USGA FY1998 EXECUTIVE SUMMARY

Project Title: Transformation of Bermudagrass for Improved Fungal Resistance

Principle Investigators: Michael P. Anderson, Arron C. Guenzi, Charles M. Taliaferro, and Jeffrey A. Anderson

University: Oklahoma State University

A major disease commonly known as spring dead spot (SDS) causes significant economic damage to bermudagrass in the Southeastern United States. The causal agent for SDS throughout most of the United States is *Ophiosphaerella herpotricha* and *Ophioshpearella korrea*. Both fungal species are very active in the fall and early spring when the temperatures are cool and moisture is plentiful. Infected areas appear as regular circular patches of dead and diseased turf that generally occurs in more mature stands of bermudagrass.

The long-term goal of this project is to increase resistance in bermudagrass turf varieties to SDS through gene transformation technology. To accomplish this objective we have three main objectives: 1) to develop an efficient and reliable transformation system for bermudagrass species, 2) to identify and isolate SDS inhibitory factors, and 3) to transform bermudagrass with the genes that code for these anti-SDS factors. This report describes the current progress and results for the development of the transformation system and the isolation and characterization of antifungal factors during FY1998.

Bermudagrass Transformation

The use of high velocity microprojectiles (Biolistics™) to deliver recombinant DNA into intact plant cells has been successfully utilized to transform many grass species, and is considered the method of choice for most grass species. Immature inflorescences of the bermudagrass cultivar 'Brazos' were used to induce the formation of embryogenic callus tissue. Brazos was chosen for this experiment because it had previously demonstrated superior growth and plant regeneration potential in tissue culture. Tissue was transformed with a plasmid containing two chimeric genes of interests, the *bar* and *uidA* genes, under the control of ubiquitin promoters. The *bar* and *uidA* genes serve as a selectable marker and reporter gene, respectively. The GUS enzyme, coded for by *uidA,* can be assayed by accumulation of fluorogenic products by providing the enzyme substrate. PAT detoxifies bialaphos, the active ingredient in the herbicide 'Liberty' in the selective media, thereby allowing transgenic cells and plants to continue to grow. Six hundred and seventy one putative transgenic plants have been recovered from this experiment. We are currently evaluating these putative transformants with PCR to determine if they contain the *bar* gene (Figure 1). PCR positive plants will be characterized by Southern analysis and enzyme assays for phosphinothricin acetyl transferase during FY1999.

Anti-SDS Proteins

Living organisms to protect themselves from pathogens, or to give them a competitive advantage for nutrients, produce many antimicrobial compounds. They range from the small molecular weight antibiotics and secondary metabolites to the larger macromolecular proteins and assorted polypeptides. Recently we discovered a bacterium that was strongly and persistently inhibitory towards O. herpotricha. The bacterium was identified to the genus taxonomic level with confidence by a GC-FAME and BIOLOG technology. This bacterium secreted many proteins into the extracellular matrix. Dialysis of the extracellular excretions suggested that the antifungal factor was a protein. Purification of the antifungal proteins on anion exchange, hydroxyapatite, and Mono Q chromatography resulted in the isolation of a 36 kD protein that is most likely expressed as multiple isoforms (Figure 2). Analysis of the purification results suggested that there are at least two distinct antifungal factors antagonistic against O. herpotricha secreted by the bacterium. Experiments are in preparation to identify, sequence, and characterize the 36 kD protein.

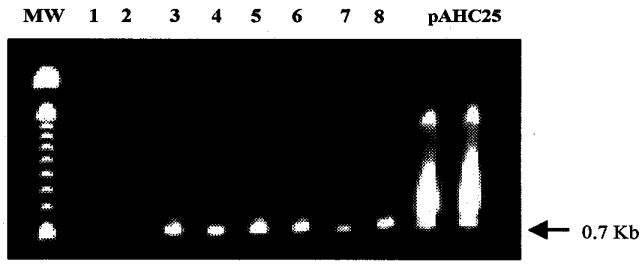

Figure 1. PCR analysis of putative bermudagrass transformants. Legend: Lane 1-Brazos; Lanes 2 to 8-putative transformants; pAHC25-plasmid used for transformations. Transformants should contain the 0.7 kb band that represents the coding sequence of the *bar* gene. In this gel, plant 2 is not transgenic and plants 3 through 8 are positive for the bar gene.

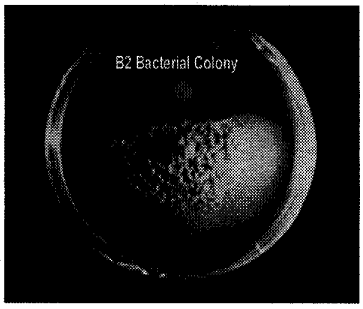

Figure 2. Inhibition of *O. herpotricha* by bacterial colony B2. One-half of the plate was inhibited by a single bacterial colony.

