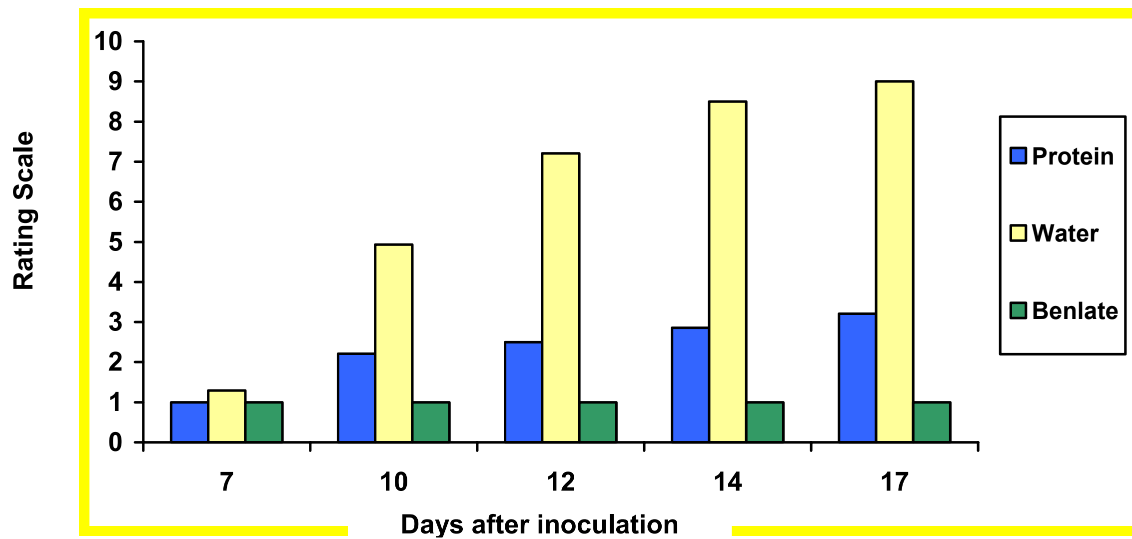

Figure 2. **Angular leaf spot disease development in artificially inoculated bean plants following treatment with crude antifungal protein preparations isolated from *C. ternatea* CIAT 20692, the fungicide benlate, or water control.**

