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ABSTRACT OF DISSERTATION

Brian Christopher King

The Graduate School

University of Kentucky

2011

T-PHYLLOPLANIN AND *CIS*-ABIENOL, TWO NATURAL PRODUCTS FROM TOBACCO HAVE BROAD SPECTRUM, ANTI-FUNGAL ACTIVITIES

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the

requirements for the degree of Doctor of Philosophy in the

College of Agriculture

at the University of Kentucky

By

Brian Christopher King

Lexington, Kentucky

Director: Dr. George J. Wagner, Professor of Plant and Soil Sciences

Lexington, Kentucky

2011

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ABSTRACT OF DISSERTATION

T-PHYLLOPLANIN AND *CIS*-ABIENOL, TWO NATURAL PRODUCTS FROM TOBACCO HAVE BROAD SPECTRUM, ANTI-FUNGAL ACTIVITIES

Tobacco phylloplanins (T-phylloplanin) are a group of closely-related glycoproteins that are formed and disposed at the interface between the plant aerial surface (the phylloplane) and the atmosphere. They are synthesized in short procumbent trichomes and are secreted to aerial surfaces where they are thought to serve the plant as a first line of defense against fungal pathogens. Here it is shown using in vitro and in planta assays that tobacco and sunflower phylloplanins have broad-spectrum antifungal activities against spores - and also hyphae for two species - of several true fungi. Field tests show that T-phylloplanin reduces diseases caused by three important fungal pathogens of turf grasses.

Tobacco phylloplanins are distinct proteins but they have properties in common with small, membrane-pore-forming, antimicrobial peptides formed by other organisms. To directly determine if T-phylloplanin has pore-forming activity we monitored conductivity change and specific ion leakage from spores and hyphae in suspension. Results indicate that phylloplanin causes fungal membrane disruption that leads to ion depletion and cell death.

Having observed broad efficacy of T-phylloplanin against spores and/or hyphae of several true fungi, but no activity towards hyphae of the oomycetes, *Pythium* and *Peronospora parasitica*, we tested for possible effects on zoospores of the latter two pathogens. T-phylloplanin was shown to be effective against their zoospores, extending the efficacy of T-phylloplanin to include water molds. In the course of these experiments we also tested the effects of the diterpene *cis-abienol* that is secreted from tall trichomes of tobaccos and found this compound impacted zoospores and could prevent black shank disease caused by P. *parasitica* when applied to soil-grown tobaccos as a root drench.

Thus, results of these studies with phylloplanins and cis-abienol, two different tobacco surface accumulated compounds are consistent with their serving the plant as first line of defense systems against a wide array of invading fungal pathogens. Phylloplanins and cis-abienol may be useful for controlling fungal diseases in tobacco float beds. The efficacy shown here for T-phylloplanin control of fungal pathogens of turf grasses in the field suggests that this natural product may find use in IPM of turf and other crops.

Keywords: Phylloplanin, *Pyricularia grisea*, *Rhizoctonia Solani*, Broad spectrum, Fungicide

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This work is dedicated to my daughter Chloe. I wish for her all the best the world has to offer and may she develop to surpass me in every way, intellectually, emotionally and spiritually.

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The following dissertation has benefited from the insights and direction of several people. I am indebted to each of my committee members for their guidance and advice. I have learned a great deal from each, and will carry their lessons with me as I pursue a rewarding career in plant science. From Dr. David Williams I have gained a much broader knowledge of the turf grasses, and a profound fascination for their extraordinary biology. Dr. Paul Vincelli has taught me a lot about fungi in general, and his passion and skill at teaching has helped to open up this area of science to me. Dr. Michael Barrett has always provided very insightful comments on my work, and has taught me how to critically analyze my own scientific research, as well as that of others. Dr. Elisa D'Angelo has conferred to me the will to keep trying, even if at first you don't succeed; some of life's best secrets are not learned in a day. I thank Dr. Paul Bertsch for his continued enthusiasm and positive words as a mentor and the opportunity to learn something beyond my world of plants and fungi. Thanks to Dr. Mark Farman for all that he has taught me and the friendship and lab space we shared. I would especially like to thank my major advisor and mentor, Dr. George J. Wagner, for providing me countless exciting opportunities to express my ideas, and for exemplifying the meaning of true scientific excellence. Every aspect of my graduate career has been molded by him, and I am a much better scientist for it. I am incredibly thankful for my wonderful daughter Chloe who follows in my footsteps as we chase our dreams. She has made my life truly complete. I am thankful for the love and support my parents, Gary and Ramona, have given me throughout my life and for having me as their son. Finally, I would like to thank the faculty, staff, and graduate students from Plant Science and Plant Pathology for their help and friendship. They are truly the best of the best, and I wish them all happiness and success.

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Chapter 1: The State of Turf and the Use of Fungicides

1.1. Introduction:

Turf grass is one of the most under rated and under researched crops. In the United States, there is an estimated 50 million acres of managed turf grass (Couch 2000). This 50 million acres is divided among tens of millions of residential lawns, >700, 000 athletic fields, 17,000 golf courses which accommodate over 26 million golfers annually, and parks and recreation facilities for citizens. The industry itself is estimated to have an economic value of nearly 40 billion dollars and provides economic opportunities for tens of thousands of seed producers, sod producers, fertilizer companies, and pesticide companies. Turf grass is useful, not only aesthetically, but practically. It provides dust control for thousands of miles of highways, purifies water and reduces runoff, absorbs carbon dioxide and produces oxygen. It also dramatically improves the mood in urban environments.

Due to the large acreage and economic value of turf grass nationally, it has become increasingly important that it be managed in an effective and sustainable, yet economic fashion. Although the application of water resources is critical, almost as costly to turf maintenance is the use of fungicides. There are several serious fungal pathogens of turf that are important, both economically and environmentally. Controlling these presents challenges because of the high cost of commercial fungicides and the need for their use in

integrated pest management (IPM) to avoid development of fungicide resistance (Couch 1995). The use of commercially available fungicides has reached the apex of viability and is viewed as being highly detrimental to the environment (Wedge 2005).

Our laboratory recently discovered phylloplanin glycoproteins that are produced from several plant species (*Nicotiana tabacum, Hellianthus annuus,* and *Datura metel*) and showed that these glycoproteins are natural, plant made fungicides that inhibit *Peronospora tabacina*, the causal agent of a devastating disease of tobacco known as blue mold (Shepherd, Bass et al. 2005; Kroumova, Shepherd et al. 2007). Our most recent research as detailed in this thesis suggests that phylloplanin from tobacco (T-phylloplanin) may have potential for use as a anti-fungal agent in IPM management of turf. If a fungicide is to be useful in an IPM strategy it should have broad utility, beyond tobacco, to help offset both cost and logistics of use. Tobacco phylloplanin (T-phylloplanin) and sunflower phylloplanin (S-phylloplanin) were found to be effective against at least one member of three of the four classes of true fungi using Petri plate assays and on plant spray experiments (See Chapter 2). Some efficacy was also shown for Zygomycetes, and for oomycetes, the later being fungi-like organisms that have some of the same wall and membrane structures (e.g. spores, fruiting bodies, etc.) and have similar biochemistry as true fungi.

After discovery of the broad spectrum fungicidal activity of phylloplanins, moving these compounds into the realm of commercial use became a center of focus. Testing T-phylloplanin in a field setting became preeminent, so in the spring of 2009 field tests were begun to test the effects of T-phylloplanin against naturally occurring diseases of turf in the field (Chapter 3). Field work was conducted using two concentrations of T-

phylloplanin on three different diseases (brown patch, dollar spot, and gray leaf spot) as well as on two grass hosts (perennial rye and creeping bentgrass). The research was conducted over two years and in most cases gave statistically significant results when compared to both water controls and commercially used fungicide controls. More research is needed and plans to start year three of field trials are already underway.

Earlier studies in our lab showed that T-phylloplanin caused ion leakage from isolated tobacco root vesicles and artificial liposomes. This, along with our recognition that T-phylloplanins share some similar physical and chemical properties with membrane-pore-forming antimicrobial peptides (AMPs), led us to investigate the possibility that T-phylloplanin, like AMPs, can cause ion leakage in fungal membranes. We devised experiments to determine directly if fungal spores and hyphae leak ions in the presence of T-phylloplanin. This was done by monitoring conductivity change in suspensions of intact, living spores and hyphae and by profiling constituent ions released in these experiments (Chapter 4).

Our studies with the true fungi (Ascomycete, Basidiomycete, Zygomycetes) and the fungi-like oomycete *P. tabacina* (which produces aerial spores) showed that phylloplanins were anti-fungal on Petri plate assays or slide germination assays, respectively. However, when we tested waterborne oomycetes like *Phytophthora parasitica* (causes lack shank of tobacco) and *Pythium* in Petri plate assays we did not observe fungistatic activity. So we decided to test the effects of T-phylloplanin on the motile spore (zoospore) stage of these water molds. Using assays designed to mimic commercial tobacco float tray systems we demonstrated efficacy against both *P. parasitica* and *Pythium* (Chapter 5). We tested another tobacco surface produced natural

product, *cis*-abienol, to determine if it might reduce severity of black shank disease of tobacco. In both a float tray mimic assay and a potted plant, root drench assay we observed black shank control.

In summary, this thesis shows that:

1) T-phylloplanin has broad use potential for controlling several major fungal diseases of turf grasses and tobacco and that it has the potential to augment or replace environmentally-unfriendly commercial fungicides.

2) T-Phylloplanin is effective in a field setting for controlling diseases of major turf grasses, setting the stage for its development as a commercially useful IPM component.

3) We show directly using living, intact spores and hyphae that T-phylloplanin causes spores and hyphae to leak endogenous ions. These results support earlier studies using model membrane systems and strongly suggest that T-phylloplanin disrupts membranes of target organisms leading to ion deprivation and cell death.

4) We discovered that *cis*-abienol, another natural product produced by tobacco, has potential for controlling two major water mold pathogens, *P. parasitica* and *Pythium.* These findings suggest that this compound may be useful for treating roots of soil-grown or float-bed-grown tobacco to control black shank disease.

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Chapter 2: Phylloplanins Reduce the Severity of Gray Leaf Spot and Brown Patch Diseases on Turfgrasses

2.1. Abbreviations:

AR-annual ryegrass (*Lolium multiflorum* cv. 'Linn'); PR-perennial ryegrass (*Lolium perenne* cv. 'Double Eagle Blend'); CB-creeping bentgrass (*Agrostis stolonifera* L. cv. 'L-93'); LWW-leaf water wash; T- LWWs, leaf water washes of tobacco and S-LWWs, leaf water washes of sunflower; T-, and S-phylloplanin, phylloplanins of tobacco and sunflower; ProtK, ProteinaseK; SHAM, Salicylhydroxamic acid.

2.2. Abstract:

Plant-produced, natural-product fungicides should be evaluated for their potential to complement or replace synthetic fungicides that are major components in the management of high maintenance turf. Tobacco and sunflower phylloplanins (T-, and S-phylloplanins) collected in leaf-water-washes (LWW) of plants, and recombinant T-phylloplanin, inhibit *Peronospora tabacina* D.B. Adam (oomycete) spore germination and reduce blue mold disease on tobacco. Here, i*n vitro* (hyphal extension) and *in vivo* studies were conducted to test the ability of T-, and S-phylloplanins to inhibit growth of the turf pathogens *Pyricularia oryzae Cavara* (Ascomycete) and *Rhizoctonia solani Kuhn.,* (Basidiomycete), and to test protection of turfgrass plants from gray leaf spot and brown patch diseases. Hyphal extension assays showed that LWWs containing T- and S-phylloplanins inhibited both pathogens. Spraying potted grass plants with LWWs containing T-, and S-phylloplanins followed by inoculation with pathogens resulted in 97% and 88% inhibition of *P. oryzae* disease and 94% and 82% inhibition of *R. solani* on annual (*Lolium multiflorum* Lam. Cv. 'Linn') and perennial (*Lolium perenne* L. cv. 'Double Eagle Blend') ryegrasses, respectively, under the conditions tested. In addition,

T- and S-phylloplanins led to 94 and 100% symptom reduction, respectively, on creeping bentgrass (*Agrostis stolonifera* L. cv. 'L-93') , for *R. solani* only). Results indicate that T- and S- phylloplanins have broad selectivity, inhibiting at least one member of the Ascomycete and Basidiomycete phyla, and may be useful as exogenously-applied or endogenously-expressed, natural-product, anti-fungal agents to protect turf, crops and other plants.

2.3. Introduction:

Pathogen species- and isolate-specificity in disease implies that endogenous resistance mechanisms occur within particular pathosystems. Plants can possess indirect and direct microbial resistance mechanisms. Indirect, generally induced, microbial inhibition can occur via recognition/signaling pathways. Resistance (R) genes encode R-proteins that recognize pathogen avirulence proteins and, through complex signaling pathways similar to those in animals, induce local and systemic defense responses within the plant (Holt, et al. 2003). Peroxidases that occur on the surfaces of certain plant tissues (roots, Quiroga et al., 2001; stigma, McInnis et al., 2006; in hydathode fluid, Grunwald et al., 2003) are generally considered PR proteins, but their functions are not well defined. Direct protection against pathogens in plants can occur through molecules such as inter-tissue defensins, trichome exudate chemicals (e.g., terpenoids, phylloplanins), and cuticular lipids (Kerstiens 1996; Shepherd and Wagner 2007). In animals, the direct, antimicrobial-peptide-based microbial resistance strategy is increasingly recognized for its importance in surface protection (Gallo and Huttner 1998). Direct microbial resistance is localized at the first point of contact, usually at the boundary between the host and the exterior environment (e.g., in plants the epidermis/air interface).