USGA FY1998 ANNUAL REPORT

Project Title: Transformation of Bermudagrass for Improved Fungal Resistance

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BACKGROUND INFORMATION

A major disease commonly known as spring dead spot (SDS) causes significant economic damage to bermudagrass in the Southeastern United States. Effected parties include owners and operators of golf courses, sport fields and residential units. The causal agent for SDS throughout most of the United States is *Ophiosphaerella herpotricha* and *Ophioshpearella korrea* (Hagan 1989). Both fungal species are very active in the fall and early spring when the temperatures are cool and moisture is plentiful. The fungi are ectotrophic, meaning that they live on the outside of roots and crowns while sapping nutrients from the plant. Infected areas appear as regular circular patches of dead and diseased turf that generally occurs in more mature stands of bermudagrass (Figure 1).

Figure 1. *O. herpotricha* infected bermudagrass. (Martin and Tisserat research plots)

High quality vegetatively propagated bermudagrasses are the most susceptible to SDS. These varieties are sexually isolated and cannot be bred for increased SDS resistance by conventional means. The most promising avenue for introducing SDS resistance into these varieties is through the genetic transformation of bermudagrass with genes that specifically attack the fungus.

Development of a reliable and efficient transformation system is a major objective of this proposal. A plant transformation system is essential for the introduction, identification, and functional analysis of agriculturally important genes. When transformation techniques are optimized, manipulation of genes influencing herbicide tolerance, insect resistance, disease resistance, drought, salinity, and low temperature tolerance can proceed. Bermudagrass biotechnology lags far behind other economically important grass species because of the lack of a transformation system. At OSU, we have already developed an efficient bermudagrass tissue culture system. However, preliminary attempts at obtaining transformed plants using the Biolistic approach have not been successful (A.C. Guenzi, C.M. Taliaferro, and M. P. Anderson, unpublished). A focused effort is now being undertaken to overcome this technological barrier.

Three methods are currently utilized for the transformation of plant cells; Biolistics™ (Sanford *et al.*, 1987), *Agrobacterium* infection (Grimsley, T. *et al.* 1987) and electroporation (Fromm, P. *et al.* 1985). Although these methods differ in the mechanism by which recombinant DNA is introduced into the plant cell, they are fundamentally the same in that they are capable of introducing foreign DNA into a living cell. There are advantages and disadvantages to each of the three techniques mentioned above. As of this time, Biolistics (the gene gun) is considered the method of choice for transforming grass species. Recently the use of *Agrobacterium* for transforming grasses has been demonstrated and shows considerable promise for grass species such as rice and corn. However, this method has not been tried on bermudagrass. Electroporation is the most speculative of the three methods. It has the advantage that you can recover transgenic plants directly from meristematic tissues without the need of a tissue culture-based plant regeneration system. Electroporation of intact vegetative tissue seems ideal for bermudagrass given its propensity for vegetative reproduction from meristematic nodes. During the past year, we have initiated experiments to evaluate Biolistics and electroporation for bermudagrass transformation. Ms. Yan Zhang was hired in May 1998 as a graduate research assistant to conduct this research in partial fulfillment of the requirements for a Ph.D. in Plant Science. One-half of her assistantship is being paid for by the USGA grant and the remainder by the Oklahoma Agricultural Experiment Station. The earlier tissue culture research and initiation of the Biolistics research was conducted by Ms. Melissa Maxwell as part of the requirements for a M.S. in Plant and Soil Sciences. Her graduate research assistantship was provided by the Oklahoma Agricultural Experiment Station.

The other major objective of this proposal is the isolation of antifungal factors that are antagonistic against *O. herpotricha*. Living organisms to protect themselves from pathogens, or to give them a competitive advantage in the competition for nutrients, produce many antimicrobial compounds. They range from the small molecular weight antibiotics and secondary metabolites to the larger macromolecular proteins and

assorted polypeptides. For example, insects produce a polypeptide called Cecropin A consisting of 37 amino acids that are strongly antimicrobial. Cecropin A functions by puncturing the cell membrane of pathogenic bacteria. This report details the discovery of a bacterium that is strongly fungistatic, and the isolation of a secreted antifungal bacterial protein that is antagonistic against *O. herpotricha*.

Many proteins are currently known to express antifungal activity. Probably the most widely utilized and studied antimicrobial proteins are chitinases (Graham and Sticklen 1994) and glucanases (Simmons 1994). Chitinases and glucanases are expressed as multiple isozyme each with a defined function and specificity. In addition to their antimicrobial role, chitinases have been shown to be overexpressed during plant acclimation to cold temperature, suggesting a role in protecting plants from freeze induced damage. Indeed, chitinases are thought to prevent damaging ice recrystalization during freeze thaw cycles common in temperate climates. Mark Gatschet isolated a specific chitinase in bermudagrass crown tissues that increased several-fold during cold acclimation, especially in a resistant variety Midiron (Gatschet, Taliaferro et al. 1996). Moreover, Baird and Martin documented an intriguing positive correlation between SDS resistance and cold tolerance in many bermudagrass varieties (Baird and Martin 1996), suggesting a common mechanism between these two stresses. Those varieties that are more cold tolerant appear to be more resistant to the fungal disease. The duel role of chitinases in disease resistance and cold acclimation may help to explain this intriguing relationship. The search for chitinases that are antagonistic against *O. herpotricha* is an objective of this work.

