

USGA Project Annual Report for 1999

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 bermudas. Improvement in resistance in these sexually isolated lines require 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 include the bar gene coupled to a constitutive ubiquitin promoter. The bar gene codes for an enzyme that metabolizes the herbicide Liberty™. After bombardment Liberty was used in the selection media to screen for resistant transgenic tissues. Over 671 potential transformants were recovered from bombarded tissues. Current efforts to screen transgenic plants using PCR and Southern hybridization techniques identified three consistent positives (P252, P303, and P319). Of the three positives, P303, showed more intense hybridization possibly indicating incorporation of multiple copies of the bar gene. Furthermore, in an herbicide swipe test P303 showed higher levels of resistance to the herbicide Liberty. Further work to exhaustively screen all 671 transformants is in progress.

A microorganism with potent activity against *O. herpotricha* was recently discovered and characterized. 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 and Biolog analysis. Activity was due to the secretion of a potent antifungal factor that was stable under the harshest conditions. Isolation of the factor using preparative SDS PAGE and reverse phase chromatography was successful. The compound was 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.

Chitinases are well known enzymes that possess potent antifungal activity. To determine if chitinases play a role in the resistance mechanism against *O. herpotricha* we inoculated pots with the fungus, grew the plants for more than a month to establish the infection and then transferred the plants to a 15 C temperature, a temperature more conducive to *O. herpotricha* growth. We developed a novel protocol to isolate individual chitinase isozymes and determine enzyme activity. Isozymes were separated according to their isoelectric point. Infection with *O. herpotricha* resulted in a doubling of basic

chitinases in resistant Midiron, but not Tifgreen. Furthermore, northern blot analysis clearly showed an increase in basic chitinase gene expression at 15 C. This data indicates that basic chitinases may have a role in disease resistance in bermuda crown tissues.

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 *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. 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 is 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.

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 chitinases (Graham and Sticklen 1994) and glucanases (Simmons 1994).

We have isolated a microorganism that secretes a factor with potent activity against spring dead spot. The activity is stable at room temperature for many months and could possibly be used against the disease organism by transforming bermudagrass with the gene that codes for the antifungal factor. In addition, the bacteria or the antifungal factor could by itself be used as a biocontrol agent.

Objectives of This Project

- 1) Develop an efficient transformation system for Bermudagrass
- 2) Isolate promising antifungal factors against the casual agents of spring dead spot.
- 3) Utilize the antifungal factors and genes to significantly reduce damage caused by fungal diseases such as spring dead spot.

Progress

Bermudagrass Transformations:

Work Performed and Results:

- 1) 1997: Ms Melissa Maxwell as part of the M. S. in Plant and Soil Science initiated the production of tissue culture material and the Biolistic bombardment of tissues. The tissues were bombarded with DNA containing a ubiquitin driven promoter and the bar gene. Brazos was chosen as a tissue source 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). We have recovered 671 putative transgenic plants from these experiments.
- 2) 1998: Hired Ms. Yan Zhang as a graduate research assistant to conduct the research in partial fulfillment of the requirements for a Ph.D. in Plant Science at OSU.
- 3) 1998-1999: Extracted DNA from putative transgenic plants. We had difficulty at this stage due to suspected high concentrations of polysaccharides and/ or phenolics in bermudagrass leaf tissues. We had to optimize the procedure using a modified CTAB DNA isolation technique to remove these contaminants. This has resulted in higher yields, but restriction digestion of the DNA is still difficult possibly due to either a high degree of DNA methylation or the presence of other contaminants. We are currently trying to improve the technique.
- 4) 1998-1999: The initial southern and PCR analysis was contradictory due to the trouble with isolating high quality DNA mentioned above. However with the improved technique we have been successful in consistently identifying three bermudagrass transformants in both PCR and Southern analysis. We are confident that these plants are transgenic. To our knowledge these are the first transgenic bermudas produced. Based on the intensity of the band from the southern analyses, P303 appears to contain multiple copies of the bar gene. It also has the greatest tolerance to the herbicide LibertyTM when assayed with the leaf swipe test.