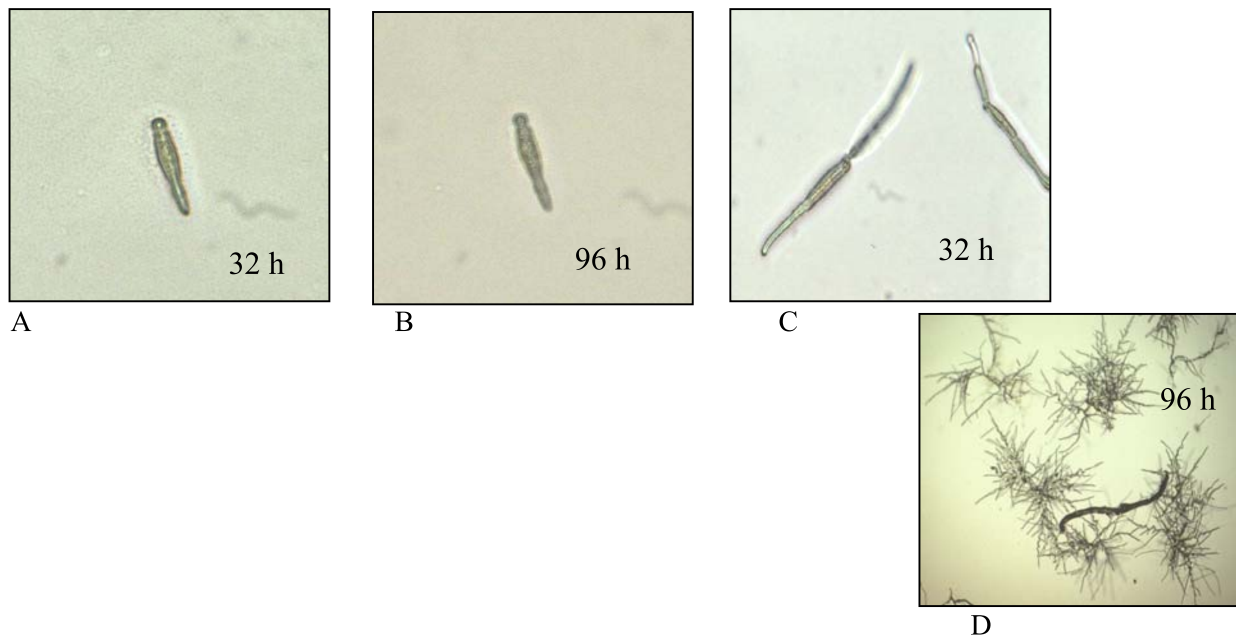

Figure 3. **Treatment of *Phaeoisariopsis griseola* conidia with the antifungal protein Finotin. Conidia failed to germinate in the presence of the antifungal protein Finotin 32 and 96 hours (A and B) after treatment, whereas those treated with sterile water germinated and grew into mycelia (C and D) .**

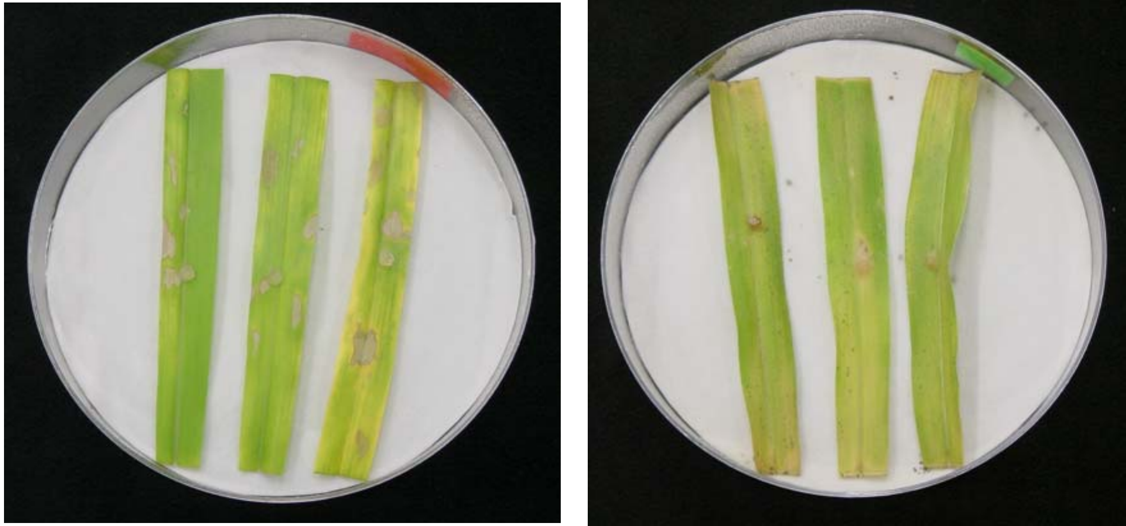

Figure 4. **Detached *Brachiaria* CIAT 36061 leaves sprayed with crude antifungal protein isolated from the forage legume *Clitoria ternatea* (L.), and subsequently inoculated with *Rhizoctonia. solani* mycelial discs (right) and control leaves (left).**

Contributors: Gustavo Segura, George Mahuku, Segenet Kelemu.

