USGA GREEN SECTION ANNUAL REPORT FOR 2000

Title: Transformation of Bermudagrass for Improved Fungal Tolerance

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EXECUTIVE SUMMARY

Spring dead spot is a major bermudagrass disease in the Southern United States. The causal agent is *Ophiosphaerella herpotricha* and *Ophiosphaerella korrea.* The most susceptible varieties include many high quality vegetatively propagated bermudagrasses. Improvement in resistance in these sexually isolated lines requires the development of genetic transformation protocols. The objectives of this work is to first develop an efficient bermudagrass transformation system, second, to isolate genes and/or factors with specific activity against the causal agent of spring dead spot and other fungal diseases, and third, to utilize these agents or genes to increase resistance to fungal diseases.

Transformation of the bermudagrass variety Brazos was performed using the Biolistics™ bombardment with DNA containing an expression cassette that included the bar gene coupled to a constitutive ubiquitin promoter. The bar gene codes for an enzyme that metabolizes the herbicide Liberty ™ (bialaphos). After bombardment, Liberty was used in the selection media to screen for resistant transgenic tissues. Exhaustive evaluation of all 671 putative transformants indicated that none of these plants were transgenic. New transformation experiments utilizing higher concentrations of bialaphos have been initiated. A gene construct with a hygromycin resistance gene is also being assembled to be evaluated for bermudagrass transformation. Screening of elite lines in the bermudagrass breeding program has identified a turf-type genotype (PCR-58) with tissue culture and plant regeneration capabilities similar to Brazos. PCR-58 will be utilized in future transformation experiments.

A microorganism with potent activity against *O. herpotricha* was recently isolated and identified. Activity of the antifungal factor was stable *in vitro* over a six-month period. The microorganism was identified to the genus level using GC-FAME, Biolog, and 16S ribosomal sequence analysis. Activity was due to the secretion of several potent antifungal factors that were stable under the harshest conditions. Isolation of the factor using preparative SDS-PAGE and reverse phase chromatography was successful. In addition, a new purification procedure was developed to permit the purification of large quantities of the bioactive substance. The active compounds were identified through mass spectroscopy and other analytical techniques. Currently we are interested in developing either the microorganism or the antifungal factor as a biocontrol agent. We also isolated eight additional endogenous microorganisms with antifungal activity from bermudagrass crown tissues. The microorganisms were identified using their 16S ribosomal sequence. Characterization of the active components is in progress. In order to study the basic biology of spring dead spot we developed a sensitive assay that is capable of quantitatively determining the relative infection state of bermudagrass crown tissues. We hope to use the assay to track the progress of infection in the field throughout the year.

A functional genomic analysis was initiated during the past year to study gene expression profiles between tolerant (OkSU 91-11) and susceptible (Jackpot) varieties when infected with *O. herpotricha.* Suppression-substractive hybridization (SSH) libraries were created to identify genes that are up or down-regulated in response to fungal infection. These clones are currently being sequenced and they will be utilized to study bermudagrass defense responses to *O. herpotricha.*

PROJECT OVERVIEW

A major disease known as spring dead spot (SDS) causes significant economic damage to bermudagrass in the Southeastern United States. The causal agent in the USA is *Ophiosphaerella herpotricha* and *Ophioshpaerella 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.

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 avenues for introducing SDS resistance into vegetatively propagated bermudagrass varieties are through genetic transformation with genes that specifically attack the fungus. Bermudagrass biotechnology lags far behind other economically important grass species because of the lack of a transformation protocol. Development of an efficient transformation system is necessary to introduce antifungal and other genes important for bermudagrass improvement. At OSU, we have already developed an efficient bermudagrass tissue culture system. A focused effort is now being undertaken to overcome the technological barriers involved in bermudagrass transformation.

There are significant problems in working with spring dead spot. The first problem is that it takes 1 to 2 years to establish infection in the field, making it very difficult to obtain significant results on a short-term basis. The second problem is that resistance to SDS is only manifest in the field, and not under controlled environmental conditions. Infection of bermudagrass under growth chamber conditions would be advantageous because it would allow us to infect the plants anytime during the year, not just in the fall. However in order to do this we would have to better understand the basic biology of the fungal-plant interaction. Ned Tissaret at KSU has encountered similar problems, and we have discussed some approaches to resolve these differences.