PROGRESS AND RESULTS

The long-term goal of this project is to increase resistance in bermudagrass turf varieties to SDS through gene transformation technology. To accomplish this objective we have divided the project into three main objectives: 1) to develop an efficient and reliable transformation system for bermudagrass species, 2) to identify and isolate SDS inhibitory factors, and 3) to transform bermudagrass with the genes that code for these anti-SDS factors. This report describes the current progress and results for the development of the transformation system and the isolation and characterization of antifungal factors during FY1998.

Bermudagrass Transformation Systems

Gene Construct. All transformation methods were evaluated utilizing derivatives of the ubiquitin promoter gene expression cassette developed in Peter Quail's laboratory (USDA-ARS, Plant Gene Expression Center, Albany, CA). This promoter induces a very high level of gene expression in grass species. All experiments were conducted using the plasmid pAHC25 (Figure 2). This plasmid contains two chimeric genes of interests, the bar and uidA genes, under the control of ubiquitin promoters. Each gene constructs contain the ubiquitin (*Ubi1*) promoter, *Ubi1* intron, coding sequences for *uidA* ß-glucuronidase (GUS)] or *bar* [phosphinothricin acetyl transferase (PAT)] and the NOS termination sequence from *Agrobacterium tumefacians*. The *bar* and *uidA* genes serve as a selectable marker and reporter gene, respectively. The GUS enzyme coded for by *uidA* can be assayed by accumulation of fluorogenic products by providing the enzyme

substrate. PAT detoxifies bialaphos, the active ingredient in the herbicide 'Liberty' in the selective media, thereby allowing transgenic cells and plants to continue to grow and divide.

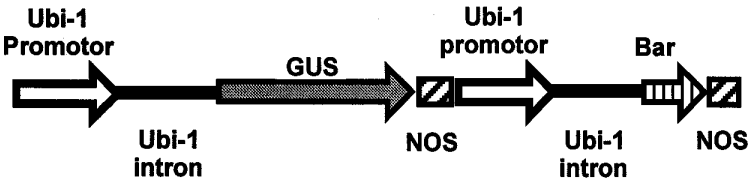

Figure 2. Chimeric gene construct used for Biolistic and electroporation transformation experiments. Legend: Ubi-1 promoter-open arrows; Ubi-1 intron-solid black line; coding sequence for ß-glucuronidase (GUS)-gray arrow; NOS termination sequence from *Agrobacterium*-diagonal hatched box; coding sequence for phosphinothricin acetyl transferase-vertical striped arrow. All symbols represent the relative size of DNA sequences illustrated.

Putative transgenic plants were analyzed at the molecular level by isolating genomic DNA and utilizing it as a template for PCR reactions with primers designed to amplify the *bar* gene. PCR products were separated and analyzed by agarose gel electrophoresis.

Biolistics. The use of high velocity microprojectiles (Biolistics™) to deliver recombinant DNA into intact plant cells has been successfully utilized to transform many grass species, and is considered the method of choice for most grass species. Immature inflorescences of the bermudagrass cultivar 'Brazos' were placed on callus induction medium (Table 1) for 4 to 7 days and bombarded with 1 µm diameter gold particles coated with pAHC25. Brazos was chosen for this experiment because it had previously demonstrated superior growth and plant regeneration potential in tissue culture (M. Maxwell, C.M. Taliaferro, and A.C. Guenzi, in preparation). Tissue was grown on non-selective medium for 1 week post-bombardment and then transferred to selection medium containing 1 mg L^{-1} bialaphos for six weeks (Table 1). Tissue growing vigorously on selection medium was transferred to plant regeneration medium containing the same concentration of bialaphos (Table 1). Shoots regenerated during the following three months were placed on rooting medium containing 3 mg L^{-1} bialaphos. Healthy regenerated plants were transferred to soil and maintained in the greenhouse. We have now recovered 671 putative transgenic plants from this experiment. We are currently evaluating these putative transformants with PCR to determine if they contain the *bar* gene (Figure 3). PCR positive plants will be characterized by Southern analysis and enzyme assays for phosphinothricin acetyl transferase.

Table 1. Tissue culture media used in the recovery of putative transgenic bermudagrass.