Previous work indicates that tobacco and sunflower plants secrete glycoprotein phylloplanins, called T-, and S-phylloplanins, respectively, to aerial tissue surfaces via specialized, aerial-surface-located trichomes (Shepherd, et al. 2005, Kroumova, et al 2007). T-, and S-phylloplanins appear to provide a first-point-of-contact, direct resistance against *Peronospora tabacina* (Shepherd, et al. 2005; Shepherd and Wagner 2007). Various plants also produce various antimicrobial peptides, e.g., defensins, thioneins, hevein, snakin, etc. (Broekaert, et al. 1997), but these are believed to be secreted to the apoplastic space and are not thought to be present on the phylloplane. Tobacco and sunflower phylloplanins can be easily washed from leaves, and such preparations (leaf water washes - LWWs) when applied to tobacco leaves together with spores suppress *Peronospora tabacina*, the oomycete that causes blue mold disease of tobacco (Shepherd, et al. 2005). These phylloplanins are also shown to suppress *P. tabacina* spore germination *in vitro*. When T-, or S-LWWs are treated with ProteinaseK (ProtK), or boiled, phylloplanin proteins and disease-control-activities are lost. Recombinant T- phylloplanin inhibits *P. tabacina* spore germination (ProtK treatment negates this activity). And, young tobacco plants suppressed in *T-phylloplanin* gene expression (using dsRNAi), and thereby having greatly reduced T-phylloplanin production, are more susceptible to blue mold (Kroumova, et al. 2007). Thus, phylloplanins in LWWs of tobacco, sunflower, and *Datura metel*, var. (L) Saff fastuosa (Kroumova, et al. 2007) are shown to confer resistance to the oomycete, *P. tabacina*. The gene encoding S-phylloplanin has not yet been cloned, but comparison of effects of LWW containing S-phylloplanin with and without ProtK treatment (or boiling) show that proteins in S-LWW are also responsible for inhibition activities (Kroumova, et al. 2007,

data presented here). Analysis of tobacco LWW for surface located, tall-trichome-secreted diterpenes and sugar esters indicates the presence of <2% of these as compared to that recovered in a methylene chloride wash of the same leaf surface-area-equivalents, and ion analysis of T-LWW suggests an ~8% (w/v) content of inorganic ions (Shepherd, et al. 2005).

Agricultural fungicides comprise ~70% of all fungicides used. Increased fungal resistance to currently available fungicides (Gisi, et al. 2007) and increasing social sensitivity to the use of chemically-synthesized fungicides has caused a need for effective natural-product fungicides. One of the most intensively fungicide-treated plant types is turfgrass (Corwin et al., 2007), because maintaining high-quality turf is one of the top priorities of golf turf managers. Gray leaf spot (caused by *Pyricularia oryzae*) and brown patch (caused by *Rhizoctonia solani*) are two highly destructive diseases of cool- and warm-season grasses. Gray leaf spot is a disease of increasing importance in the United States, e.g., it continues to be a chronic problem in St. Augustine grass (*Stenotaphrum secundatum*) (Uddin, et al. 2003; Jo, et al. 2005) and it is reported to be a serious problem in common cool season grasses; including annual ryegrass (*Lolium multiflorum*), perennial ryegrass (*Lolium perenne*) (Uddin, et al. 2003), and tall fescue (*Schedonorus phoenix*). This disease is particularly devastating because there are limited options for chemical control, and resistance (some strains) to commonly used strobilurin fungicides has been documented (Vincelli and Dixon 2002). *Pyricularia oryzae,* which is distributed over a wide geographic region, kills the plant through severe leaf blight.

Rhizoctonia disease of turfgrasses (brown patch) is caused by *Rhizoctonia spp.* which are present in practically all soils throughout the world (Parmeter 1970). *Rhizoctonia solani* infects most turfgrasses, causing foliar and seedling blights, and damping off.

The most widely used preventative fungicides for control of gray leaf spot and Rhizoctonia diseases include QoI fungicides (strobilurin chemicals including azoxystrobin, trifloxystrobin and pyraclostrobin), and the benzimidazole thiophanate-methyl. QoI agents inhibit the Q site of Cytochrome bc1 in the mitochondrial Complex III (Brent and Hollomon 2007). A potential problem with their use is the development of resistance (Brent and Hollomon 2007), e.g., resistance to QoI fungicides in *P. oryzae* isolates from turfgrass was first reported in 2001 (Farman 2001). Thiophanate-methyl resistance has been found in *M. oryzae* isolates from rice (Kim 2009).

The objective of this work was to investigate the potential of T-, and S-phylloplanins contained in T-, and S-LWWs for inhibiting turfgrass diseases that are caused by the Ascomycete, *P. oryzae* and the Basidiomycete, *R. solani* using hyphal extension, Petri plate assays, and *in planta* disease assays.

2.4. Materials and Methods:

2.4.1. Fungal cultures:

Single-spore cultures of *P. oryzae* (lpky98-1) were obtained from Dr. Mark Farman, University of Kentucky Plant Pathology Department. Cultures were grown at

23°C on oatmeal agar media as described by Valent, et al. (1984), under constant light of 20 watt Vita-lux© fluorescent grow lamps (Sylvania, St. Danvers, MA). Plates were overgrown within 20 days. A single isolate (hyphal tip method) (Brown 1924) of *R. solani* from creeping bentgrass was provided by Dr. Paul Vincelli at the University of Kentucky, Plant Pathology Department. *R. solani* was grown at 23°C under constant light as above on standard PDA media made from Difco^{TM} PDA agar mix. These cultures overgrew the plates in about 10 days. Standard sub-culturing methods were used to keep mycelia viable. Long-term storage was on sterilized Whatman #1 filter paper disks placed on actively growing cultures of the fungi until the mycelia completely covered the disks. The disks were removed, air dried, and placed into 2ml cryovials and stored at -20°C.

2.4.2. LWW (phylloplanin) Preparation:

N. tabacum, cv. T.I. 1068 LWW containing T-phylloplanin shown to consist of 4 related glycopeptides (Kroumova et al., 2007) was collected by washing 120 total, stalk-cut field-grown plants (early flowering stage) in 64 liters of water (20 second wash/plant). *Helianthus annus* L., cv. 'Dove Hybrid Black Oil' LWW was washed (20 seconds) from leaves of 7 week old greenhouse-grown plants. LWWs were filtered through glass wool to remove particulate debris and frozen at -40°C until lyophilized. To make stock solutions lyophilized powders were re-suspended in water to concentrations of 112 mg/ml (T-phylloplanin) and 19 mg/ml (S-phylloplanin), unless otherwise noted, and centrifuged to remove insoluble materials. Total protein equivalents were determined to be 3.73 and 0.63 mg/ml for tobacco and sunflower, respectively (Pierce BCA^{TM} protein assay, Thermo Scientific). SDS-PAGE analysis showed these phylloplanin preparations to be similar to those described earlier (Shepherd, et al. 2005;

Kroumova, et al. 2007). The method for treatment of LWW's with ProteinaseK to destroy/greatly reduce phylloplanin and subsequent verification of phylloplanin proteolysis by SDS-PAGE was as previously described (Kroumova, et al. 2007). To estimate leaf area equivalents of LWWs (for Figure 1 experiments) all leaves of typical tobacco or sunflower plants were traced on uniform weight paper, tracings were cut out and weighed to determine total leaf area of a typical plant. Total surface area equivalents of LWWs were determined by multiplying surface area/typical plant times the number of plants washed for a preparation (Kroumova et al., 2007).

2.4.3. Control Chemical Fungicides:

Commercial fungicides used as positive inhibition controls in *in vitro* hyphal inhibition assays were pyraclostrobin ([methyl 2-[1-(4-chlorophenyl) pyrazol-3-yloxymethyl]-N-methoxycarbanilate]), Insignia™ (ornamental label rate used - EPA# 7969-184, BASF, Research Triangle Park, N.C.) and the azoxystrobin ([methyl (2E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy] phenyl}-3-methoxyacrylate]), Heritage TL™(EPA# 100-1191, Syngenta, Greensboro, N.C.). Only the later was used in plant treatment assays. For Insignia™ the label rate for brown patch is 10 oz in 100 gallons at 20% a.i.. For a stock, 1.8µl of Insignia™ was diluted in 10ml to make a 0.027pg of a.i. per µl. In hyphal extension assays we used 20 µl per 1 cm diameter paper disk, which equals 0.54 pg of a.i. per disk. For Heritage ^{TL} the label rate (for brown patch & gray leaf spot) is 2fl oz per 1000 sq. ft^2. (0.8 lbs. of a.i. per gallon). This equals to 95.9 µg per µl on a 1cm round paper disk. We diluted 2µl of Heritage ^{TL} in 10ml of water to yield 0.019 µg per µl. Where used, we applied 20µl per paper disk or 0.38 µg of a.i. per disk. For in plant assays we sprayed 10ml per pot. Using 2µl of Heritage ^{TL} in 10ml as a stock, we applied 191.8 µg of a.i. per pot.

2.4.4. *In vitro* Hyphal Inhibition Assays:

P. oryzae and *R. solani* were grown on oatmeal and PDA agar media, respectively, but the assay method (modified from Dhingra and Sinclair, 1995) was the same for both pathogens. Agar media were aseptically inoculated with 5mm x 5mm pieces of actively growing culture, placed directly in the center of a 95x15 mm Petri plates containing appropriate media. Plates were then placed under constant light (Vita-lux© fluorescent grow lights) at 25°C. After ~48 hours, the growing fungal mat was ~1.0cm in diameter. At this time, 1.0 cm diameter, sterile paper disks of Whatman #1 filter paper were placed at the 0, 90, 180, and 270 degree positions on the agar surface, near the plate edge. Samples (20 µl) containing T-or S- LWW (3.73mg protein/ml of T-, or 0.63 mg protein/ml S-LWW) in sterile water, T- or S-LWWs treated with ProtK, chemical fungicides, or water/ProtK control were added directly to the disks. Preparation of the water/ProtK control was as previously described (Kroumova, et al., 2007). Hyphal growth was checked twice per day to observe growth and sporulation (*P. oryzae* only), as it approached the region of the treatment discs. The zone of inhibition was measured as millimeters distance between the arc representing the zone of inhibition and the nearest edge of the paper disc, in line with the center of the plate. Independent experiments were conducted 3 times, 1 plate per treatment with three replications.

Hyphal inhibition assays to test for possible SHAM effects were also performed as described by (Vincelli, 2002) comparing the presence and absence of salicylhydroxamic acid (SHAM, Cat# 156576, MP Biomedicals Solon, OH). For these tests, 5 ml of 100 µg/ml SHAM, in water or water alone, were spread on the surface of agar medium in Petri plates and allowed to dry before application of paper discs and samples. Independent experiments were conducted 3 times, 1 plate per experiment.

2.4.5. Plant materials:

Annual ryegrass, *Lolium multiflorum* cv. 'Linn' (designated AR), perennial ryegrass, *Lolium perenne* cv. 'Double Eagle blend' (PR), and creeping bentgrass, *Agrostis stolonifera* L. cv. 'L-93' (CB) were used in this study. Break-away, 18-unit pack, plastic containers (each unit henceforth referred to as a pot, 9x6x6 cm) were filled with sterile Pro Mix (Kimbert, Riviere-Ouelle, Quebec, Canada), and then thoroughly watered. Each 18-unit pack was seeded with one species only. Seeds were placed on Pro Mix surfaces at a rate of ~ 50-60 seeds per unit, then covered with ~5 mm of Pro Mix, and lightly misted. The containers were placed in a 23°C growth chamber, under 24 hours of light, with humidity domes. After germination (4-7 days), the domes were removed and plants were grown for another 3 weeks until optimal growth was established (clipped 2x during this period). Fertilizer (20-20-20) was applied once per week at a rate of 3.7 g per liter, 20 ml per pot. Individual pots were inoculated and used after their 5th week of growth.

2.4.6. Plant Pretreatments Prior to Inoculations:

All grass types had their respective treatments (10 ml/pot, using a glass atomizer spray apparatus and air pressure) applied 20 minutes before inoculation, during which time leaves became visibly dry. Treatments included: 1) a water-treated, un-inoculated control; 2) Heritage TL™ with inoculation; 3) T-or S- LWW with inoculation; 4) T- or S-LWW treated with ProtK, with inoculation; 5) water control with inoculation. Independent experiments were made 3 times, 1 pot per treatment.

2.4.7. Inoculation of Plants:

A Petri plate culture of *P. oryzae* was grown (above fungal growth conditions) until sporulation occurred (~ 7 days). After addition of sterile water and gentle agitation of the agar surface with a sterile glass rod, conidia were collected by decantation. The conidial suspension was centrifuged at ~140 x g for 3 minutes, and the pellet was washed 3X with distilled water, followed by centrifugation, before being diluted with sterile water to a concentration of 1.0 x 10^5 conidia per milliliter (measured with a hemocytometer). The suspension was applied to plants (pretreated as above) using a glass atomizer spray apparatus and air pressure as above. AR- and PR-containing pots were placed into 3 liter individual polyethylene bags, and while holding open the top, 10ml of inoculum containing suspension was sprayed onto test grasses to completely cover leaf blades. Bags were sealed with twist ties and placed in the dark at 23°C for 48 hours and then exposed to a 12/12 hour light cycle at 23°C for up to seven days, or until symptoms of gray leaf spot had developed.