Previously, incubation of infected plants under continuos cool temperatures (12 to 15°C) in the growth chamber has failed to induce resistance. One possible reason for the absence of resistance in growth chamber infected plants may be due to the lack of appropriate environmental queues. Queues such as low temperatures, day length and light quality may influence the induction of SDS resistance mechanisms. Exposure to low temperatures may induce tolerance to SDS in resistant types. Tolerances to SDS and cold temperatures have been shown to be positively correlated. Resistance to cold usually means resistance to SDS. Low fall temperatures may stress crown tissues and make them more susceptible to damage by opportunistic fungi. We yet to expose our infected plants to a significant cold stress (4°C for 4 days), and this could be the reason why we have yet to induce resistance in growth chamber grown plants.

The second possibility for the discrepancy between growth chamber and field may grown plants may be due to the differences in endogenous microorganisms. Endogenous microorganisms may be an important factor to SDS resistance. Last year, we identified 8 endogenous or crown associated organisms with anti-SDS activity. Of the 8 organisms only one was from the resistant line. This refutes the hypothesis that differences in endogenous microorganisms contribute to resistance to SDS. However, one problem with last year's data may have been in the surface sterilization of the crown tissues. We used a recommended procedure, but were unsure of its effectiveness in eliminating all surfaces or crown associated

microorganisms. This year we will repeat the endogenous microorganism isolation procedure with better surface sterilization technique to make sure we are looking at endogenous organism rather than rhizosphere associated organisms.

The isolation of antifungal factors may lead to development of resistant transgenic bermudagrass, or the development of an effective biocontrol agent. Many compounds and proteins are currently known to express antifungal activity. Probably the most widely utilized and studied antimicrobial proteins are the chitinases and glucanases. In an attempt to characterize these proteins we isolated and separated bermudagrass crown chitinases in infected and noninfected tissues and then determined their antifungal activity against *O. herpotricha*. We have yet to find any chitinase isozyme that possess direct anti-SDS activity. However, reports in the literature suggest that chitinases and glucanases work together synergistically, so we may have to isolate both enzymes, test them individually and in combination for a final resolution of this question.

We have put a lot of effort into characterizing a potent antifungal bacterium that secretes an antifungal factors against *O. herpotricha*. The active component is stable at room temperature for many months under the harshest conditions. We have identified the bacteria using three analytical techniques. Using a new purification technique we found and identified additional antifungal components. Some of these factors appear to be novel structures. This bacteria may be developed into an agent that can be used to control spring dead spot in the field.

Our final research area is directed toward dissecting molecular responses of bermudagrass to infection by *O. herpotricha*. New tools in functional genomics allow for identification and characterization of hundreds of genes which are up and down-regulated in response to fungal infection. The detailed analysis of bermudagrasses responses to infection should provide insights into designing future resistance mechanisms or strategies.

USGA funding for this research has been supplemented with funding from the Oklahoma Agricultural Experiment Station, the National Science Foundation, and the Environmental Institute at Oklahoma State University.

RESEARCH PROGRESS

I. Bermudagrass Transformation and Tissue Culture

- M. Maxwell, R. Edwards, Y. Zhang, C. Sweeney, A. Guenzi, and C. Taliaferro

Work Performed and Results:

Transformation. Over the past three years, we have recovered and characterized 671 putative transformants by Biolistics™. Tissues were bombarded with DNA of a chimeric gene construct containing the ubiquitin (Ubi-1) promoter and coding sequence for phosphinothricin acetyl transferase (bar) (Figure 1). Brazos (a forage-type bermudagrass) was chosen because it had previously demonstrated superior growth and plant regeneration potential in tissue culture. After thorough evaluation of these materials at the biochemical and molecular levels, we have concluded that none of these plants are transgenic. We initiated a new