Step	Basal Medium	Plant Growth Regulators	Bialaphos Concentration
Callus Initiation	Murashige & Skoog, 60 g L^{-1} sucrose	1.5 mg L^{-1} 2,4-D	None
Callus Maintenance	Murashige & Skoog, 60 g L^{-1} sucrose	1.5 mg L^{-1} 2,4-D	None
Selection of Transgenic Tissue	Murashige & Skoog, 60 g L^{-1} sucrose	1.5 mg L^{-1} 2,4-D	1 mg L^{-1}
Shoot Regeneration	Murashige & Skoog, 30 g L^{-1} sucrose	1 mg L^{-1} Zeatin 1 mg L^{-1} IAA	1 mg L^{-1}
Root Regeneration	One-half strength Murashige & Skoog, 30 g L^{-1} sucrose	None	3 mg L^{-1}

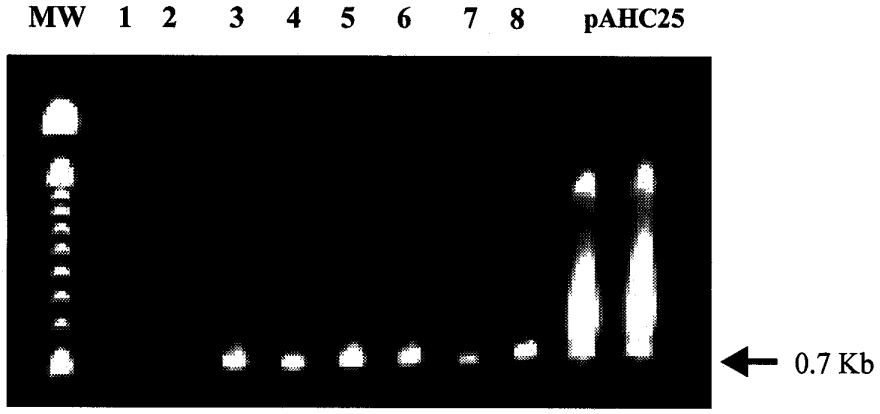

Figure 3. PCR analysis of putative bermudagrass transformants. Legend: Lane 1-Brazos; Lanes 2 to 8-putative transformants; pAHC25-plasmid used for transformations. Transformants should contain the 0.7 kb band that represents the coding sequence of the *bar* gene. In this gel, plant 2 is not transgenic and plants 3 through 8 are positive for the bar gene.

Electroporation of Intact Plant Tissues. Electroporation is the most widely used transformation technique for microbial and mammalian cells. Electroporation consists of subjecting cells to an electric field, which results in "pores" being formed in the cell membrane. Pore formation is rapidly reversible and the cell survives. A cell which is immersed in DNA and subjected to electroporation will take-up large quantities of DNA from the solution. This is a very exciting because it bypasses the problems usually associated with the tissue culture regeneration systems. In bermudagrass, as in all grass species, only certain cultivars respond well to tissue culture. Furthermore, regeneration through tissue culture frequently introduces genetic changes that must be eliminated. This technique has the potential to allow us to improve all current bermudagrass cultivars, especially those high quality vegetatively propagated and sexually isolated bermudagrasses that are in demand. Direct transformation through genetic engineering may be the only way of improving these high quality bermudagrass cultivars. Our objective is to develop a direct transformation procedure based on bermudagrass' ability to reproduce from aggressively growing stolons. Our experimental approach is to sample nodes from stolons, surface sterilize the tissue, place the nodes into an electroporation chamber, transform with pAHC25, and then induce plant regeneration in the presence of bialaphos. We have now established a procedure for surface sterilizing nodal tissue. It consists of: 1) a 12 h soak in 0.4 g L^{-1} Terrachlor; 2) a 10 min. soak in 0.04% $HgCl_2$; and 3) a 5 min. soak in 20% Clorox. This treatment eliminates fungal contamination and results in approximately 40% of the nodes being able to regenerate into plants. The growth inhibition of nodes by bialaphos was also investigated. Nodes were sampled from Tifgreen, surface sterilized, and placed on medium consisting of one-half strength Murashige & Skoog basal salts and 0.2 g L^{-1} Phytagel with varying concentrations of bialaphos. Results from this experiment are summarized in Table 2. After 14 days in culture, 38% of the nodes developed into plants in the absence of bialaphos. All concentrations of bialaphos inhibited the regeneration from nodes, however 2 mg L^{-1} with a 14-day exposure appears to be sufficient selection pressure to prevent non-transformed tissue from developing into plants.

Table 2. Growth inhibition of bermudagrass nodes by bialaphos

	Bialaphos Concentration (mg L^{-1})						
Day	0	1	2	3	4	6	8
	-------% of Nodes Developing into Plants --------						
7	24*	14	5	1	0	0	0
14	38	9	0	0	0	0	0

* Mean value for four replications with 20 nodes per replication.

Isolation of anti-SDS Inhibitory Factors

Isolation of antifungal factors took two approaches. The first involved the discovery and identification of microorganisms inhibitory towards *O. herpotricha*, and the isolation and characterization of the antifungal inhibitors. The second approach involved the isolation of potential antifungal chitinases antagonistic against *O. herpotricha*. In this approach, we will use resistant bermudagrass and other plant species as tissue sources.