R. solani Petri plate cultures were left to grow until sclerotia developed (~9 days). A 1x1x0.2 cm piece of agar from a sclerotium-producing culture was placed on a toothpick and suspended 2.5 cm above the surface of the soil in the middle of pots containing growing AR, PR, and CB. Pots were then placed into 3 liter polyethylene bags and misted with sterile water until all leaf surfaces were wet. The bags were then sealed with twist ties and placed in the dark at 23°C for 36 hours, then exposed to a 12/12 hour light regime at 23°C and checked daily until disease symptoms appeared. Independent *in planta* experiments were made 3 times, 1 pot per treatment.

2.4.8. Disease Assessment Analysis:

The experimental design for *in planta* experiments was completely randomized. Disease severity was rated by whole pot average visually on a scale of 0-5 where: 0, 1, 2, 3, 4, 5 represented no disease, 20%, 40%, 60%, 80% disease and complete plant death, respectively.

Turfgrass health was assessed visually using several criteria; density, color, uniformity, and the presence or absence of disease. Disease severity ratings were made 3 days after the first appearance of symptoms. Similar disease ratings were found by 3 individuals, but statistical evaluations were made using the ratings of one observer.

2.4.9. Statistical Analyses:

Data from hyphal extension experiments (except SHAM tests) and *in planta* assays were analyzed using PROC GLM of SAS (SAS Institute, Cary, NC). The non-parametric Tukey's method of analysis was used. Means were separated by F-protected HSD tests.

2.5. Results and Discussion:

2.5.1. Hyphal Inhibition Assays:

Hyphal extension assays showed that T-phylloplanins and the chemical control Insignia^{TM} inhibited growth of both *P. oryzae* and *R. solani*. As shown in Figure 1A (one of 3 replicate assays) hyphal growth was inhibited (zones of inhibition delineated

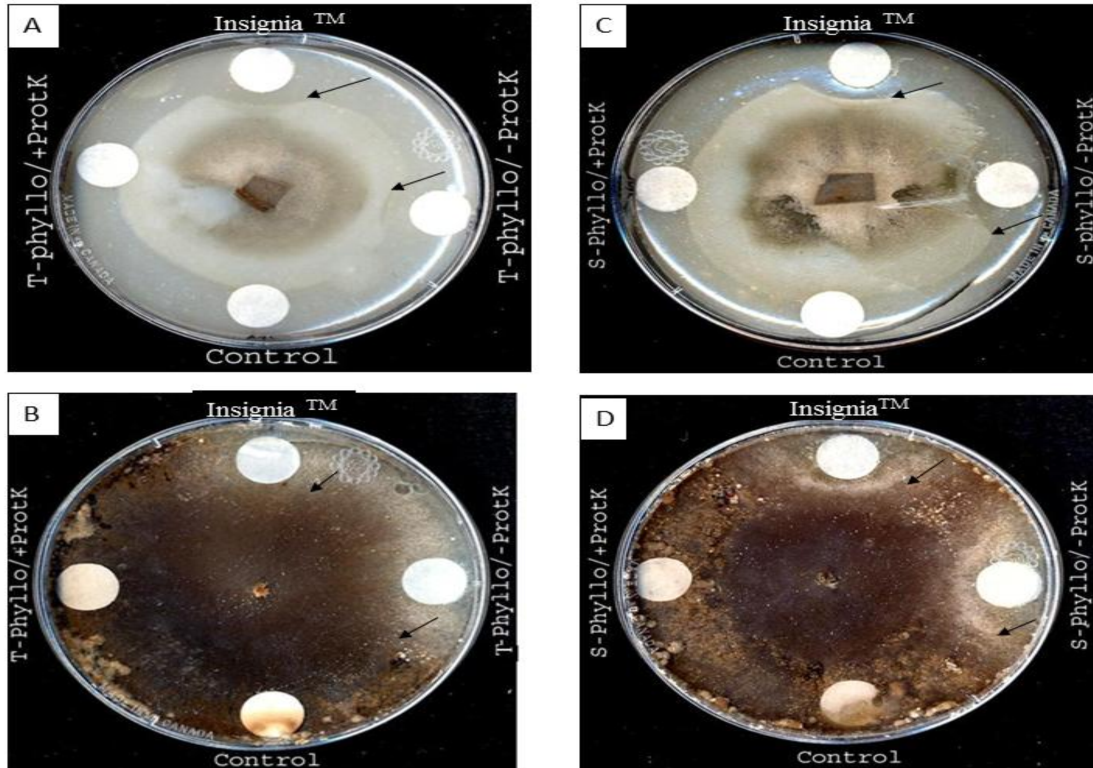
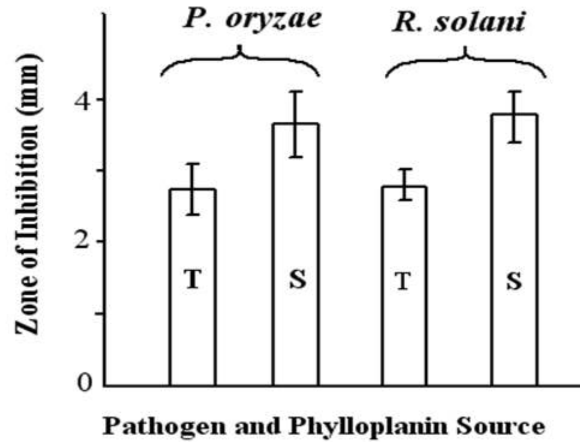


Figure 1 ***In vitro*, hyphal inhibition, Petri-plate, assays of T-LWW (T-phylloplanin) effects (A and B) and S-phylloplanin effects (C and D) on growth of *Pyricularia oryzae* (A and C), or *Rhizoctonia solani* (B and D). Insignia^{TM} (upper most filter paper discs) was used as a positive protection control. Water (lower most disk) was used as a solvent control. LWWs containing T-, or S-phylloplanins were applied in water. The disc containing phylloplanin pre-treated with ProteinaseK to destroy phylloplanins is at the left in each case, and that with phylloplanin not treated with ProteinaseK is at the right. ProteinaseK controls (incubation of ProtK with water only) showed no inhibition (not shown). A clear zone of inhibition (arrows) was observed with Insignia^{TM} and intact T-, or S-LWWs containing phylloplanins, in all cases.**

with arrows) by T-phylloplanin and by the commercial fungicide Insignia™ (labeled for use against *P. oryzae and R. solani*,). When T-phylloplanin in LWWs was destroyed by incubation with ProtK, hyphae inundated the sample disk, as was the case with water controls. No protection was observed with the ProtK control (ProtK incubation in water alone), demonstrating no effect on fungal growth of treatment with this insoluble protease in water (data not shown). This control is required because earlier (and again here, see Figure 3, below) we observed that a small amount of soluble ProtK enzyme is released from acrylic beads during incubation with LWWs, and this is not entirely removed upon centrifugation of beads (Shepherd, et al. 2005). However, the amount released apparently does not affect hyphal growth in this *in vitro* assay, or in *in planta* assays (see below). It was shown earlier that the solubilized, trace ProtK had no impact on *P. tabacina* spore germination, or *in planta* blue mold disease on tobacco (Shepherd, et al. 2005). S-phylloplanin gave protection against *P. oryzae* that was similar to that found with T-phylloplanin (Figure 1C). Results of hyphal extension assays monitoring growth of *R. solani, in vitro* are shown in Figures 1B and D. As observed with *P. oryzae*, intact T-, and S-LWWs (containing T-, and S-phylloplanins), and Insignia™ showed inhibition of *R. solani* hyphal growth. Quantification of results in Figure 1 is shown in Figure 2.



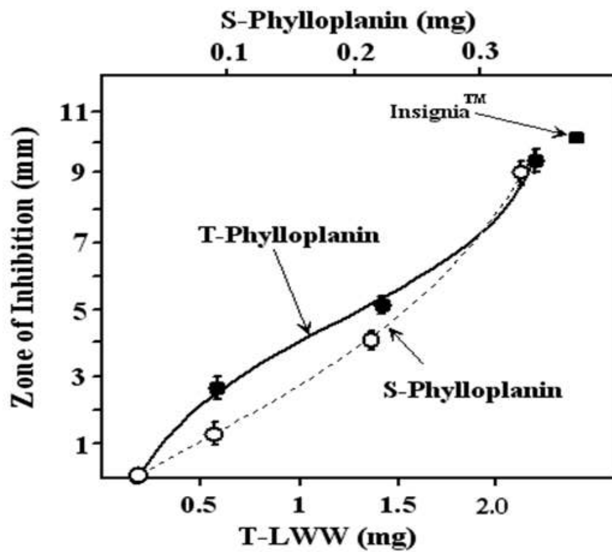


Figure 2 Quantitative representation of zones of inhibition in *Pyricularia oryzae* and *Rhizoctonia solani in vitro* hyphal inhibition assays, and LWW protein concentration dependence of inhibition responses. Data of Figure 2A show means and standard deviations from zone of inhibition measurements (no ProtK treatment), comparing T-, and S-phylloplanin on a leaf surface area equivalency basis (see Kroumova et al., 2007). Results represent data from the experiment of figure 1 and that from two additional experiments. T and S designations represent T-, and S-LWWs, respectively. Figure 2B shows the magnitude of the zones of inhibition versus LWW protein concentrations.

Since cultures shown in Figures 1A and 1C (and similarly Figures 1B and 1 D) were started simultaneously it is possible to quantitatively compare impacts of T-, versus S-

phylloplanins for their ability to inhibit *P. oryzae* and *R. solani* hyphal growth. As shown, the average widths of zones of inhibition from three experiments (adjusted to equal surface-area-equivalents of LWWs used) indicate that inhibition activity of S-phylloplanin is greater than that of T-phylloplanin, on a surface-area-equivalent basis. Similar results were found earlier when inhibition activities of T- and S-LWWs were compared in blue mold inhibition assays (Kroumova et al., 2007). In the course of our work numerous SDS-PAGE experiments comparing protein silver staining of T-, and S-LWWs prepared from identical surface equivalents indicated that there is much less total protein in LWWs of S-phylloplanin (Kroumova, Shepherd et al. 2007, and see below) than in T-phylloplanin preparations. This comparison suggests that S-phylloplanin is more active than T-phylloplanin on a surface area equivalency and protein basis, at least for the tissues from which they were prepared (young plants for S-phylloplanin and mid-maturity plants for T-phylloplanin).

To summarize, Figure 1A-D data show that T- and S-phylloplanins are clearly active in inhibiting hyphal growth of *P. oryzae* and *R. solani*, *in vitro*. Figure 2 shows the concentration dependence relationship between the widths of the zones of inhibition versus protein concentration of LWWs. Dilutions of T-, and S-phylloplanin stock solutions were used to obtain these data. Earlier we showed that % inhibition of *P. tabacina* spore germination and *in planta* disease inhibitions were phylloplanin protein concentration dependent (Shepherd, et al. 2005). As found earlier with *P. tabacina* for T-, S- and *Datura*-phylloplanins (Kroumova, et al. 2007), there appears to be a threshold concentration of T-, and S-phylloplanin (no activity below 0.25mg of T-phylloplanin or 0.05 mg S-phylloplanin) for obtaining inhibition of *P. oryzae* and *R. solani* hyphae

(Figure 1). We note that recent results indicate that the mode of action of phylloplanins in inhibiting both spores and hyphae is via ion leakage (unpublished). Thus phylloplanins and the positive inhibition control used here (Insignia ™ - inhibits mitochondrial respiration) have different modes of action, so Insignia™ serves as an independent positive control.