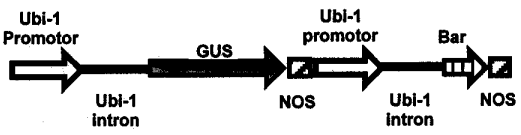

Figure 1. Chimeric gene construct used for Biolistic transformation experiments. Legend: Ubi-1 promoter-open arrows; Ubi-1 intron-solid black line; coding sequence for ß-glucuronidase (GUS)-gray arrow; NOS termination sequence from *Agrobacterium*-diagonal hatched box; coding sequence for phosphinothricin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

set of transformation experiments during the summer of 2000 utilizing a new gene construct which contains the ubiquitin/bar chimeric gene as well as the reporter gene construct containing the green fluorescence protein (GFP) coding region under the control of the Ubi-1 promoter (Figure 2). This will allow us to non-destructively assay, by fluorescence microscopy, for transgenic cells during tissue culture selection and regeneration. We have also increased the concentration of bialaphos (Libertry™) in the selection media from 1 mg/L to 3 or 6 mg/L. Approximately 6,000 inflorescences pieces have been bombarded with the GFP/bar gene construct during the summer of 2000. We have also recently obtained the gene for hygromycin resistance and will assemble a gene construct to evaluate this selectable marker for bermudagrass transformation during 2001 (Figure 2).

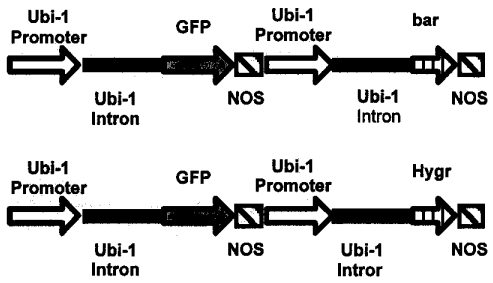

Figure 2. Chimeric gene constructs being used for new Biolistic™ transformation experiments. Legend: Ubi-1 promoter-open arrows; Ubi-1 intron-solid black lines; coding sequence for green fluorescence protein (GFP)-gray arrow; NOS termination sequences from *Agrobacterium*-diagonal hatched box; coding sequence for selectable markers hygromycin (Hygr) or bialaphos (bar) resistance - vertical striped arrow. All symbols represent the relative size of DNA sequences illustrated.

Tissue Culture. We are continuing to evaluate turf-type bermudagrasses for their tissue culture response. Seven elite lines from the breeding program were evaluated on three tissue culture media for callus induction and plant regeneration potential (Table 1). Experimental line PRC-58 demonstrated excellent callus induction and plant regeneration potential. Its tissue culture response is similar to Brazos. This genotype will be used in transformation experiments during the summer of 2001.

Table 1. 2000 Bermudagrass tissue culture test

Genotype	Medium*	No. Explants	No. Calli Induced	Embryogenic Calli (%)	Total No. Shoots
PRC-7	1	108	29	0	0
	2		47	0	0
	3	70	55	0	0
Beijing-CNT	1	100	21	0	0
	2		23	0	0
	3	100	73	0	0
94 Cross	1	100	25	0	0
	2		10	0	0
	3	100	44	0	0
7100-W-92 (#3)	1	100	41	0	0
	2		44	0	0
	3	100	53	0	0
7100-W-92 (#4)	1	100	20	5	4
	2		30	0	0
	3	100	33	6	1
A12185 U-3	1	100	9	0	0
	2		15	0	0
	3	100	42	0	0
PRC-58	1	130	48	39	101
	2		71	32	97
	3	150	145	48	402

* Medium 1 = 60 g/L sucrose; Medium 2 = 60 g/L sucrose for two weeks then transferred to 30 g/L sucrose; Medium 3 = 30 g/L sucrose.

II. Antifungal Factor Isolation and Characterization

- T. Duran, J. Enis, C. Anderson, and M. Anderson

Introduction. We discovered a very potent bacterium that showed remarkably stable and potent activity against *O. herpotricha*. We decided to identify and isolate the causal agent and to try to see if this bacterium can be used as a biocontrol agent. We also hypothesize that endogenous bacteria may play a role in SDS resistance, so we decided to isolate, identify and test these organisms for antifungal activity.