Antifungal Factor Isolation and Characterization

Note: Information in this section relating to the discovery and characterization of the antifungal factor 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 2). We decided that it would be worth pursuing since inhibition was very strong and stable for several months.

- 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 3).
- 4) 1999: Developed purification system for the antifungal factor. Purified the antifungal substance to homogeneity by a combination of preparative SDS PAGE electrophoresis (Figure 4) and reverse phase HPLC chromatography (Figure 5).
- 5) 1999: Identified antifungal factors partial structure using mass spectroscopy and amino acid analysis.

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. *Bacillus* species are widely known for producing antimicrobial compounds including the peptides: surfactins, iturins and lichenysins. Our antifungal factor was purified to homogeneity as indicated above by preparative SDS PAGE electrophoresis and reverse phase HPLC. 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 9 amino acid residues. Amino acid analyses showed 7 amino acid residues, including: 1 valine, 1 aspartic acid, 1 glutamic acid and 4 leucines. 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.

Surfactin is a cyclic heptapeptide with a 14-carbon beta hydroxy fatty acid linked to leucine 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 14-carbon cyclic fatty acid structure. Surfactin is known to possess potent antibiotic properties and is the most powerful surface acting agent known to man. Surfactin disrupts membrane structure and alters cellular activity by forming ion channels in membranes. Surfactin is synthesized by a multienzyme 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.

Chitinase Isozymes and Infection with SDS

Work Performed:

- 1) 1998: Developed a chitinase purification system using organically synthesized chitin from treated crab shell substrate.
- 2) 1998: Developed a sensitive and 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 (Figure 6).
- 5) 1999: Performed northern blot analysis in infected tissues to determine expression pattern of recently isolated basic chitinase gene (Figure 7).

Results of first replication study:

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.

Future Plans:

- 1) Complete in-depth biochemical and molecular analysis of the 671 putative transgenic plants recovered from Biolistic bombardment.
- 2) Submission for publication research relating to the development of the first bermudagrass transformation system.
- 3) Initiation of transformation experiments to introduce potential antifungal genes into bermudagrass. These genes include the basic bermudagrass chitinase gene recently

cloned at OSU (Cht1), a rice chitinase gene from the Salk Institute, and a glucanase gene from the Noble Foundation. All genes will be expressed using the constitutive ubiquitin promoter.

- 4) Confirm structure of antifungal factor. This will require selective hydrolysis of the lactone ring and amino acid sequencing from the C terminal end.
- 5) Submission for publication research relating to the purification and characterization of the antifungal factor.
- 6) Initiate experiments to determine if the Bacillus bacteria and / or surfactin could be used as a biocontrol agent in the field. This may take several years to determine due to the slow growth and development of *O. herpotricha*.
- 7) Investigate patent possibilities for the use of surfactin and/or Bacillus bacteria in the control of SDS and other microbial diseases.
- 8) Develop an assay to quantitate *O. herpotricha* levels in the field and in tissues using DNA hybridization methodology. This is necessary to correlate the level of infection with potential resistance factors.
- 9) Complete 2nd and 3rd replication of chitinase activity assays in the growth chamber. These should be completed by next summer (year 2000).
- 10) Submit for publication analysis of chitinase isozyme activity and SDS infection.
- 11) Initiate experiments to determine if chitinase isozyme activity varies in crown tissue in resistant and susceptible bermudas in the field during fall and spring warm up.
- 12) Isolate additional microorganisms with antifungal activity against *O. herpotricha* using a newly conceived and novel screening procedure.
- 13) Determine if the recently isolated basic chitinase gene codes for an isozyme with *in vitro* activity against *O. herpotricha*

Experimental Data



Figure 1. 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 β -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.