Activity 2. Association of bacteria with *Brachiaria* genotypes

Characterization of endophytic bacteria isolated from *Brachiaria*

Rationale

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

Many plant-growth-promoting bacteria (PGPB) that include a diverse group of soil bacteria are thought to stimulate plant growth by various mechanisms such as plant protection against pathogens, providing plants with fixed nitrogen, plant hormones, or solubilized iron from the soil. Three bacterial isolates 01-36062-R2, 02-36062-H4, and 03-36062-V2 were isolated from *Brachiaria* CIAT 36062 in roots, leaves and stems, respectively, that tested positive for sequences of the nifH gene (the gene that encodes nitrogenase reductase) [IP-5 Annual Report 2003]. As stated in the 20003 Annual Report, the fatty acid analysis matched the bacterium coded 03-36062-V2 with *Flavimonas oryzihabitans* at 0.887 similarity index. *F. oryzihabitans* has been described as a plant growth promoting rhizobacterium in graminicolous plants. The analysis matched isolate 02-36062-H4 with *Agrobacterium rubi* at 0.845 similarity index. The name *A. rubi* is synonymous to *Rhizobium rubi*. The match using fatty acid data of the isolate 01-36062-R2, however, was not conclusive, matching it with *Leclercia adecarboxylata*, *Klebsiella pneumoniae*, and *Enterobacter cloacae*, at 0.879, 0.841, and 0.820 similarity index, respectively. Of these, *E. cloacae* has been described as one of the dominant endophytic bacteria isolated from citrus plants (Araújo et al., 2002. Applied and Environmental Microbiology 68:4906-4914). A nitrogen-fixing endophytic strain of *Klebsiella pneumoniae* (Kp342) has been ioslted from a nitrogen-efficent line of maize (Chelius and Triplett, 2000. Applied and Environmental Microbiology 66:783-787). This strain has been described to have a very broad host range and is capable of colonizing the interior of many plants with fewer than 10 cells in the inoculum (Dong et al., 2003. Plant Soil 257:49-59). More recently, endophytic colonization and nitrogen fixation in wheat were demonstrated upon inoculation with *Klebsiella pneumoniae* strain Kp342 (Iniguez et al., 2004. Molecular Plant Microbe Interaction 17:1078-1085).

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

Materials and Methods

Marking bacterial cells for antibiotic resistance: An overnight culture of bacterial cells (strain 01-36062-R2) were plated on nutrient agar medium containing rifampicin (50 µg/ml) and incubated at 28 C for 48 h. Individual colonies which appeared on the medium were transferred on to freshly prepared medium containing the same concentration of rifampicin. The growing colonies were transferred on to freshly prepared medium containing 50µg/ml rifampicin. The same process was repeated until a mutant bacterium was obtained which grew the same on

rifampicin-containing medium at a concentration of 50 µg/ml as well as on medium containing no rifampicin. Dilution series of the mutant bacterium were plated on nutrient agar medium with and without rifampicin to determine that the mutant grew equally on both media. Growth curves of the mutant bacterium were also conducted in nutrient broth media with and without rifampicin. The growth of the rifampicin-resistant mutant strain was determined in comparison with that of the original isolate from which the mutant was derived. In addition, nested PCR amplifications were conducted on both the marked mutant and the original bacterial isolate to make sure that the *nifH* gene sequences can be detected in both.

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

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

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

Results and Discussion

Bacterial cells were re-isolated from inoculated plants (hybrid *Brachiaria* CIAT 36061; cv. Mulato) on nutrient agar medium containing rifampicin (50 µg/mL) as late as 75 days after inoculation. No bacterial cells that can grow on the rifampicin-containing media were isolated from control plants. These rifampicin-resistant bacterial colonies also tested positive for sequences of *nif*H **(Figure 1)**. In summary, we took a bacterial strain isolated from what appeared to be a nitrogen-efficient *Brachiaria* CIAT 36062 and that tested positive for *nif*H sequences, marked it for resistance to the antibiotic rifampicin at 50 µg/mL, introduced it to cv. Mulato and re-isolated it 75 days after inoculations, indicating that the bacterium was established in artificially inoculated plants of cv. Mulato.