Note: Information in this section relating to the discovery and characterization of the antifungal factor and bacterium may be of proprietary value. Please keep confidential.

Work Performed:

1) 1998: Discovered a microorganism that inhibits the *in vitro* growth of *O. herpotricha* (Figure 3).

2) 1998: Identified the bacteria to the genus level. Beyond the genus level there was considerable uncertainty.

3) 1998: Developed assay to detect *O. herpotricha* inhibition (Figure 4).

4) 1999: Developed purification system for the antifungal factor. Purified the antifungal substance to homogeneity by a combination of preparative SDS PAGE electrophoresis and reverse phase HPLC chromatography (Figure 5).

5) 1999: Identified antifungal factors partial structure using mass spectroscopy and amino acid analysis.

6) 2000: Developed new purification system to isolate large amounts of antifungal agent.

7) 2000: Identified additional antifungal factors with the new purification system

8) 2000: Initiated *in vivo* testing of antifungal agent against *O. herpotricha* and other fungal species.

9) 2000: Isolated and identified eight new species of bacteria with antifungal activity against *O. herpotricha*.

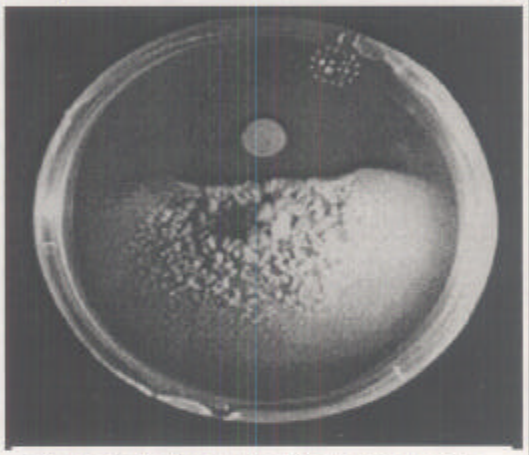

Figure 3: Antifungal Bacteria colony growing on plate inoculated with O. herpotricha. The large cleared zone indicates the extent of the inhibition.

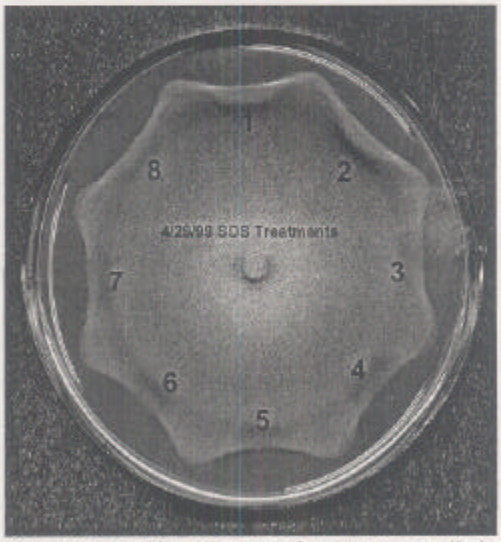

Figure 4. Antifungal assay of treatments applied to outwardly growing fungal mat of *O. herpotricha*. The cleared zones around the periphery indicate inhibition zones. The assay results are only qualitative in nature.

Results. The unknown bacterium was subjected to GC-FAME and Biolog analysis and was found to be a member of the gram-positive *Bacillus* genus. The closest related species were either the

B. subtilis or *B. coagulans*, though the match was not very good suggesting an atypical or uncharacterized member of the genus. Sequence analysis of the 16S ribosomal sequence confirmed the identity of the bacteria to be *Bacillus* species. *Bacillus* species are widely known for producing anti-microbial compounds including the peptides: surfactins, iturins and lichenysins. A molecular weight around 1000 was indicated by SDS PAGE electrophoresis. Mass spectrometer analysis of the pure and active fraction off our reverse phase column gave a molecular weight between 1022 and 1058. MS/MS fragmentation yielded a potential peptide with a mass consistent with nine amino acid residues. Amino acid analyses showed 7 amino acid residues, including: 1 valine, 1 aspartic acid, 1 glutamic acid and 4 luecines. Analyses of the literature revealed that surfactin very closely matches the molecular weight derived from the mass spectrometer analysis and is identical in amino acid composition. We conclude with a high degree of probability that the antifungal factor is either surfactin or a very close analog.