Anti-SDS bacteria. The discovery of anti-SDS bacteria began with the development of the antifungal assay, a technique for the isolation of factors antagonistic against *O. herpotricha*. After much experimentation we settled on an assay where we inoculated the center of a 150 mm PDA agar plate (Figure 4) with *O. herpotricha* (generously donated by Ned Tisserat). As the fungus grows outward from the central well, it forms a hyphal mat covering about 2/3 of the agar surface. At this time, we added individual fractions containing our test samples to the perimeter of the plate. As the fungal hyphae continue to extend toward the edges, they encounter the antifungal compounds and their growth is slowed or stopped. The assay generally takes from 2 days to one week to develop to the point where you can observe the cavity-like inhibition zones. The degree of inhibition is reflected in the size of the inhibition zone. The major disadvantage of this assay is that it takes so long to develop and results are at best semiquantitative. However, the primary advantage is that results truly reflect fungal growth inhibition.

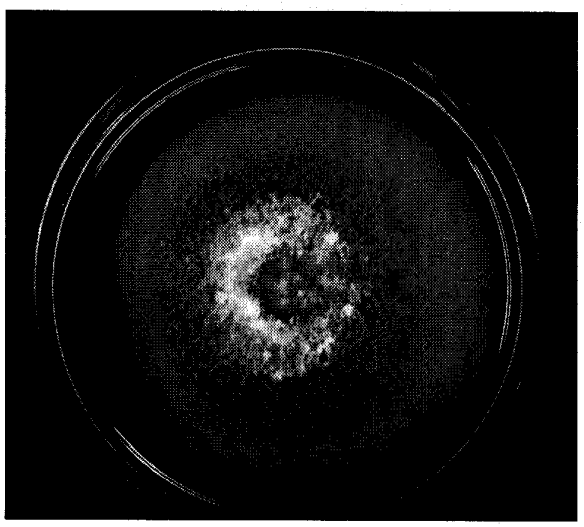

Figure 4. A typical example of the antifungal assay. Inhibition zones are evident in the lower left and upper right of the plate.

While developing the assay, we chanced upon a couple of plates that contained bacterial colonies inhibitory to *O. herpotricha*. Several of the colonies showed extensive and persistent inhibition for many months (Figure 5). One of these bacterial colonies B1 was subcultured until single bacterial colonies were obtained. All subcultures of colonies B1 showed equal fungal inhibitions. B1 was selected for the isolation of the potent antifungal factors based on the degree and persistence of the inhibition observed in our antifungal plates. The single colony of bacteria inhibited the growth of the fungus for many months. Since then, at least 9 other microbial cultures were found to inhibit *O. herpotricha*.

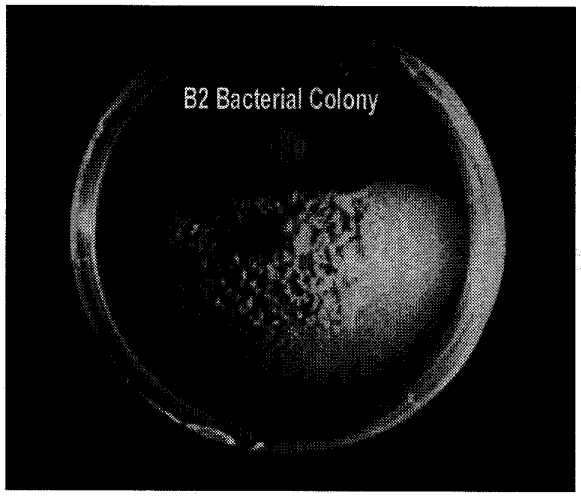

Figure 5. Inhibition of *O. herpotricha* by bacterial colony B2. One-half of the plate was inhibited by a single bacterial colony.

A national diagnostic laboratory using gram stain, GC FAME and Biolog techniques tentatively identified the B1 bacterial strain. Comparing BI microbial test characteristics with thousands of other bacterial species characteristics in an extensive database completed identification. Identification was significant at the genus level, but conflicted at the species level, where the significance of the match was not particularly good.

Examination of the inhibition zone showed a darkened area extending across the hyphal front. Within this darkened zone, the fungi appear to be excreting material in microscopic globules (Figure 6). In untreated areas, the he excretions were not evident. These globules may represent seepage caused by fungal cell rupture, or they may contain antimicrobial secretions excreted by the fungus which function in fungal defense. The interplay between the fungus and the bacteria is probably complex.