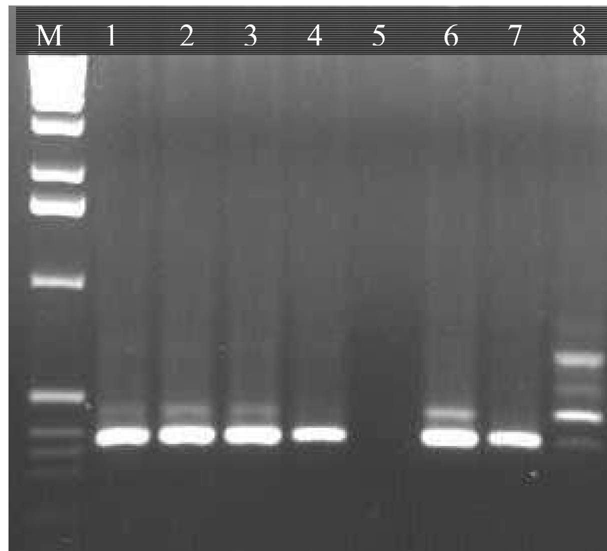

Figure 1. Nested PCR analysis of rifampicin-resistant bacterial colonies reisolated from artificially inoculated CIAT 36061 (cv. Mulato) plants, for *nif*H gene sequences. Lanes 1-4 are rifampicin-resistant independent bacterial colonies re- isolated from Mulato plants 23 days after inoculations (lanes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

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

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

Contributors: Raul Sedano, Carolina Zuleta, Segenet Kelemu

Activity 3. Publications, book chapters, conferences and workshops.

Refereed Journals

Chakraborty, S., Ghosh, R., Ghosh , M., Fernandes, C. D., Charchar, M. J. and Kelemu, S. 2004. Weather-based prediction of anthracnose severity using artificial neural network models. *Plant Pathology* 53:375 –386.

Dongyi, H. and Kelemu, S. 2004. *Acremonium implicatum*, a seed-transmitted endophytic fungus in *Brachiaria* grasses. Plant Disease 88:1252-1254.

Kelemu, S., Cardona, C. and Segura, G. 2004. Antimicrobial and insecticidal properties of a protein isolated from seeds of the tropical forage legume *Clitoria ternatea* (L.). (Abstract) *Phytopathology* 94:S50.

Submitted

Kelemu, S., Changshun, J., Guixi, H. and Segura, G. 2004. Genetic transformation of the tropical forage legume *Stylosanthes guianensis* with a rice-chitinase gene confers resistance to *Rhizoctonia* foliar blight disease. *Plant Pathology*.

Accepted

Kelemu, S., Cardona, C. and Segura, G. 2004. Antimicrobial and insecticidal protein isolated from seeds of *Clitoria ternatea* (L.), a tropical forage legume. *Plant Physiology and Biochemistry*.

Invited 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. 2004. Weather dependency of anthracnose and risk mapping. ***In:*** S.Chakraborty (editor) High-yielding anthracnose-resistant S*tylosanthes* for Agricultural systems. Chapter 20, pp 203, ACIAR, Australia.

Chakraborty, S., Fernandes, C.D., Charchar, M.J., Weeds, P.L. and Kelemu, S. 2004. *Colletotrichum gloeosporioides* diversity at centres of origin in Brazil and Colombia. In: S.Chakraborty (editor) High-yielding anthracnose-resistant *Stylosanthes* for Agricultural systems. Chapter 15, pp.165, ACIAR, Australia.

Kelemu, S., Miles, J. W. and Rao, I. M. 2004. Biotic and abiotic constraints to *Stylosanthes* production. In: S.Chakraborty (editor) High-yielding anthracnose-resistant *Stylosanthes* for Agricultural systems. Chapter 8, pp 97. ACIAR, Australia.

Conference and Workshop Proceedings

Kelemu, S. 2004. The Role of Agricultural Biotechnology in Crop Protection. Paper for the Workshop on The Ethiopian Agricultural Biotechnology Initiative, 6-8 July 2004, Addis Ababa, Ethiopia.

Other Publications (Newsletter articles)

In Press

Kelemu, S., Lascano, C., Miles, J., Rao, I. and Horne, P. 2004. Bringing an African pasture grass home: The zebra's staple, the vaquero's joy, the Bantu's hope. *Spore*.

Project Staff List

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