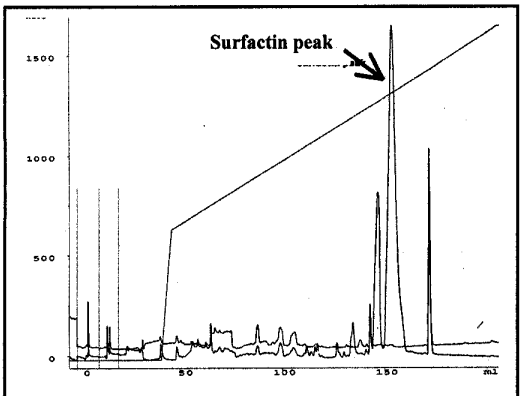

Figure 5. HPLC profile of surfactin eluted off a c18 reverse phase HPLC column. Elution was performed with a 40% to 95% acetonitrile gradient over 180 ml volume.

Surfactin is a cyclic heptapeptide with a 13, 14 or 15 -carbon beta hydroxy fatty acid linked to luecine and glutamic acid residues forming a lactone ring. The discrepancy between the 9 residues found in the MS/MS fragmentation pattern and the 7 in the amino acid analysis is due to the mass of the cyclic fatty acid structure. All three isoforms of the surfactin were present in our HPLC profiles. Surfactin is known to possess potent antibiotic properties, and is the most powerful surface acting agent known. Testing of all three isoforms showed that all of them had some activity against the casual agent of spring dead spot. Surfactin disrupts membrane structure and alters cellular activity by forming ion channels in membranes. Surfactin is synthesized by a multi-enzyme complex and therefore is not coded directly by a single gene. There are no reports of production of transgenic plants using genes for surfactin, or other similar peptides due to the difficulty in regulating transcription and translation of the gene products.

We have isolated additional antifungal compounds using our new and improved purification technique. The three factors were identified by mass spectroscopy with some unusual features not previously found in the scientific literature for like compounds indicating that we are probably dealing with a unique strain of bacteria. We are going to seek licensing for commercial purposes this year.

The most promising use of the antifungal microorganism is as a biocontrol agent against spring dead spot. Accordingly, Dr. Dennis Martin established field plots for SDS inoculation that will be ready some year 2002. We plan on using these plots for several purposes: 1) to test the efficacy of the biocontrol agent in the field, 2) to assay for the fungus using our newly developed assay, and 3) to sample bermudagrass crown tissues for endogenous microorganisms with activity against *O. herpotricha*. The plots will be inoculated in the summer of 2001 and since it takes a year to establish the infection, we won't be able to test the biocontrol agent until 2002. In the mean time, we hope to develop a growth chamber test for SDS infection.

Future Work:

1) Develop assay to monitor biocontrol agent in the field. (2000-2001)
2) License biocontrol agent to a private company. (2000-2001)
3) Submit first paper on discovery and characterization of biocontrol agent. (2001)
4) Test biocontrol agent in the field. Monitor antifungal bacteria in the field during trials. May take several years. (2002-2005)
5) Characterize additional antifungal agents from endogenous microflora. (2001-2005)

III. Chitinase Isozymes and Infection with SDS

- C. Anderson and M. Anderson

Introduction. Chitinases and glucanases are known antifungal proteins that are induced in response to pathogen invasion and low temperature stress. This project seeks to determine if bermudagrass chitinases have antifungal activity against *O. herpotricha*, and if their expression is induced during infection with *O. herpotricha*. As indicated above we will have to infect the plants in the field.

Work Performed:

1) 1998: Developed a chitinase purification system using organically synthesized chitin from treated crab shell substrate.
2) 1998: Developed a sensitive and semi-quantitative chitinase assay using radioactively labeled chitin.
3) 1998: Developed a chitinase isozyme purification system using a granulated gel isoelectric focusing system.
4) 1999: Inoculated both resistant and susceptible bermudagrass with *O. herpotricha* and incubated bermudagrass at 15°C. Measured individual chitinase isozyme activity to determine if activity is enhanced by fungal infection. Activity was determined by our quantitative chitinase assay for each isozyme. Results are described below from the first replication study, and the second replication is currently in progress.
5) 1999: Performed northern blot analysis in infected tissues to determine expression pattern of recently isolated basic chitinase gene (Figure 6).