2.5.2 Phylloplanins are Protective Factors in LWWs:

The SDS-PAGE, silver stained gel of Figure 3 shows the impact of ProtK

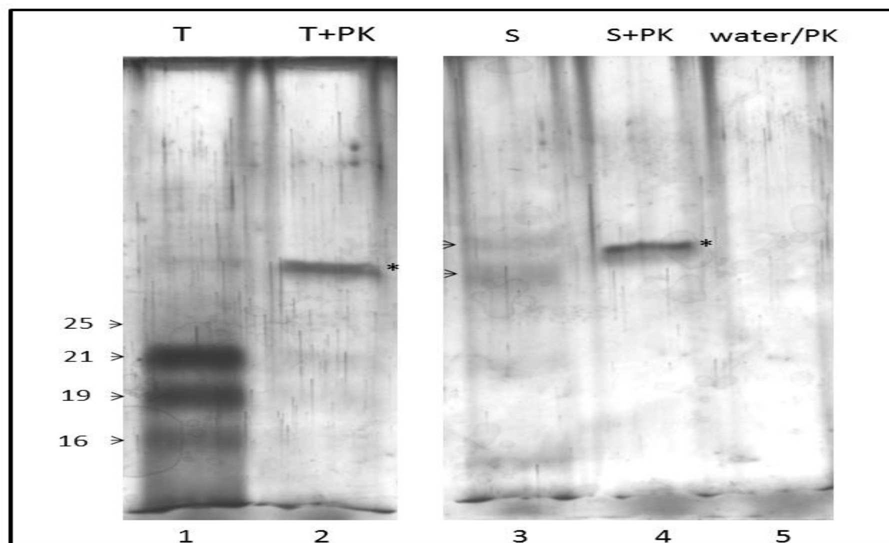


Figure 3 SDS-PAGE shows: lane 1 (left), the 4 characteristic 4 bands (arrows, 25, 21, 19, 16 kDa) of T-phylloplanin in T-LWW, (T); lane 2, T-LWW after treatment with ProtK (T+PK); lane 3, the two major bands (arrows) of S-phylloplanin in S-LWW (S); lane 4, S-LWW water treatment with ProtK (S+PK); lane 5, water/PK control (PK control).

treatment on T-, and S-phylloplanins contained in LWWs, and thus verifies the efficacy of ProtK treatment as a control in fungal inhibition assays of Figures 1, (and also 5 and 6, see below). Lane 1 (left) of this silver-stained SDS-PAGE gel shows the 4 characteristic protein bands of T-phylloplanin (16, 19, 21, and 25kD – may be variants in glycosylation,

also in some preparations the 25kD band may not be visible, Kroumova, et al. 2007). The degradation of these proteins upon ProtK treatment is seen in Lane 2. The only protein band present in lane 2 (and lane 4) has the well-established molecular size of soluble ProtK (28.9 kDa), and is concluded to represent enzyme solubilized from acrylic beads during incubation with either T-, or S-phylloplanin. This band also occurs when bovine serum albumin is treated with ProtK beads. Interestingly, the apparent ProtK solubilization observed here does not occur when insoluble ProtK is incubated with water only (lane 5). Lane 3 shows S-phylloplanin of S-LWW and lane 4 the loss of protein in the region of 35-38kD (asterisks) upon ProtK treatment. The results of Figure 3 are consistent with our observation that S-LWW contains less phylloplanins per unit leaf surface area than tobacco (compare lanes 1 versus 3), and that phylloplanins of these two plants differ in molecular weight as observed earlier (Shepherd, et al. 2005). However, like T-phylloplanin, S-phylloplanin also appears to be highly glycosylated (Kroumova, et al. 2007). Most important to this report, Figure 3 demonstrates that T- and S-phylloplanins are completely or substantially digested by ProtK and therefore this control can be used to demonstrate that protein of LWWs (phylloplanins) are responsible for anti-fungal activities observed.

Related to the use of ProtK as an indicator that phylloplanins are responsible for anti-fungal activity of LWW's is the observation that LWW prepared from tobacco cv. T.I. 1112 (closely related to T.I. 1068) also inhibits *P. oryzae* and *R. solani*, in a ProtK-dependent manner (data not shown). Tobacco T.I. 1112, like T.I. 1068, synthesizes phylloplanins and possesses short glandular trichomes, but lacks tall, glandular trichomes that produce diterpenes or sugar esters (not shown). Thus, we conclude that residual

diterpenes and sugar esters in T-LWW from T.I. 1068 are not principally responsible for antifungal activities observed here. Similar results were observed in blue mold disease assays (Shepherd et al., 2005).

Results showing the lack of impact of SHAM on the ability of T- and S-phylloplanin to inhibit *P. oryzae* and *R. solani* hyphal extension are shown in Figure 4. SHAM did not impact inhibition by Heritage™ either, presumably because fungi were sufficiently inhibited at the Q site of respiration, and alternate oxidase, if contributing, did not provide sufficient ATP in the presence or absence of SHAM. These results are consistent with a mechanism of action for T-phylloplanin that is different from that of the strobilurin commercial fungicides used as the positive inhibition controls in the experiments presented here. Thus, no involvement of the alternate oxidase pathway is indicated for T-phylloplanin. We also note that the QoI fungicide, Heritage™ was used in the experiments of Figure 4, the commercial fungicide used for *in planta* assays described below.

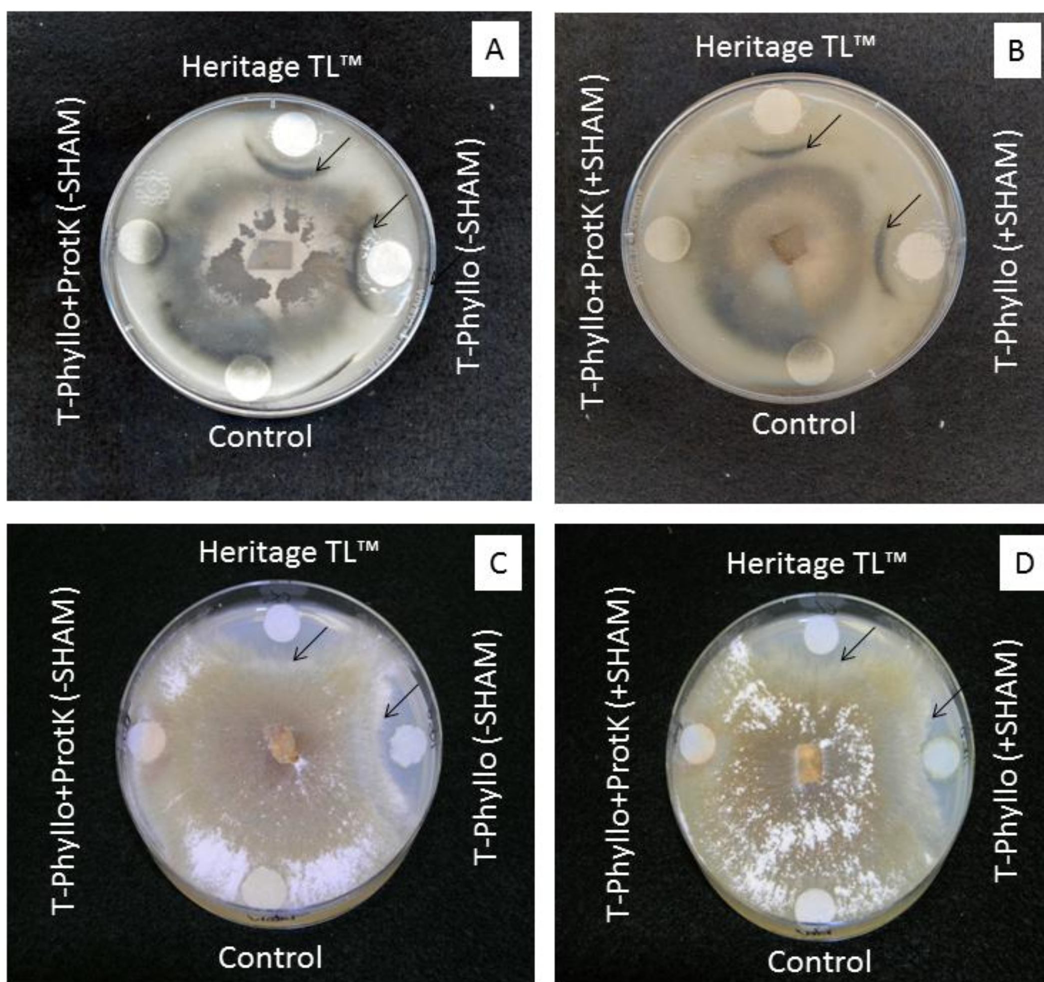


Figure 4 *In vitro*, hyphal inhibition, Petri-plate, assays showing the lack of impact of SHAM on T-LWW (T- phylloplanin) inhibition of *Pyricularia oryzae* (A and B), or *Rhizoctonia solani* (C and D). Heritage TL^{TM} (upper most filter paper discs) was used as a positive inhibition control. Water (lower disc) was used as a solvent control. The disc containing T-LWW pre-treated with ProteinaseK to destroy phylloplanins is at the left in each case, and that with T-LWW not treated with ProteinaseK is at the right. ProteinaseK controls showed no inhibition (not shown). A clear zone of inhibition (arrows) was observed with Heritage TL^{TM} and intact T-phylloplanin containing LWW in all cases.

2.5.3 *In-Planta* Disease Assays: T-phylloplanin containing LWW:

Impacts of spraying AR and PR with T-phylloplanin (or control solutions) prior to inoculation with *P. oryzae* or *R. solani* (also CB, with *R. solani*) are shown in Figure 5 (one of 3 replicate experiments shown). All panels (A to E) show five pots: 1. Water control, without inoculation; 2. Heritage™ with inoculation; 3. T-LWW (T-phylloplanin) with inoculation; 4. ProtK treated T-LWW with inoculation; and 5. Water control with inoculation. Water/ProtK controls were made in several experiments, and disease severities were like that of water alone treatments (not shown). Figure 5A shows that T-LWW application prior to spraying with *P. oryzae* spores (treatment 3), like Heritage™ (treatment 2) protected AR plants, while spraying with treatments 4 and 5 did not protect against this disease. Insets in 5A and 5B show typical leaf blades from pots 1 to 5, and show disease symptoms typical of *P. oryzae* on AR and PR leaves, respectively, in treatments 4 and 5. Panels C, D, and E of Figure 5 show results for *R. solani* infection assays on AR, PR, and CB, respectively. Insets in 2.5C and 2.5D show typical leaf blades from pots 1 to 5, and describe disease symptoms typical of *R. solani* on AR and PR leaves, respectively. In 2.5E, note the excessive damping off near the soil, and lesser leaf color in treatments 4 and 5, as compared to treatments 1 to 3. These results regarding *in planta* disease protection are consistent with results of *in vitro* assays (Figure 1), and show that pre-treatment of grasses with T-LWW containing T-phylloplanin protects AR and PR against gray leaf spot disease, and AR, PR, and CB against brown patch disease. The continued culture of the plants shown in Figure 5 resulted complete death of plants with treatments 4 and 5, and disease did not develop after treatments 1 to 3. This suggests that inocula of both fungi were unable to invade plants treated with intact T-phylloplanin, and lost viability before growth of new, presumably unprotected

tissue. However, further study is needed to determine if resistance would persist on phylloplanin treated plants under conditions of higher disease pressure. Since plants were sprayed with a large volume (10 ml) of *P. oryzae* spore suspension in water, or 10 ml water after applying the agar piece containing *R. solani* after phylloplanin treatments, phylloplanins were apparently not easily washed off, and appeared to have adhered to plant aerial surfaces to allow protection. We note that in preliminary experiments we observed some protection against *P. oryzae* on AR and PR using 10 ml of 0.373 mg protein/ml of T-LWW (1/10th the concentration used here). Concentration requirements for adequate protection are currently being investigated in field trials.