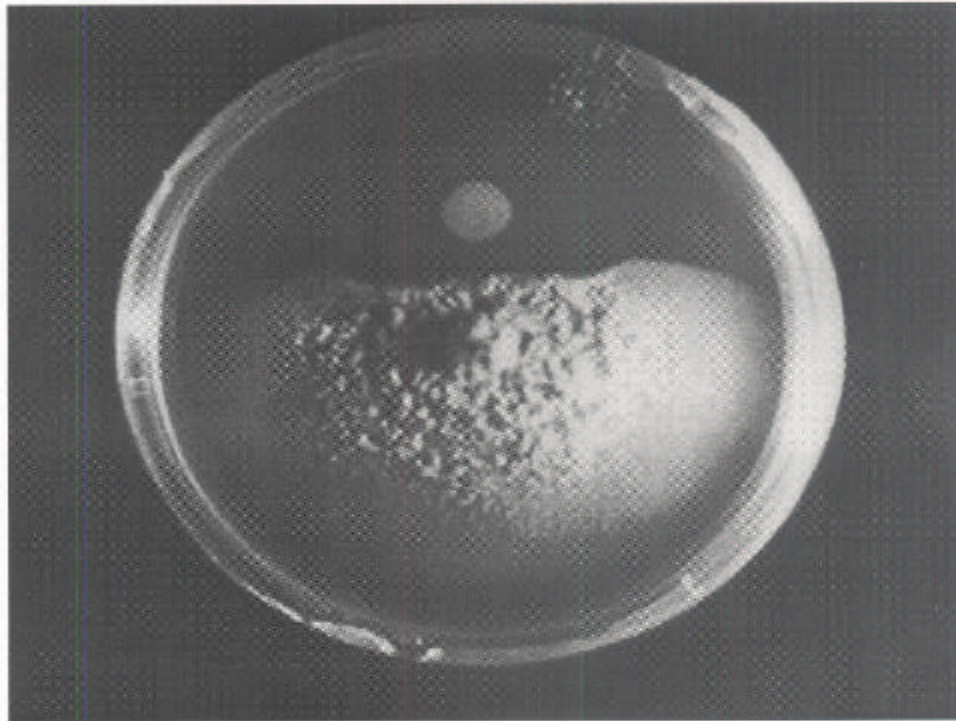


Figure 2: Antifungal Bacteria colony growing on plate inoculated with *O. herpotricha*. The large cleared zone indicates the extent of the inhibition.