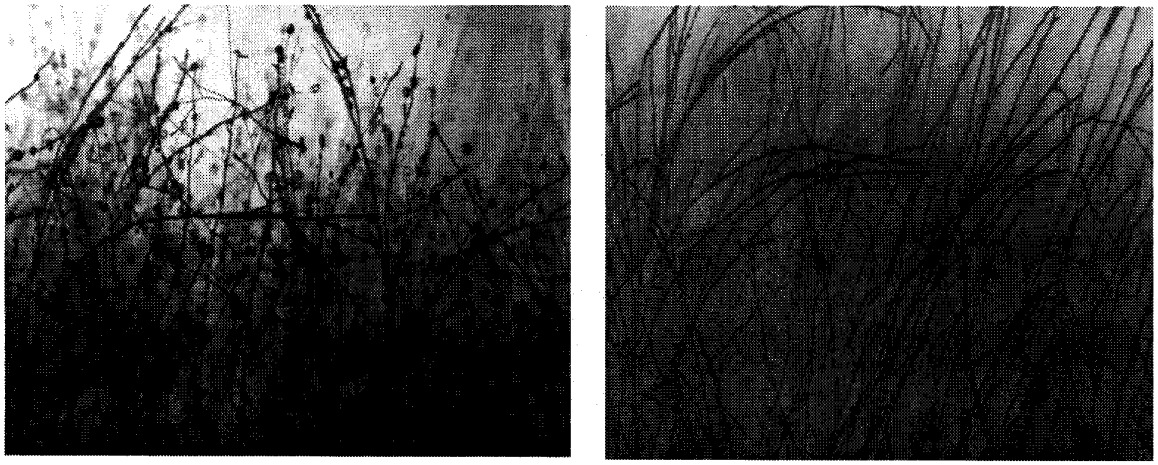

Figure 6. The inhibition zone under the microscope at approximately 45 power treated (left) and not treated (right) with antifungal compounds.

Up to this point, inhibition was only examined in our agar antifungal plates, so we wondered if the antifungal factors could be effective in a tissue environment. We grew the bacteria in liquid media and inoculated sterilized oats with the fungus. After allowing several days for the fungus to establish itself, we either inoculated the flasks with liquid media alone or with bacteria in liquid media. The oat hulls after several weeks inoculated with the media alone showed extensive fungal growth, while the flasks with the bacteria plus media were without the fungus. Moreover, limited growth of *O. herpotricha* was present even after 10 months after bacterial inoculation, indicating that the bacteria are persistent in competition with the fungus in a tissue source (Figure 7).

Figure 7. Growth of *O. herpotricha* on sterilized oats laced with (left flask) or without (right flask) antifungal bacteria.

Purification of antifungal factor. Purification of the antifungal factor is an essential step leading to identification and characterization of the inhibitory factor. Purification is often a very difficult step especially since little is known concerning the chemical nature of the antifungal factor. Knowledge of these characteristics is essential for developing a rapid efficient isolation scheme. Moreover, our dependence on our slow but effective antifungal assay made it difficult to quickly purify the antifungal factor. We tried to develop more rapid assays, but because of peculiar features of the fungus, all of these turned out to be less reliable. The use of the slow assay also caused concern about denaturation of the antifungal factor during extended purification. Steps were taken to ensure that denaturation was minimized.

The bacterial cells from B1 were used to inoculate liquid media. The media was grown for two days until the flask was noticeably turbid. Pelleting the cells by centrifugation and filtering through a 0.22 micron filter produced a cell free filtrate. After centrifugation and filtration, the filtrate contained the extracellular bacterial secretions. To determine if the antifungal factor was a large macromolecule protein, we dialyzed the filtrate through an 8,000 MW cut-off membrane. Antifungal activity was retained after dialysis indicating that the antifungal factors had molecular weights above 8,000. Concentration of antifungal compounds in filters with a cutoff of 50,000 molecular weight showed extensive activity, also suggesting a high molecular weight. This finding was significant in that it increased the probability that the antifungal factors were proteins. The gene coding for the protein could theoretically be used to transform bermudagrass.

Characterization of the purification parameters took some time. Chromatographic characterization involves studying the binding and elution properties of the antifungal protein on a variety of chromatographic matrixes. At each stage of the purification some proteins were bound to the chromatography column and then selectively eluted into separate fractions. By using a variety of matrixes, a biochemist can retain the desired proteins while eliminating all the others. After chromatography, fractions are collected and analyzed for antifungal activity using the antifungal assay. SDS-PAGE electrophoresis was used to analyze individual proteins in each fraction. Eventually our goal was to eliminate all proteins without antifungal activity and retain those with antifungal activity. After several purification stages we finally met our goal of purifying an antifungal protein to homogeneity. In our preliminary characterization, we used anion exchange, hydroxyapatite, concavalin A, and gel filtration chromatography. Our final purification technique used anion exchange, hydroxyapatite chromatography to purify the proteins to homogeneity.

Purification began by growing the bacteria in liquid media, followed by centrifugation and filtration to remove the bacterial cells and retain the extracellular proteins. The bacterial filtrate was loaded onto an anion exchange column and eluted off with a salt gradient into 20 fractions. Each fraction was analyzed by SDS PAGE for protein content and for antifungal activity (Figure 8a and b). The results showed that many proteins were extruded into the media by the bacteria. When fractionated by the anion exchange chromatography the fractions 1-4 and 9-20 all contained antifungal activity, indicating that there were at least several proteins or protein isoforms present in the extracellular secretions with antifungal activity against *O. herpotricha*.