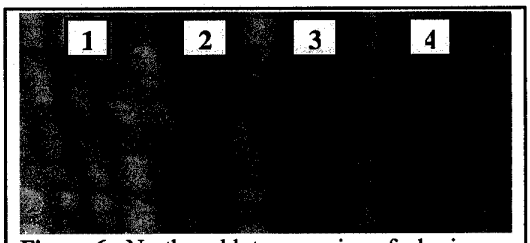

Figure 6: Northern blot expression of a basic bermudagrass chitinase gene upon infection with *O. herpotricha* at 15 C. Chitinase expression increases dramatically in both Midiron and Tifgreen crown tissues, but more so in resistant Midiron. **Legend**: 1 = Tifgreen control; 2 = Midiron control; 3 = Tifgreen infected; 4 = Midiron infected.

6) 2000: Found that growth chamber samples from resistant and susceptible lines are equally infected. No difference due to resistance.
7) 2000: Decided to perform activity estimates in the field due to the problems associated with infection in growth chamber tissues.

Results. Total chitinase activity across the isozyme profile was very similar in both treated and control crown tissues of resistant Midiron and susceptible Tifgreen. Midiron showed a doubling of basic chitinase activity upon infection with a concomitant decrease in acidic chitinase activity. The increase in basic chitinase and decrease in acidic chitinase activity was not apparent in susceptible Tifgreen. Northern blot analysis of basic chitinase expression from crown tissues showed a strong induction upon infection with *O. herpotricha* at 15°C. These results indicate that basic chitinase expression is increased in response to infection, particularly in resistant variety Midiron. This may indicate that the basic isozyme functions in the resistant defense response to *O. herpotricha* infection. The above data was derived from growth chamber tissue where the resistant and susceptible lines were recently shown to be equally infected by our new assay. It is apparent that in order to determine the true relationship between chitinase activity and SDS infection we need to use field-infected tissues.

Future Work:

1) Develop assay for glucanase activity in bermudagrass crown tissues (2001).
2) Chitinase and glucanase activity study on field grown tissue (2002-2004).

IV. Development of an Assay for *O. herpotricha* in the Field (New Project)

- J. Hironaka and M. Anderson

Introduction. In order to study the basic biology of SDS infection it is imperative to develop an assay to quantitatively determine the level of infection. Accordingly, we have developed a highly sensitive RT-PCR assay for detection and semi-quantification of *O. herpotricha* in living tissues. This assay could also be used in a breeding program to more rapidly screen bermudagrass lines for SDS infection.

Work performed. Developed and tested a highly sensitive RT-PCR assay for *O. herpotricha* in infected tissue samples living.

Results. The assay performed as expected in a highly sensitive manner. Growth chamber infected material from resistant and susceptible Midiron and Tifgreen, respectively, was strongly positive for *O. herpotricha*. Uninfected material (control tissue) showed very little or no response. Infection in resistant material was similar to susceptible material.

Future Work. Use the assay to monitor *O. herpotricha* levels in field-infected tissues throughout the year. (2001-2004)

V. Carbohydrate Content of Bermudagrass Crown tissues (New Project)

- J. Enis, N. Mannes, and M. Anderson

Introduction. Carbohydrates in bermudagrass crown tissues are known to be important in cold tolerance mechanisms in plants. Sugars are thought to protect sensitive metabolic components from freeze injury. Specific sugars such as raffinose and stachyose were shown to increase in cold acclimated alfalfa crowns. Carbohydrate status may also be important for SDS resistance. We decided to investigate the carbohydrate status of a SDS resistant and susceptible bermudagrass line during cold acclimation.

Work Performed. Experiments were initiated during 2000 to examine carbohydrate levels in bermudagrass crown tissue and its relationship to SDS resistance in the growth chamber and in the field samples.