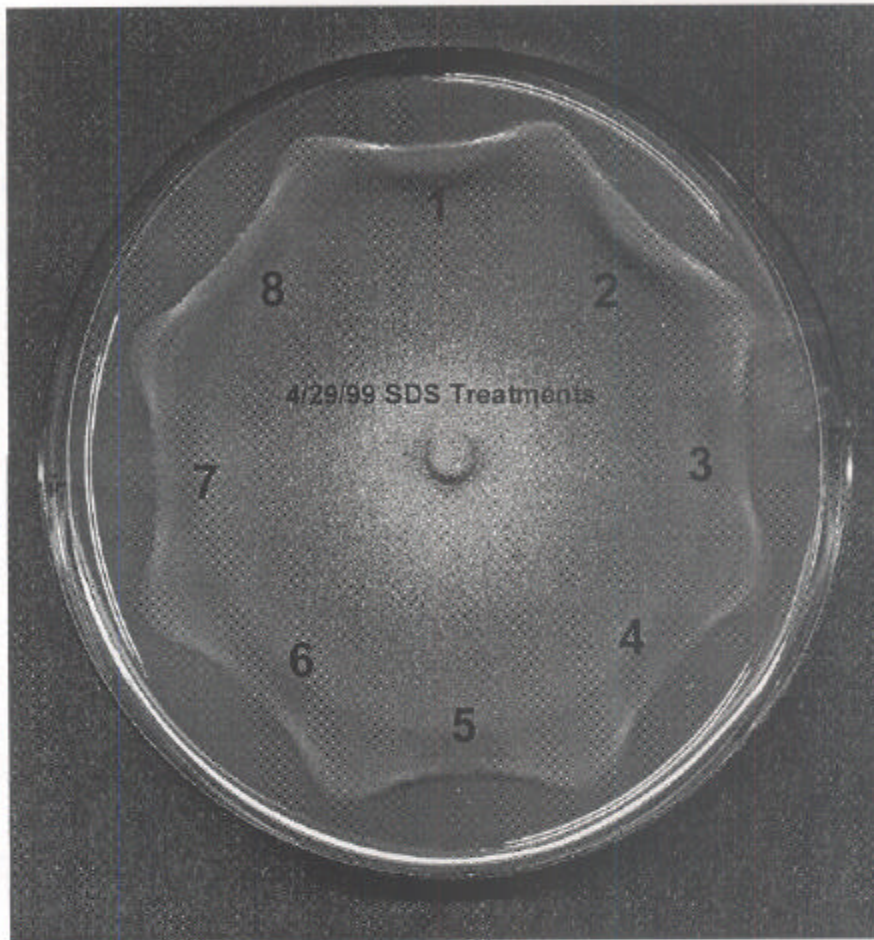


Figure 3. Antifungal Assay of treatments applied to outwardly growing fungal mat of *O. herpotricha*. The cleared zones around the periphery indicate inhibition zones. The assay results are only qualitative in nature.

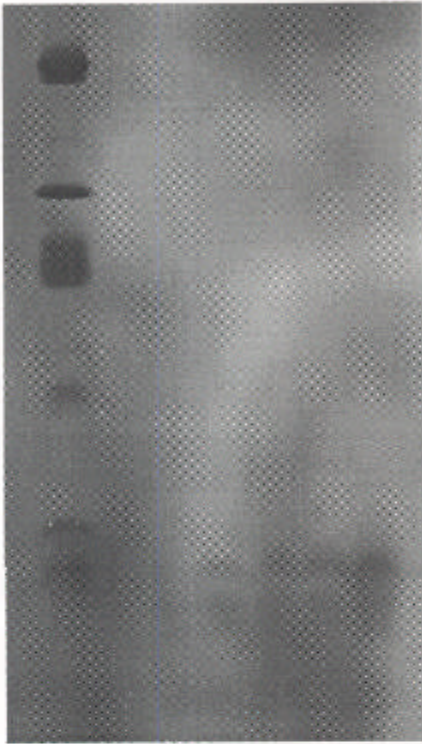


Figure 4. Tricine SDS PAGE gel of fractions off the Bio Rad SDS Prep Cell. Lowest molecular wt marker is near 1 kD as is the weight of the surfactin band. The lightness of the staining of this small peptide is due to the lack of retention of silver ions during silver staining.