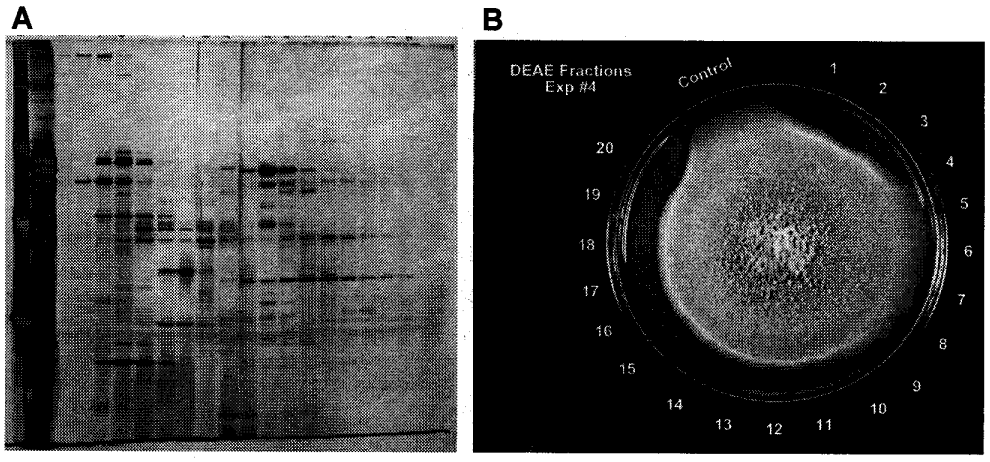

Figure 8. A) SDS PAGE gel of MW markers (lane 1), concentrated bacterial proteins (lane 2) and anion exchange fractions in the following order 1-8, 9 and 10 combined, 11-12 combined, and 13-20. B) Antifungal assay of anion exchange fractions plus a salt control.

Antifungal fractions 13-14 were especially interesting since they contained a prominent band above 50,000 MW. Previous data indicated that at least one antifungal factor should have a molecular weight above 50,000. These fractions were combined and loaded onto a hydroxyapatite column and protein was eluted with phosphate buffer gradient. Much of the antifungal activity did not bind to the column, but flowed through. Some activity was found in fractions 1-6 of the HTP column, but not as much as in the unbound fractions. The flow-through was then loaded onto a higher resolution mono Q column and fractionated with a salt gradient (Figure 9).

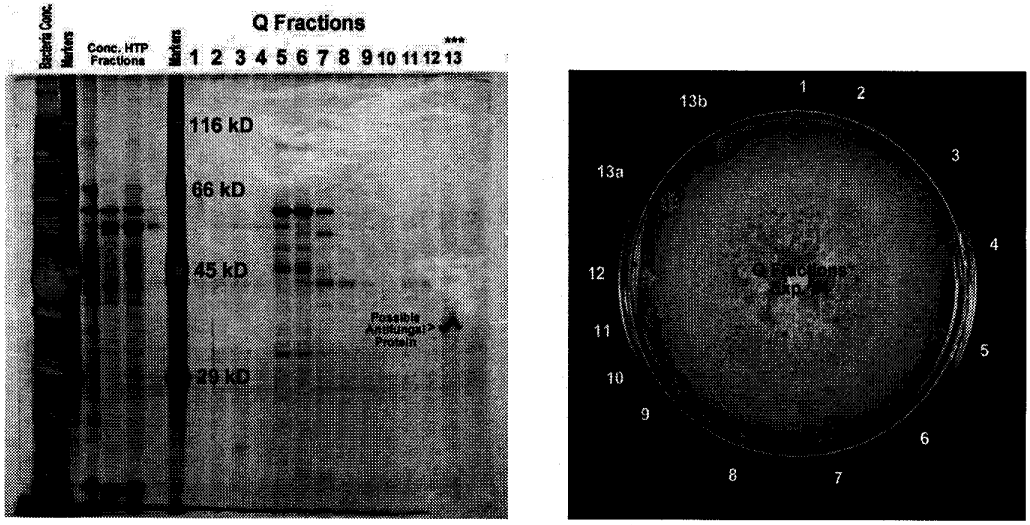

Figure 9. Q column fractionation of flow through from HTP column. SDS-PAGE gel of Q column fractions with the corresponding antifungal assay. Note the antifungal assay has some contaminating bacteria from the unsterilized chromatographic fractions. These bacteria are sometimes present in our chromatographic fractions and are seldom

if ever antifungal towards O. herpotricha. *** Indicates very active against O herpotricha.

Fraction number 13 showed the highest activity towards O. herpotricha. This fraction was nearly homogeneous for a prominent band with a molecular weight around 36 KD. These results indicated that there is a high probability that this protein has strong antifungal activity. Fraction 13 was rechromatographed on the Q column (Figure 8) using a shallower gradient for better resolution. SDS PAGE gel loaded with fractions from the second mono Q chromatography step showed a band at 36 kD extending across 10 fractions. This indicated that the 36 kD protein probably comes in several isoforms. The multiple isoform theory may explain the why many fractions were found in the first anion exchange fractionation (Figure 6a) (fractions 9-20 contain a 36kD protein). However, it cannot explain the antifungal activity in anion exchange fractions 1-4, which supports the conclusion that there are at least two antifungal proteins secreted by B1. It will be interesting to see if the multiple forms differ in antifungal activity or if only one form shows activity.