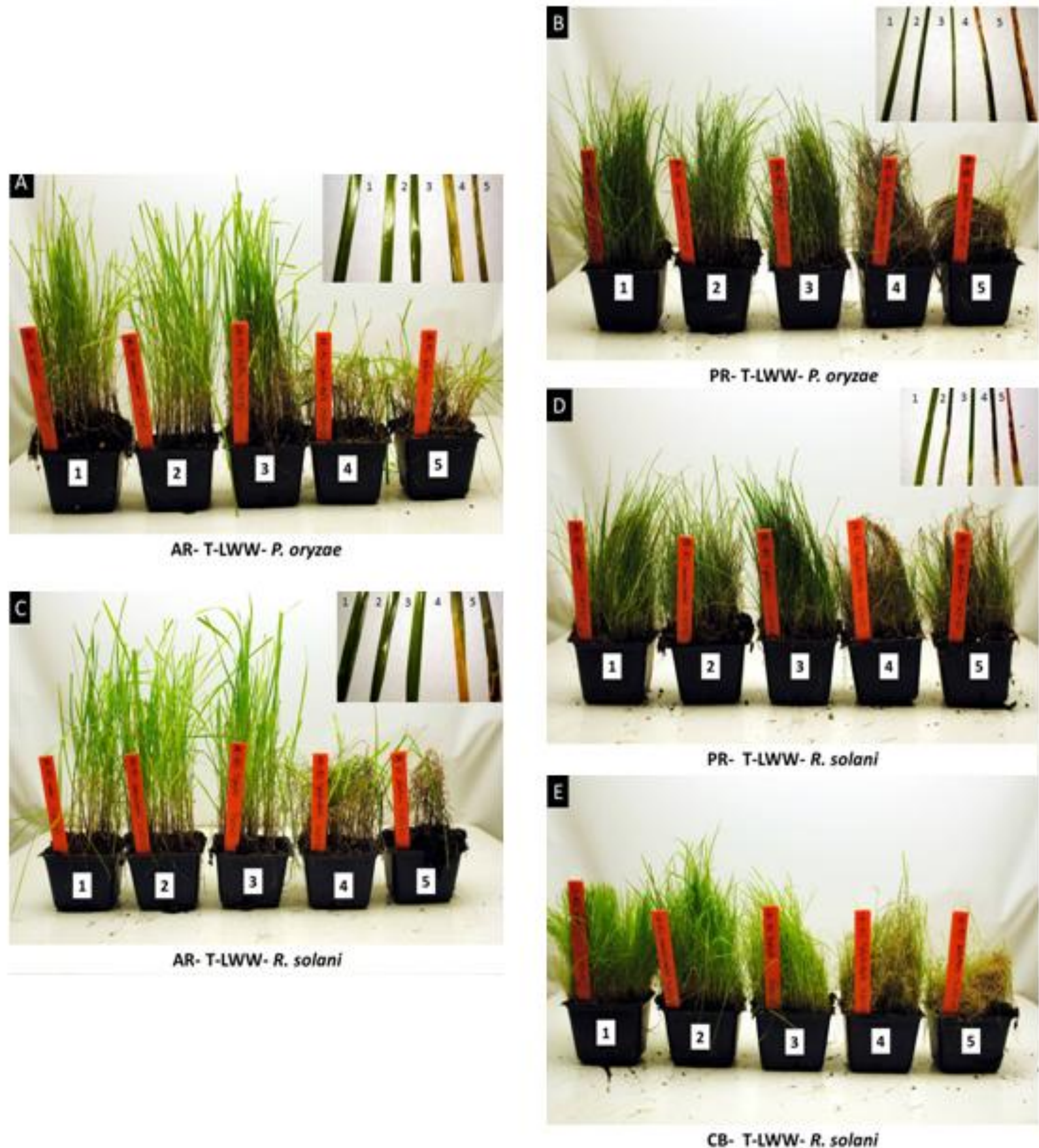


Figure 5 Effects of spraying LWW containing T-phylloplanin on grasses prior to inoculation with conidia of *Pyricularia oryzae,* or hyphae of *Rhizoctonia solani*. AR is annual ryegrass, PR is perennial ryegrass, and CB is creeping bentgrass. T-LWW is tobacco LWW containing T-phylloplanin. Treatments for pots labeled 1 through 5 were: 1) water only, no inoculation control; 2) Heritage TL™ with inoculation; 3) T- or S-LWW with inoculation; 4) T- LWW treated with ProtK, with inoculation; 5) water control with inoculation. Note the healthy growth of grass in treatments 1 to 3 and deterioration of plants in pots 4 and 5. Insets show symptoms typical of diseases on AR leaf blades of treatments 4 and 5, and healthy leaf blades of treatments 1 to 3. A = annual ryegrass/T-LWW/*P. oryzae*, B = perennial ryegrass/T-LWW/*P. oryzae*, C = annual

ryegrass/T-LWW/*R. solani*, D = perennial ryegrass/T-LWW/*R. solani*, E = creeping bentgrass/T- LWW/*R. solani*.

2.5.4 *In planta* Disease Assays: S-phylloplanin containing LWW:

Similar experiments to those described in Figure 5 were made using S-LWW containing S-phylloplanin (Figure 6, one of 3 replicate experiments shown). As shown in Figure 6A, S-phylloplanin protected AR from gray leaf spot disease. Similarly, S-phylloplanin protected PR from *P. oryzae* (Figure 6B). Panels 6C, D, and E show the impacts of S-phylloplanin containing LWW (and controls) on brown patch disease in AR, PR, and CB, respectively. Results of *in planta* assays of Figure 6 are consistent with phylloplanin protection observed with *in vitro* assays of Figure 1. Continuation of *in planta* assays beyond the time shown in Figures 5 and 6 resulted in death of plants not treated with intact phylloplanins (treatments 4 and 5). Intact phylloplanin treated plants flourished, despite the fact that they were only treated once prior to infection.

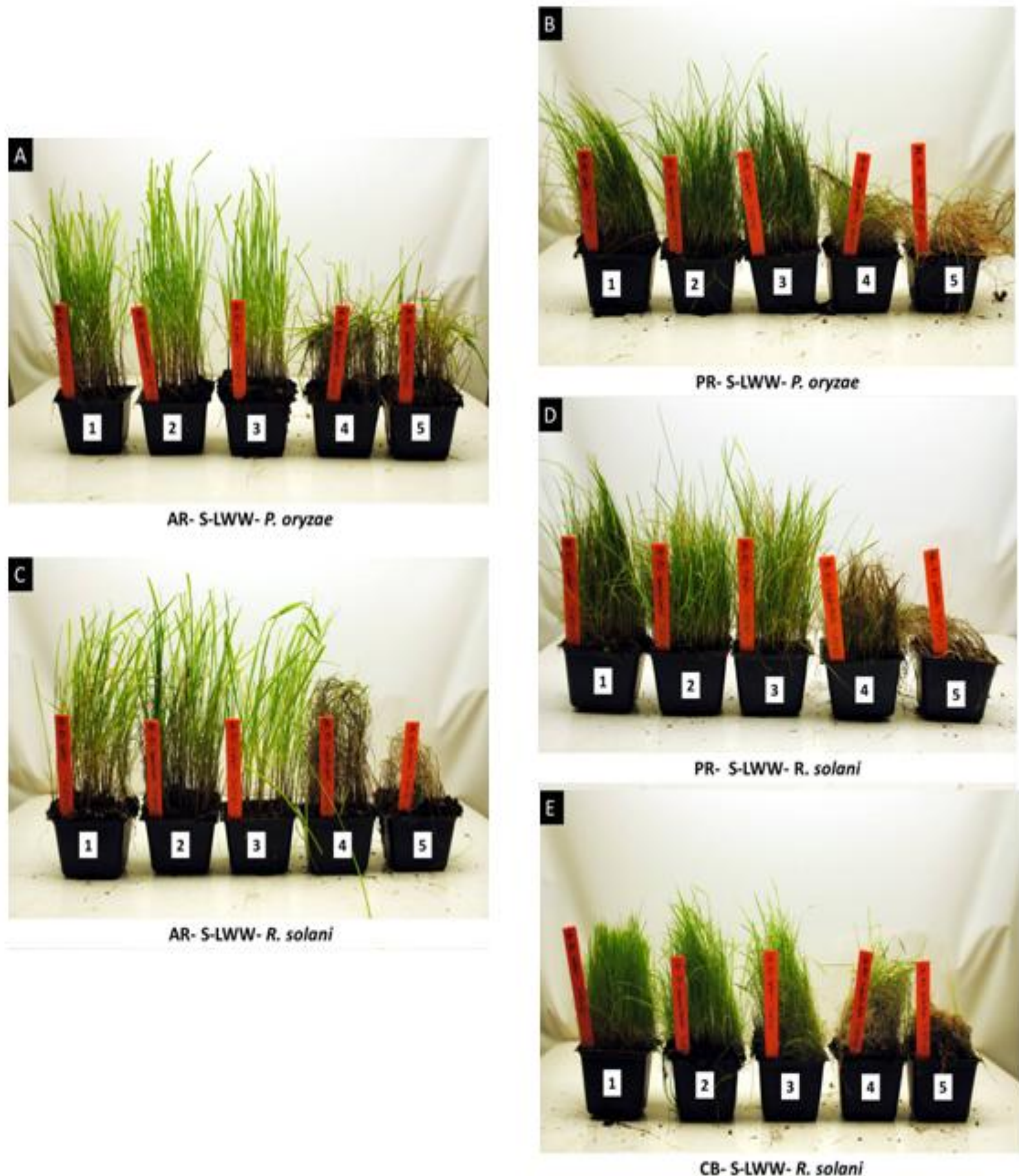


Figure 6 Effects of spraying LWW containing S–phylloplanin on grasses prior to inoculation with conidia of *Pyricularia oryzae* or hyphae of *Rhizoctonia solani*. Designations are as described in the legend of Figure 5. A = annual ryegrass/S-LWW/*P. oryzae*, B = perennial ryegrass/S-LWW/*P. oryzae*, C = annual ryegrass/S-LWW/*R. solani*, D = perennial ryegrass/S-LWW/*R. solani*, E = creeping bentgrass/S-LWW/*R. solani*

2.5.5 Statistical Analyses:

Statistical analyses of disease severities observed in experiments of Figures 4 and 5 are represented by data in Table 1, (utilized results of all 3 replicates). Rating data were analyzed using the Tukey's analysis as described in the methods section. There were no significant differences among runs of the experiments (Table 2). All data reported are the means of three runs. Additionally, there were no significant interactions among the grass species or pathogen species tested. Hence, data reported are for each individual pathosystem (e.g., *R. solani* infecting perennial ryegrass).

2.6. Conclusions:

Results show that leaf water washes of tobacco and sunflower which contain phylloplanins inhibit hyphal growth of *P. oryzae* and *R. solani* in *in vitro* assays, and that spraying plants with LWWs containing T- or S-phylloplanins prior to inoculation provide protection against these pathogens on annual and perennial ryegrasses, and also protection against *R. solani* on creeping bentgrass. T-phylloplanin glycoproteins are well studied while S-phylloplanin(s) are not yet as well characterized. Nevertheless, proteins of both T- and S-LWWs are shown to be active in the disease protections observed. The results add to earlier demonstrations that T- and S-phylloplanins provide partial protection against blue mold disease of tobacco caused by the oomycete, *Peronospora tabacina*. Thus, results to date demonstrate the ability of T- and S-phylloplanins to protect plants against diseases caused by at least one member of three major phyla of fungi/fungi-like pathogens, an Ascomycete, a Basidiomycete, and an oomycete. We recently showed *in vitro* that T-phylloplanin provides resistance against the Zygomycetes *Rhizopus stolonifera* (not shown). Thus, at least one representative of all four of the

fungi/fungi-like classes is affected by at least T-phylloplanin. In other experiments we have also observed inhibition of a mixed fungal population consisting of primarily penicillum (Ascomycete) species. The extent of persistence of protection observed here is not inconsistent with phylloplanin occurrence in the hostile environment of the aerial plant surface and suggests that these phylloplanins may be useful as surface protectants against fungi pathogens in high maintenance turf grass settings.

2.7. Acknowledgements:

We acknowledge Dr. Paul Vincelli and Dr. Mark Farman for generously supplying disease organisms and for advice on culturing organisms, Ed Dixon, Etta Nuckles, and Douglas Brown for advice on maintaining organisms, and Dr. Paul Vincelli for reviewing the manuscript.

Table 1 Mean disease severity, with statistical analysis for: grass type (AR, PR, CB), pathogen, and phylloplanin type following treatment with water, commercial fungicide, phylloplanin, and proteinase K treated phylloplanin. Means are pooled from three separate experiments, 1 pot per treatment.

		AR		PR		CB
Phylloplanin type[†]		***P. oryzae***	***R. solani***	***P. oryzae***	***R. solani***	***R. solani***
Tobacco	Water	0	0	0	0	0
	Heritage TL[‡]	0	0	0.3333	0	0
	T-phyllo[§]	0.6667	1	0.3333	0	0.6667
	T-phyllo+Prot K[¶]	3	3.3333	3.3333	4.3333	3.6667
	Infection[#]	4.3333	5	4.3333	4.6667	5
	$LSD_{0.05}$	0.876	1.6486	2.0276	0.9635	0.892
	CV	29.092	46.907	43.128	18.976	16.941
Sunflower	Water	0	0	0	0	0
	Heritage TL[‡]	0.3333	0	0.3333	0.3333	0
	S-phyllo[§]	1	0.3333	0.3333	0.6667	0.3333
	S-phyllo+Prot K[¶]	3.3333	3.333	3.3333	3.6667	3.3333
	Infection[#]	4.6667	4.6667	4.3333	5	5
	$LSD_{0.05}$	1.2394	0.9095	1.7081	2.4156	0.892
	CV	35.265	28.983	36.332	44.294	18.244

[†]Two separate types of phylloplanin were used with identical treatment and pathosystems

[‡]Heritage TL was used as chemical control, azoxystrobin applied at 6g a.i. ha^{-1}

[§]T- and S-phyllo was applied at 3.73 mg/ml and 0.63 mg/ml respectively

[¶] T- and S-phyllo+Prot K was applied at 3.73 mg/ml and 0.63 mg/ml respectively

[#]Infection was applied at this rate

Table 2 Analysis of variance of hyphal extension assays and in planta assays. For Hyphal extension assays means of the zones of inhibition from three separate experiments, 1 plate per experiment were used. For in planta assays mean disease severity from three separate experiments, 1 pot per treatment were used.

Tobacco Phylloplanin

	df	Hyphal Extension		*in Planta*				
				AR		PR		CB
		P. oryzae	*R. solani*	*P. oryzae*	*R. solani*	*P. oryzae*	*R. solani*	*R. solani*
Replication	2	NS†	NS	NS	NS	NS	NS	NS
Treatment	4	***	***	***	***	***	***	***

Sunflower Phylloplanin

	df	Hyphal Extension		*in Planta*				
				AR		PR		CB
		P. oryzae	*R. solani*	*P. oryzae*	*R. solani*	*P. oryzae*	*R. solani*	*R. solani*
Replication	2	NS†	NS	NS	NS	NS	NS	NS
Treatment	4	***	***	***	***	***	***	***

*Significant at the $p \leq 0.05$ level.

**Significant at the $p \leq 0.01$ level.

***Significant at the $p \leq 0.001$ level.

†NS= not significant.