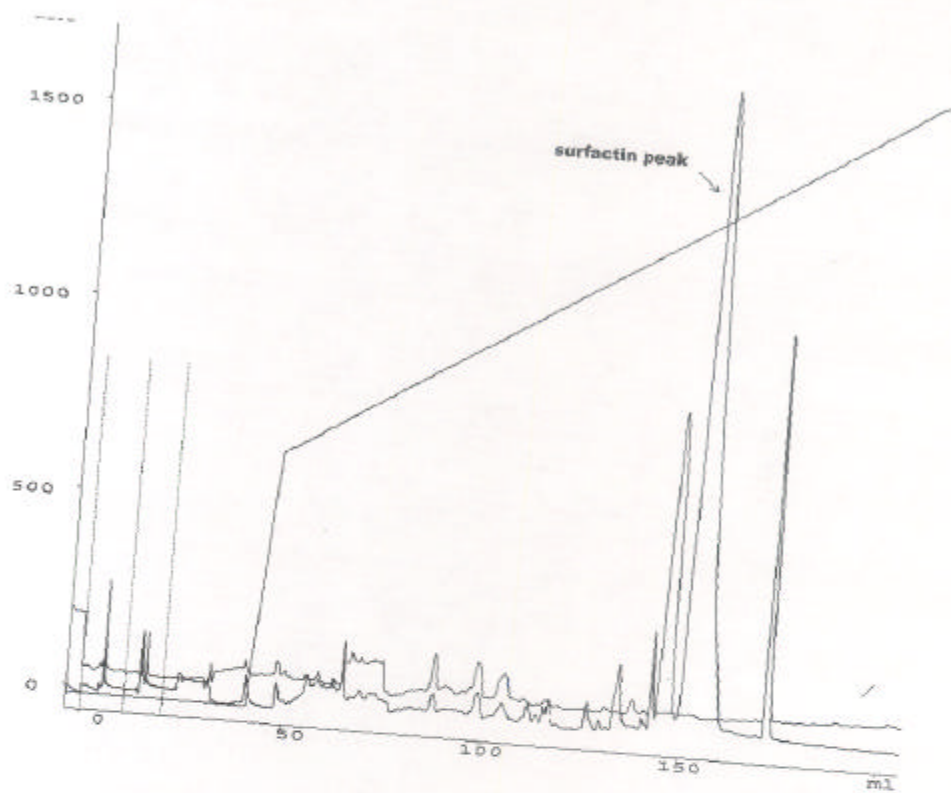


Figure 5. HPLC profile of surfactin eluted off a c18 reverse phase HPLC column. Elution was performed with a 40% to 95% acetonitrile gradient over 180 ml volume.