Results. In the first replicate experiment, SDS resistant material showed little change in specific carbohydrates during acclimation. However, the SDS susceptible line showed a dramatic rise in sucrose concentration during acclimation. We are repeating the experiment to confirm this trend, and are looking carbohydrate levels in field tissue. This may help explain the reason for susceptibility. Possibly susceptible lines produce more sucrose, which serves as a food source for opportunistic fungi. We have to check to see if this runs true for other resistant and susceptible lines of bermudagrass.

Future Plans:

1) Examine specific sugars from first experiment during a 28-day cold acclimation treatment. (2001)
2) Examine specific sugars from a range of resistant and susceptible bermudagrass lines during cold acclimation. (2001)
3) Examine specific sugar profiles in field grown tissues for changes in carbohydrate status. (2000-2002)

VI. Detection of Fungal Induced and Repressed Genes from Bermudagrass using Subtractive Hybridization Coupled to cDNA Microarray Analysis (New Project)

- Y. Zhang, A. Guenzi, M. Anderson, C. Taliaferro, and D. Martin

Introduction: The goal of this new research project is to dissect molecular mechanisms of interactions between bermudagrass and *O. herpotricha.* In this project, we are identifying fungal-induced gene transcripts by creating normalized cDNA libraries by use of the PCR-based suppression-subtraction-hybridization (SSH) procedure. Gene expression profiles between tolerant and susceptible bermudagrass varieties will be monitored by cDNA microarray analysis. Full-length cDNAs will be cloned by the rapid amplification of cDNA ends (RACE) technique. Results from this project will improve our knowledge on bermudagrass defense responses to fungal infection. Insights into the infection process will help develop chemical or biotechnology strategies to control Spring Dead Spot. Fungal-induced transcript sequence data of bermudagrass will also be informative to researchers working with fungal diseases of other turfgrasses.

Hypothesis: Tolerant and susceptible varieties differ in gene expression profiles in response to fungal infection.

Objectives:

1) Construct normalized PCR-based subtraction libraries from both tolerant (OkSU 91-11) and susceptible (Jackpot) varieties
2) Monitor the expression profile of differential expressed transcripts from subtraction libraries by cDNA microarray
3) Isolate and characterize fungal induced cDNAs from tolerant and susceptible cultivars by RACE and sequencing

Preliminary Results:

1) mRNAs were isolated from crown tissues of infected and non-infected tissues of OkSU 91-11 and Jackpot.
2) One subtraction library was established from fungal infected resistant tissue (tester) against fungal infected susceptible tissue (driver)
3) In this subtraction library, 221 clones were obtained from forward subtraction and 89 clones were obtained from reverse subtraction
4) All the clones were characterized by Colony PCR method, 90% of the colonies contained inserts, which average 600 bp in size (Figure 7)

Future Research. During the next year, we will sequence clones in the SSH libraries to potentially identify up or down regulated genes. Unique clones will be microarrayed to investigate gene expression profiles between tolerant and susceptible cultivars. Clones, which demonstrate a profile associated with tolerance, will be used in 5'RACE to obtain full-length cDNAS or used to screen genomic libraries.

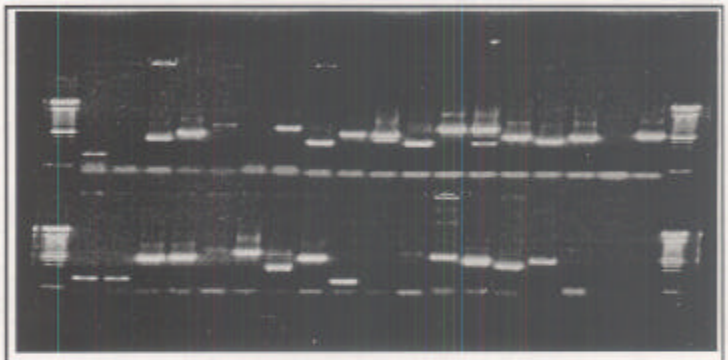

Figure 7: Colony PCR results by electrophoresis (1.5% agarose gel in 1 x TAE). Size of the inserts are compared with 100 bp DNA ladder. Average insert size is approximately 600 bp