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Chapter 3: Fungicidal Activity of Tobacco Phylloplanin on Field-grown Turf

3.1. Abbreviations:

AUDPC, area under disease progress curve; DAA, days after application; AR-annual ryegrass (*Lolium multiflorum* Lam.cv. 'Linn'); PR-perennial ryegrass (*Lolium perenne* L.cv. 'Double Eagle blend'); CB-creeping bentgrass (*Agrostis palustris* L.cv.'L-93'); BP-brown patch (*Rhizoctonia solani* Kuhn); DS-Dollar spot (*Sclerotinia homeocarpa* F. T. Bennett); GLS-Gray leaf spot (*Pyricularia oryzae* Cavara); T-phyllo, T-phylloplanin (from tobacco); ProtK- ProteinaseK; LWW-leaf water wash

3.2. Abstract:

T-phylloplanin, a natural product derived from tobacco (*Nicotiana tabacum* L.), was evaluated for the fungistatic control of the turf pathogens, brown patch (*Rhizoctonia solani* Kuhn) (BP), dollar spot (Sclerotinia homeocarpa F. T. Bennett) (DS) and gray leaf spot (*Pyricularia oryzae* Cavara) (GLS) on creeping bentgrass (*Agrostis palustris* L.cv. 'L-93') (CB) and perennial ryegrass (*Lolium perenne* L.cv. 'Double Eagle blend') (PR) turf managed as either a golf putting green or golf fairways, respectively. T-phylloplanin was compared with two commercial fungicides labeled for treatment of the above diseases. Both preventative and curative studies were conducted. T-phylloplanin treatments provided statistically similar results to commercial fungicides and in some cases provided improved disease control compared to the commercial formulations. T-phylloplanin concentration dependence was indicated when two rates (112 and 56 mg a.i/ft^2) were applied every two weeks for 8 weeks. It was concluded that T-phylloplanin was, in some cases, statistically more effective at preventing disease than curing disease, and that T-phylloplanin was as effective as commercial fungicides for preventative control.

3.3. Introduction:

Two caveats of commercial fungicides for controlling turf pathogens are their high cost, and the need to use these in integrated pest management strategies to avoid development of fungicide resistance (Couch 1995). It has been said the available fungicides have reached the apex of their viability and the pinnacle of detriment to the environment (Wedge 2005). Thus there is a need to develop alternative fungicides, ideally compounds that are more environmentally friendly, yet are efficacious and minimally prone to resistance development.

It is estimated that turf grass covers 50 million acres in the U.S., with an estimated value of $40 billion annually (Peshin, Dhawan et al. 2009). Brown patch, caused by the soil borne organism *Rhizoctonia solani,* is a disease which is widely dispersed throughout the soils of the world. This disease is most common in mid to late summer when there are extended periods with high humidity and temperatures. Brown patch mainly affects cool season turf grasses in the United States and has a propensity for bentgrass (*Agrostis sp*.), ryegrass (*Lolium sp*.), annual bluegrass (*Poa annua* L.), and tall fescue (*Festuca arundinacea* Schreb.) (Danneberger, Vargas et al. 1981). Symptoms of brown patch are variable according to environmental conditions. On wet, low mowed turf gray or purple smoke rings can be observed, but in dry and hot conditions the typical brown patches of infected turf are observed (Fidanza and Dernoeden 1996). Brown patch is a foliar disease and can infect the crown, but not the roots of turf grasses. In this study we evaluated

brown patch on creeping bentgrass under high heat, high humidity, regular low mowing height, high fertilization, and frequent irrigation.

Sclerotina homeocarpa, which is the causal agent of the disease dollar spot, is another common and widely problematic pathogen of turf. This fungus has also been classified as *Lanzia spp.*, *Moellerodiscus spp.*, or *Rutstroemi spp.* However, general convention and fungicide labeling have kept the older taxonomic classification. Dollar spot is found in turf from temperate to subtropical climates all over North America, Europe, East Asia, Australia, South America, and Africa (Couch 1995). Dollar spot disease favors temperatures between 60 and 90 F (15 to 32 C), and host species include (but are not exhaustive) Kentucky bluegrass, annual bluegrass (*Poa annua*), perennial ryegrass (*Lolium sp*.), creeping bentgrass (*Agrostis sp*.), velvet bentgrass (*Agrostis canina)*, bermudagrass (*Cynodon dactylon)*, zoysiagrass (*Zoysia japonica)*, seashore paspalum (*Pasplaum vaginatum)*, centipedegrass (*Eremochloa ophiuroides)*, and fine fescue (*Festuca sp.).* Symptoms include small circular bleached color patches that can increase to the size of a silver dollar, hence the common name (Massie and Cole 1969). The patches can become depressed and coalesce. Grayish-white, fuzzy mycelia are commonly present when the disease is active and humidity is high. Dollar spot is a foliar disease which does attack turf crowns or roots if left untreated. Dollar spot was evaluated on the same creeping bentgrass plots as the brown patch trials described above, using the same agronomic practices. Gray leaf spot caused by the pathogen *Pyricularia grisea/oryzae* , which has a close phylogenic relative known as *Cercospora zeae-mayd* (maize infecting variety), and is a particularly destructive anamorph of the rice blast disease known as *Magnaporthe grisea (*Farman *2002). P. grisea* infects foliar tissues of

turf grass and affects mainly annual and perennial rye grasses and tall fescue. The disease is of particular significance on the warm season St. Augustine grass and has been common in the Southern United States since first being reported in 1971 (Farman 2002). Recently infections have been found as far north as Long Island, the lower Hudson Valley of New York State, and Pennsylvania. This disease is of great concern to high maintenance turf managers and less of a concern to homeowners. The disease favors temperate conditions in the months of August-September, with warm night time temperatures and humid mornings with hot days (Moss and Trevathan 1987). Gray leaf spot progresses quickly and can infect large areas of turf in just a few days (Trevathan, Moss et al. 1994). Another problem with gray leaf spot is that it has shown fast mutation resulting in acquired fungicide resistance in the field which is challenging for turf managers which rely on fungicides to control the disease, as there are few fungicides which work effectively from one year/season to another(Vincelli and Dixon 2002).

Because most of the fungicide research that has been done on these diseases has tested the efficacy and longevity of commercial fungicide formulations, the objectives of this research were (1) to test the efficacy of the natural product fungicide T-phylloplanin on brown patch and dollar spot in stands of creeping bentgrass and gray leaf spot on perennial ryegrass under both preventative and curative programs and (2) to investigate concentration dependence of T-phylloplanin and (3) to compare the effectiveness of T-phylloplanin with commercial fungicide formulations.

3.4. Materials and Methods:

3.4.1. Preparation of T-phylloplanin:

N. tabacum, cv. T.I. 1068 LWW containing T-phylloplanin was collected by washing 120 field-grown plants (early flowering stage) in 64 liters of water (~20 second wash). Leaf water wash was filtered through glass wool to remove particulate debris and frozen until lyophilized. To prepare T-phylloplanin solutions lyophilized powders were re-suspended in water to concentrations of 112 mg/ml (high) and 56 mg/ml (moderate), respectively, and centrifuged to remove insoluble materials. Total protein equivalents were determined to be 4.5 and 2.24 mg/ml for high- and medium-concentrations, respectively (Pierce BCA™ protein assay, Thermo Scientific). SDS-PAGE analysis showed these T-phylloplanin preparations to be similar to those described earlier (Shepherd, et al. 2005; Kroumova, et al. 2007).

3.4.2. Test Sites:

The following sites were used in the spring/summer seasons of 2009 or 2010, or both, depending upon the occurrence of natural disease. All experiments of 2009 were repeated, but data was only collected where disease was present. All 2009 experiments were curative except for PRGLS-P_M because the expectation of gray leaf spot infection at this site was good. However, disease was not present at this site in 2010. All sites in this study contained monocultures of the respective turf mentioned below, and plots were selected for uniformity of grass stand.

Creeping bentgrass cv. 'L-93'/brown patch/curative/Spindletop farm (CBBP-Cs)

Creeping bentgrass cv. 'Sandhill'/brown patch/curative/croquet court (CBBP-Cc)

Creeping bentgrass cv. 'Sandhill'/brown patch/preventative/croquet court (CBBP-Pc)

Creeping bentgrass cv. 'L-93'/dollar spot/curative/Spindletop farm (CBDS-Cs)

Perennial ryegrass cv. Double Eagle blend/grey leaf spot/curative/Spindletop farm (PRGLS-Cs)

Perennial ryegrass cv. unknown/grey leaf spot/preventative/Marriot Golf Course (PRGLS-Pm)

Experiments with brown patch were conducted on a croquet court located at the UK Agricultural Experiment Station in Lexington, Fayette County, KY. The soil on the croquet court was a Maury silt loam (fine, mixed, mesic typic Paleudalf) and conventionally managed as a golf putting green. Plots were mowed at 4.7 mm height 5 days per week. Dollar spot was evaluated on the same plots. Additional experiments to evaluate brown patch and dollar spot on creeping bentgrass were made at the Spindletop farm using turf managed as a golf fairway. Height of cut was 1.6 cm and the turf was mowed 3 days per week. The soil was a USGA specification sand-based system. Gray leaf spot trials were performed on perennial ryegrass under standard management as a golf fairway with a mowing height of 1.6 cm. Two sites were used in 2009, at the UK Agricultural Experiment Station and at the Marriott Golf Course, Lexington Kentucky. The soil at both sites was a Maury silt loam. Irrigation at all sites was applied to prevent visible drought stress and during daytime hours in an effort to enhance disease development. Fertility was normal for the individual management regimes (putting green or fairway, native soil or sand-based, bentgrass or perennial ryegrass). No special fertility treatments were applied. Specific treatment dates for commercial fungicides and T-phylloplanins for both years are listed in Tables 1 and 2. There was no inoculation

with pathogens, thus all disease resulted from natural inoculum. Preventative trials were begun before natural infection was observed.

3.4.3. Treatment applications:

Treatments included a: a high concentration of T-phylloplanin (112 mg ai/ft^2), half concentration of T-phylloplanin (56 mg ai/ft^2), water control, and chemical control (specific to turf and fungal pathogen being studied). Heritage TL™ with the active ingredient Azoxystrobin, methyl (E)-2-{2-[6-(2-cyanophenoxy) pyrimidin-4-yloxy]phenyl}-3-methoxyacrylate, 8.8% (EPA Reg. No. 100-1191, Syngenta Crop Protection, Inc., P.O. Box 18300, Greensboro, North Carolina 27419-8300) was applied at the label rate of 1 FL. OZ. per 1000ft^2. 2.5 ml were diluted into 600ml and this was sprayed on to each series of plots used for Brown patch.

Cleary's 3336^F ™ with the active ingredient Thiophanate-methyl (dimethyl 4,4'-o-phenylenebis[3-thioallophanate]) , 41.25% (EPA Reg. No. 1001-69, Cleary's Chemical Corporation, 178 Ridge Road, Suite A, Dayton, NJ 08810-1501) was applied at the label rate of 4 FL OZ per 1000 ft^2 Used for Dollar spot and Gray leaf spot .

All treatments were applied using a CO_2-powered boom sprayer with two Teejet #8004 flat fan nozzles at a spray pressure of 207 kPa and a carrier rate of 486 L ha^{-1}. Experimental designs for all tests were randomized complete blocks with five replications of treatments. Plots were arranged in a 5 x 5 block design, each plot was 4 x 4 ft^2, one treatment per plot, and randomized.

3.4.4. Data collection and analysis:

The response variable measured was disease severity which was determined by visual estimation of the percentage of total plot area affected by the specific disease being evaluated. Mean percent disease severity among replications was averaged over each week of data collected over the observation period during the season conducive to natural infection, for each disease/grass type. Mean percent disease severity was plotted for each week after the start of the trial for the preventative trial, and after first signs of disease for curative trials. The areas under the resulting curves were calculated by the equation AUDPC (area under disease progress curve):

$$\mathbf{AUDPC} = \sum_{i=1}^{n-1} [(X_{i+1} + X_i)/2](t_{i+1} - t_i)$$

Where AUDPC is the area under the disease progress curve, X_i = percent disease severity at the *i*th observation, t_i = days at the *i*th observation, and *n* = number of observations (Woosley, Williams et al. 2003). These data are presented as area under the disease progress curve (AUDPC) in units of %-days. If the raw percentage of disease severity declines measurably over time, AUDPC values in successful treatments will decrease in relation to unsuccessful treatments and untreated controls. Hence, AUDPC data are useful as an expression of conversion of mixed percent disease severity to higher percent disease severity alone after several treatment applications with commercial fungicides and the natural product fungicide (T-phylloplanin).

Data were analyzed using PROC GLM of SAS (1999-2010), and means were separated by Fisher's protected LSD tests with $\alpha=0.05$.

3.5. Results and Discussion:

Rates of and frequency of applications are shown in table 3. Experimental start and end times are shown in table 4. AUDPC data for 2009 and 2010 are presented in Table 5. In 2009, generally, both high and moderate concentrations of T-phylloplanin were statistically more effective than water control in reducing or preventing the fungal diseases studied. These results are consistent with data obtained in *in vitro* (Petri plate hyphal extension assays) and sprayed potted plant assays conducted in the laboratory which showed that T-phylloplanin at the concentrations used here were effective in inhibiting the diseases studied. The grass types and commercial fungicides used here were also used in those studies (King, et al., 2011).