Infected Bermudagrass 15 C

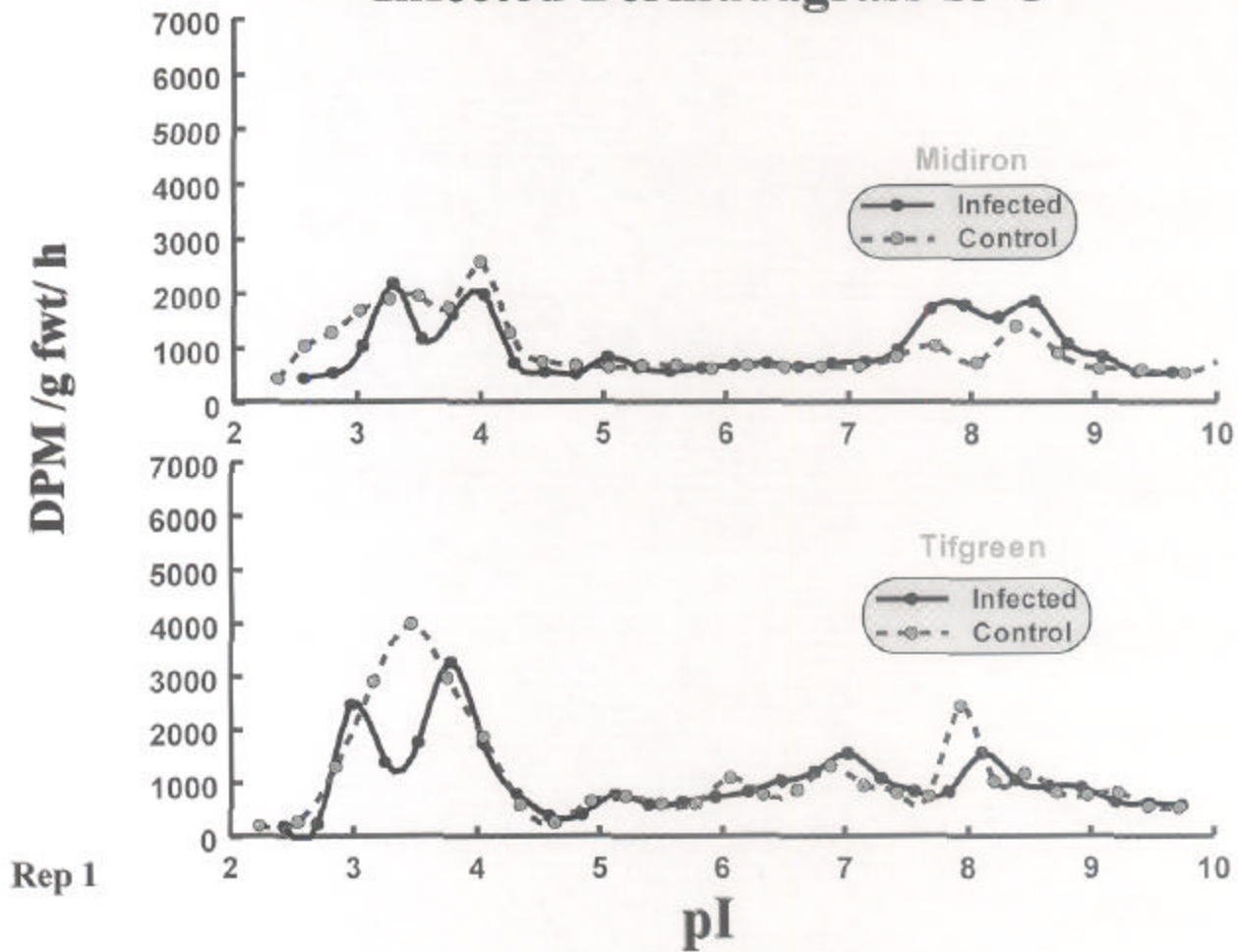
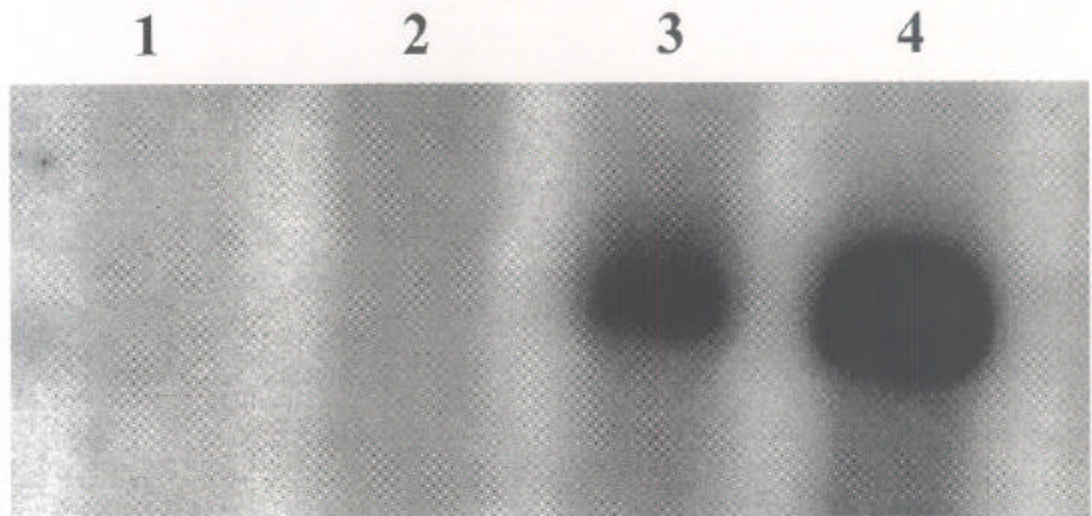


Figure 6: Chitinase activity in bermudagrass crown tissues infected with *O. herpotricha* at 15 C. Basic chitinases show an doubling in activity upon infection in resistant midiron but not Tifgreen. Data from 1st replication only.



- 1= Tifgreen, control
- 2= Midiron, control
- 3= Tifgreen, SDS-treated
- 4= Midiron, SDS-treated

Figure 7: 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 (Benildo de los Reyes, 1999)