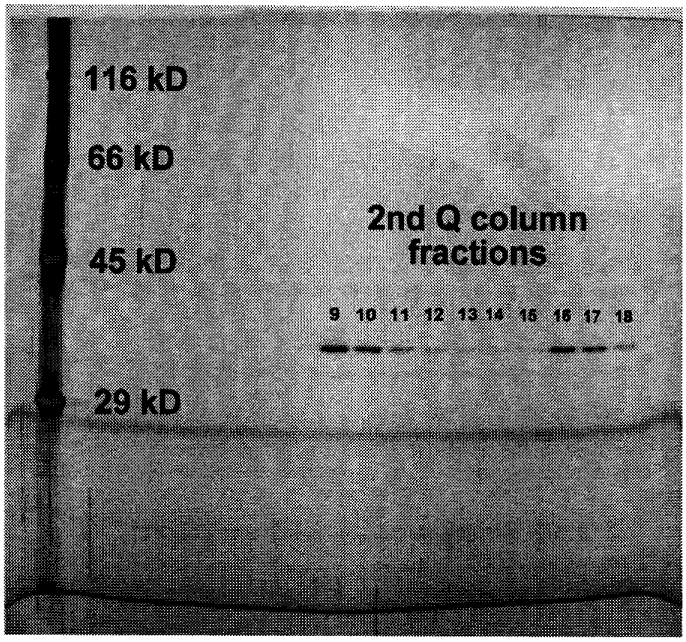

Figure 10. SDS PAGE electrophoresis gel of the second fractionation on the mono Q column.

Summary of results from the antifungal bacteria work:

1) At least 9 microorganisms antagonistic against *O. herpotricha* were discovered.
2) The bacteria B1 was identified with confidence to the genus level.
3) The antifungal factors are most likely proteins.
4) The antifungal activity is stable in the extracellular environment for many months.
5) There are multiple antifungal factors excreted into media by the bacteria.

6) One very promising 36 kD protein with several isoforms was purified from the media by anion exchange, hydroxyapatite and mono Q chromatography.

Development of Techniques for Analyzing Chitinase Expression in Bermudagrass

Chitinases are known to be antagonistic against many pathogens. They act by degrading the chitin contain cell wall of fungi and bacteria. The objective of this study was to isolate chitinases antagonistic against O. herpotricha. The discovery of the antifungal bacteria diverted our attention from our search for antifungal chitinases. However, we still want to pursue our search for antifungal chitinases in bermudagrass or other plant tissue sources, so we continued with the development of techniques to isolate and characterize these chitinases.

The first step in our study involved the development of techniques to characterize chitinase activity in the crown tissues of bermudagrass roots. These included:

1) Effective methods for extracting chitinase enzymes from tuber-like crown tissues of bermudagrass. To do this we used potato tuber as an abundant and readily available tissue source, and food preservation technology to eliminate effects of oxidative metabolism during the extraction process. Our extraction protocol virtually eliminated the protein cross-linking usually associated with polyphenol oxidase oxidation of phenolics.
2) Sensitive methods for assaying chitinase activity in small bermudagrass crown tissues. To do this we utilized tritiated chitin as a substrate for our chitinase assay. We also modified the technique so that we could process many samples at a time.
3) Effective methods for separating chitinase isozymes. In order to search for individual antifungal chitinases we need to separate the isozymes at high resolution. We originally utilized a granulated gel matrix technique developed by Radola ((Radola 1973)). This method was effective in separating basic and acidic isozymes. However, with the purchase and availability of a high-resolution chromatography system by the Biochemistry Department we may switch to high-resolution anion exchange chromatography.
4) Once the antifungal chitinases are separated, we will purify them to homogeneity. To do this we synthesized a chitin affinity column out of crab shell chitin. This column was effective in purifying chitinases to homogeneity in a single step.

The objective of this work with chitinases is to purify selective antifungal chitinases from resistant bermudagrass, or from another plant tissue source rich in antifungal chitinases. The development of the above techniques will allow us to extract, assay, separate isozymes and purify antifungal isozymes rapidly and efficiently. Experiments are in progress as of this time to accomplish this objective.

RESEARCH GOALS FOR FY 1999

Transformation Research

1. Conduct in-depth biochemical and molecular analyses of the 671 putative transgenic plants recovered during the past year by use of Biolistics.

2. Establish electroporation parameters required for maximum uptake of plasmid DNA into cells of bermudagrass nodes.

3. Attempt to produce transgenic bermudagrass by electroporation of nodes.

Antifungal Bacteria Research

1. Determine if the purified 36 kD protein has antifungal activity against *O. herpotricha* and *korrae*.

2. Sequence the N terminal end of the 36 kD protein.

3. Identify the protein based on amino acid sequence.

4. Isolate 2 to 5 other antifungal proteins from the extracellular bacterial filtrate.

5. Pull out and sequence the full-length gene using cDNA library or PCR technique.

6. Extract, purify, sequence and test for antifungal activity chitinase isozymes from resistant bermudagrass crown tissues, or other plant sources rich in chitinase activity.