Table 3 Rates and Frequency of Application of Treatments

Treatment	Rate	2009					2010		
		CBBP-C_C	CBBP-P_C	CBDS-C_S	PRGLS-C_S	PRGLS-P_M	CBBP-C_C	CBBP-P_C	CBDS-P_C
	kg/ha								
High T-phylloplanin	11.81	1 and 7	1, 7, 14, 21		1, 7, 14, 21	1 and 7	1, 7, 16, 21,	7, 16, 21,	1 and 7
Moderate T-phylloplanin	5.905	1 and 7	1, 7, 14, 21		1, 7, 14, 21	1 and 7	1, 7, 16, 21,	7, 16, 21,	1 and 7
Water	NA	1			1, 7, 14, 21	1 and 7	1 and 16	1 and 16	1
Heritage TL*	0.61	NA	NA	NA	NA	1 and 7	NA	NA	NA
Clearys 3336**	9.17	1 and 7	1, 7, 14, 21		1, 7, 14, 21	1 and 7	1, 7, 16, 21,	7, 16, 21,	1 and 7

CBBP-C_C=Brown Patch curative, CBBP-C_C=Brown Patch curative, CBBP-P_C=Brown Patch Preventative,

CBDS-C_S=Dollar Spot Curative, CBDS-P_C=Dollar Spot Preventative, PRGLS-C_S=Gray Leaf Spot Curative,

PRGLS-P_M=Gray Leaf Spot Preventative.

*Heritage TL (azoxystrobin) 0.8 lb. a.i./gal. product EPA Reg. No. 100-1191

**Clearys 3336 (Thiophanate-methyl) 4.0 lb. a.i./gal. EPA Reg. No. 1001-69

Table 4 Experimental start and end dates

	Experiment Start/End Date							
	2009					2010		
	CBBP-C_C	CBBP-P_C	CBDS-C_S	PRGLS-C_S	PRGLS-P_M	CBBP-C_C	CBBP-P_C	CBDS-P_C
Started	8/21/2009	6/25/2009	6/25/2009	8/21/2009	9/19/2009	6/21/2010	6/21/2010	7/12/2010
Ended	9/21/2009	8/11/2009	8/11/2009	9/19/2009	10/5/2009	8/10/2010	8/10/2010	7/26/2010

CBBP-C_C=Brown Patch curative, CBBP-C_C=Brown Patch curative, CBBP-P_C=Brown Patch Preventative,

CBDS-C_S=Dollar Spot Curative, CBDS-P_C=Dollar Spot Preventative, PRGLS-C_S=Gray Leaf Spot Curative,

PRGLS-P_M=Gray Leaf Spot Preventative.

Table 5 Means of area under the disease progress curves for brown patch, dollar spot, and grey leaf spot on creeping bent grass and perennial rye for all treatments in 2009 and 2010

		AUDPC							
		2009					2010		
Treatment	Rate	$CBBP\text{-}C_C$	$CBBP\text{-}P_c$	$CBDS\text{-}C_S$	$PRGLS\text{-}C_S$	$PRGLS\text{-}P_M$	$CBBP\text{-}C_C$	$CBBP\text{-}P_c$	$CBDS\text{-}P_C$
	kg/ha	% days							
High T-phylloplanin	11.8	742.9	613.2	639.3	145.9	453.6	208.4	33.3	0
Moderate T-phylloplanin	5.91	639.5	742.2	830.5	125.3	685	566.6	40.5	7
Water	NA	641.5	967.6	1013	236	987.4	1637.2	1437.5	384.4
Heritage TL*	0.61	618.4	789.4	NA	NA	857.6	118.8	161.5	NA
Clearys 3336**	9.17	NA	NA	539.1	10.8	NA	NA	NA	10.5
Pr > F		0.1235	0.0895	<0.0001	0.0579	0.0539	0.0003	<0.0001	0.0098
LSD (0.05)		240.14	208.19	146.75	125.57	417.88	465.29	310.91	192.27
C.V.		27.713	19.476	13.159	65.805	39.841	54.122	57.271	114.964

$CBBP\text{-}C_C$=Brown Patch curative, $CBBP\text{-}C_C$=Brown Patch curative, $CBBP\text{-}P_c$=Brown Patch Preventative,

$CBDS\text{-}C_S$=Dollar Spot Curative, $CBDS\text{-}P_C$=Dollar Spot Preventative, $PRGLS\text{-}C_S$=Gray Leaf Spot Curative,

$PRGLS\text{-}P_M$=Gray Leaf Spot Preventative.

*Heritage TL (azoxystrobin) 0.8 lb. a.i./gal. product EPA Reg. No. 100-1191

**Clearys 3336 (Thiophanate-methyl) 4.0 lb. a.i./gal. EPA Reg. No. 1001-69

The data for the 2009 brown patch curative experiment (CBBP-Cc) suggested that the water control, chemical control and the moderate T-phylloplanin treatments performed statistically similar to the high T-phylloplanin treatment (Table 5). In all tests with one exception (all grasses, diseases, and sites) high T-phylloplanin performed better than the water control in 2009 and 2010. The exception, the 2009 brown patch curative test (CBBP-Cc) indicated that the water control, chemical, and moderate T-phylloplanin treatments performed better than the high T-phylloplanin treatment (see below). Data also show that in some cases T-phylloplanin out performed commercial fungicides in curing the fungal diseases present. For example, in 2009, T-phylloplanin out performed commercial fungicide in Dollar spot (CBDS-C_S) statistically and both gray leaf spot (PRGLS-Pm) trials came close to the $P<0.05$ (0.0579 and 0.0539 respectively).

Data of Table 5 are illustrated in Figures 7-10. Brown patch data collected from the croquet court in 2009 (CBBP-Cc) showed that all treatments including water, were not significantly different in providing preventative control (Figure 7A). The commercial fungicide control Heritage TL was also ineffective as a curative treatment in this experiment. These results may have been due to extremely high disease pressure in this test. During the fourth week, temperature and humidity dropped significantly, as did the percent of disease observed in the plots. In the fourth week, T-phylloplanin and chemical control plots showed lower mean disease severity (Figure 10, week 4). In contrast, when this experiment was repeated in the same location and with the same treatment parameters in 2010, high T-phylloplanin was as effective as Heritage TL, while disease in the water control plots was high (Figure 7B). Mean disease severity on the fourth week was 5% as compared to 4% for Heritage TL and 47% for water. In 2009 an additional

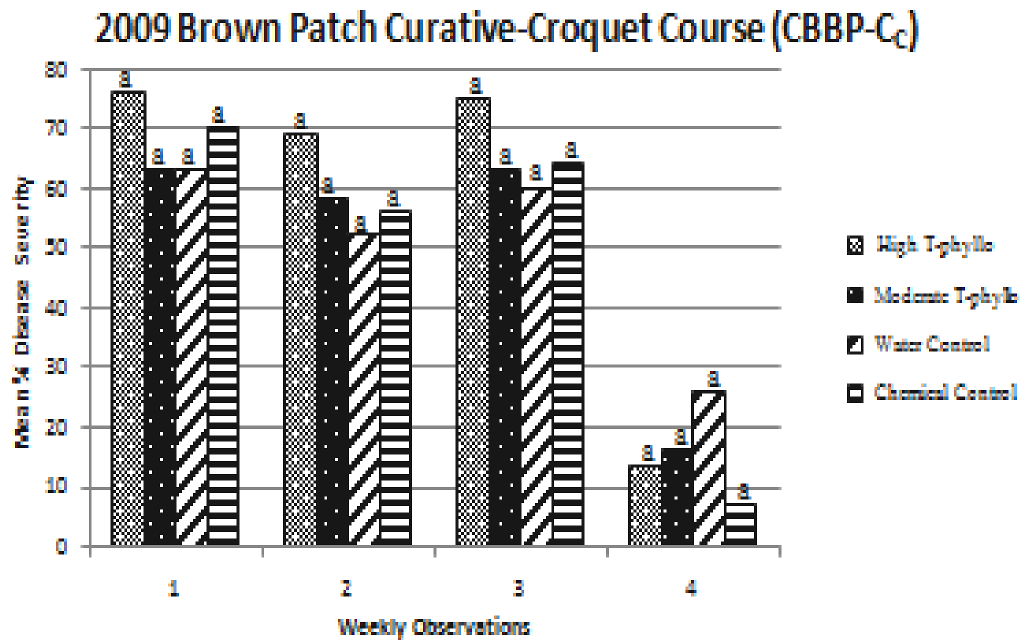
location was chosen that was expected to have lower disease pressure so that we could compare T-phylloplanin treatments with commercial fungicide (Figure 8A). The data obtained at this location (CBBP-Cs) showed no significant difference between the high or medium concentration of T-phylloplanin and Heritage TL in controlling brown patch.

In 2010 a preventative experiment (CBBP-Cs) was performed to test the effectiveness of T-phylloplanin on brown patch, on the croquet court (Figure 8B). The data show an increasing mean % disease severity over the 6 week period of the study in the water control, as compared to a relatively stable mean percent disease severity with both T-phylloplanin treatments and the Heritage TL treatment, with disease severity in the water control reaching >85% in week 6. We note that T-phylloplanin out-performed Heritage TL as a preventative treatment in this trial, with mean disease severity values of 2, 3, and 11% for high, medium T-phylloplanin, and Heritage TL, respectively.

Dollar spot occurred in 2009 and 2010 at the same location as described above for brown patch (CBBP-Cs), Figure 8A). Data for 2009 are presented in Figure 9A, (CBDS-Cs). Results with high T-phylloplanin were statistically similar to those obtained with Cleary's 3336 treatment, as a curative treatment for dollar spot; however, in this test Cleary's 3336 appeared to out-perform T-phylloplanin on 8 out of the 10 observation dates. In 2010 a preventative experiment (CBDS-Pc) was performed to compare the efficacy of T-phylloplanin versus Cleary's 3336 against dollar spot (Figure 9B). Data show that there were no significant differences between either concentration of T-phylloplanin and Cleary's 3336 for effective prevention of dollar spot, while disease occurred in the water control.

In 2009 gray leaf spot disease trials were conducted at two locations (Figures 10A and 10B). The trial at the Spindletop farm (PRGLS-Cs), Figure 10A) showed that both high and moderate T-phylloplanin concentrations and the Heritage TL treatment gave statistically similar results. Disease severity increased over time in all tests, but both high and medium T-phylloplanin concentrations and Heritage TL had lower mean % disease severity ratings as compared to water control. Results for the preventative trial comparing T-phylloplanin and Heritage TL for gray leaf spot in 2009 at the Marriot Golf Course (PRGLS-Pm) are shown in Figure 10B. Data show conflicting results in that the high T-phylloplanin treatment was similar to water control while the moderate concentration gave similar results to Heritage TL. In this case, a very low disease pressure coupled with heavy rain may have led to the results observed. An attempt was made to repeat this test in 2010, but no disease occurred due to environmental conditions.

A



B

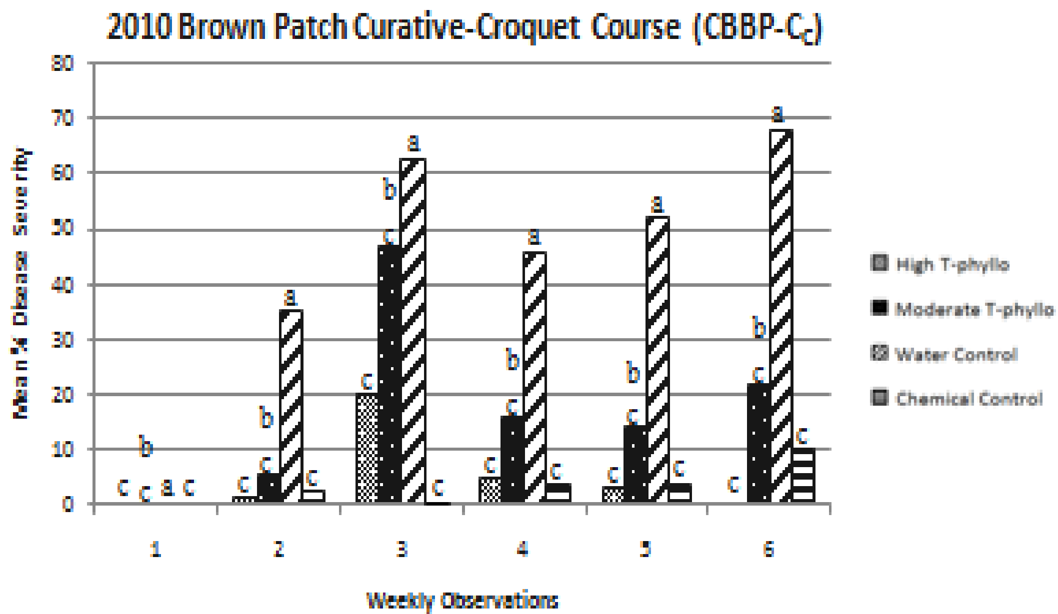
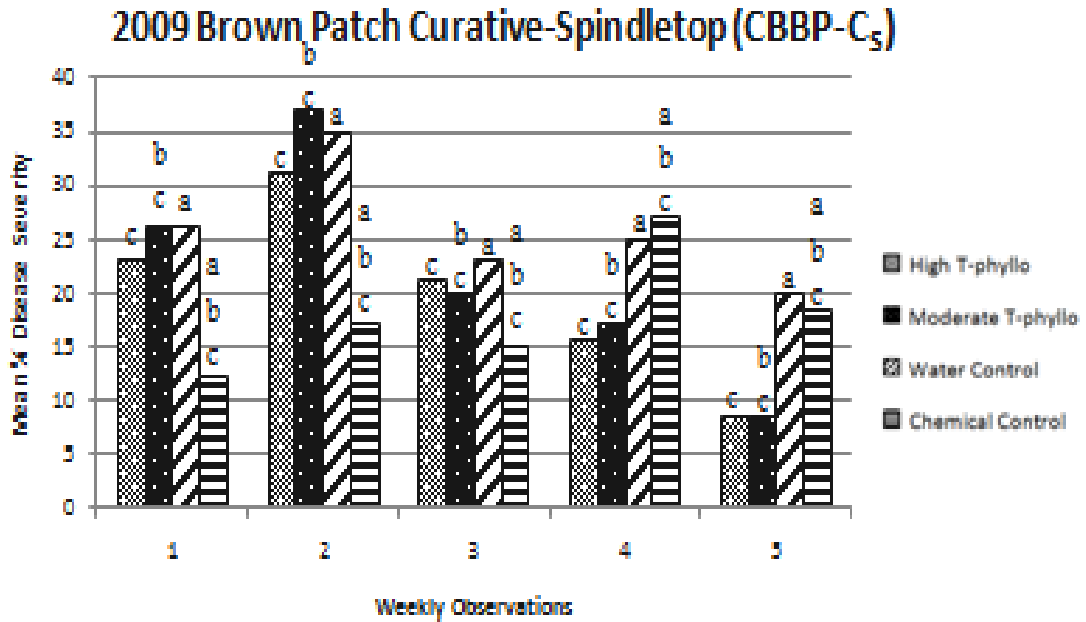


Figure 7 Mean % disease severity of brown patch, both curative, over 6 weeks of observations. Bars labeled with the same letters within both treatment and observations were not significantly different ($P \geq 0.05$). 7A shows 2009 data and 7B shows 2010 data from the same location using the same treatments.

A



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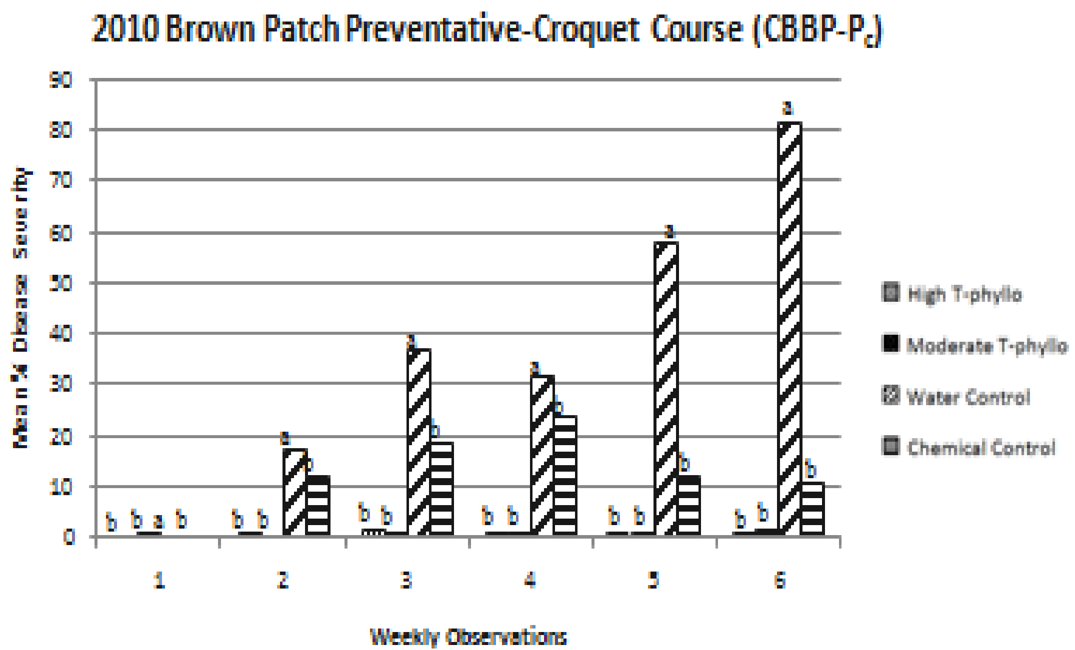


Figure 8 Mean % disease severity of brown patch, both curative, over 6 weeks of observations. Bars labeled with the same letters within both treatment and observations

were not significantly different ($P \geq 0.05$). 8A shows 2009 data and 8B shows 2010 data from the same location using the same treatments.

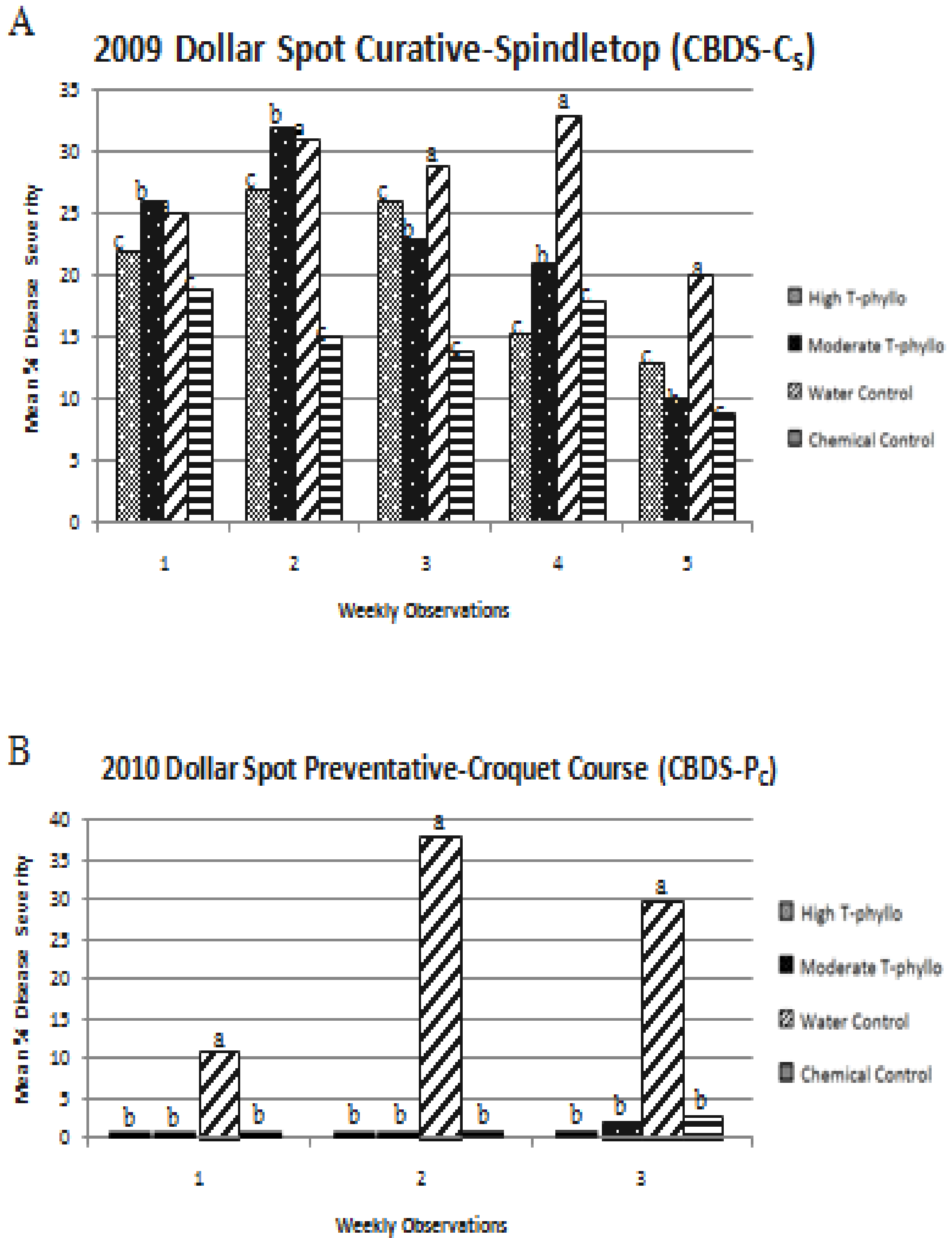
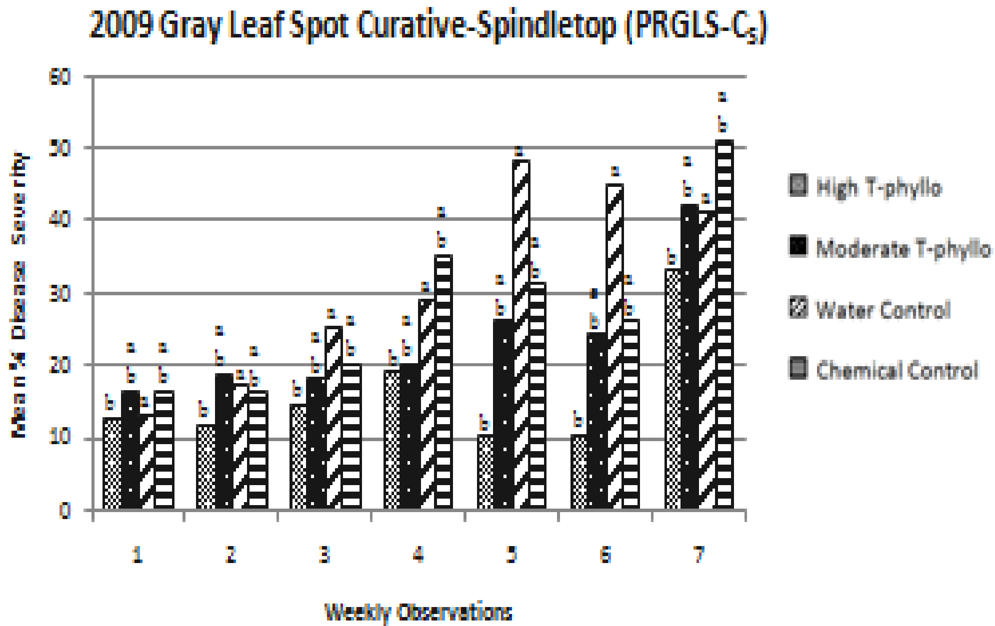


Figure 9 Mean % disease severity of dollar spot, curative or preventative respectively, over 3 weeks of observations. Bars labeled with the same letters within both treatment

and observations were not significantly different ($P \geq 0.05$). Figure9A shows Dollar spot data from 2009 at the Spindletop field station. Figure 9B shows Dollar spot data from 2010 at the croquet course.

A



B

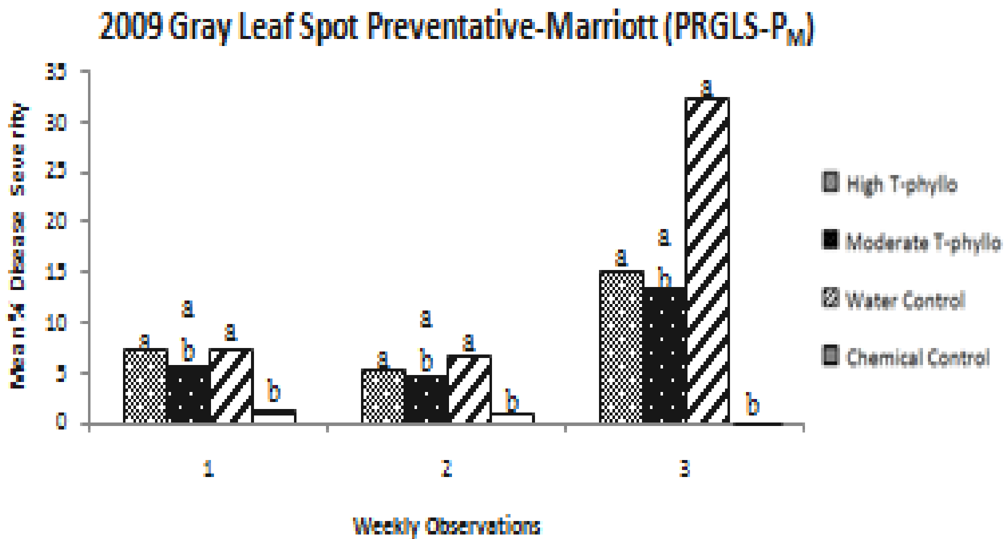


Figure 10 Mean % disease severity of gray leaf spot, curative or preventative respectively, over 3 weeks of observations. Bars labeled with the same letters within both

treatment and observations were not significantly different ($P \geq 0.05$). A)/B) Figure10A shows gray leaf spot data from 2009 at the Spindletop field station. Figure 10B shows gray leaf spot data from 2010 at the croquet course.

3.6. Conclusion:

In conclusion, the high concentration of T-phylloplanin used was as effective as the Heritage TL chemical control in preventative treatment for brown patch disease in the field trials conducted. In the case of curative experiments, Heritage TL appeared to be more effective than high T-phylloplanin. We speculate that this is so because T-phylloplanin is more effective in destroying (ion depletion) brown patch hyphae before these enter the plant. It is not expected that T-phylloplanin can penetrate the plant cuticle. T-phylloplanin appeared to be more effective for all the diseases studied. This is consistent with the apparent pore-forming mechanism for T-phylloplanin (Chapter 4). For dollar spot, high T-phylloplanin was nearly as effective at curing and more effective at preventing this disease in the field plots tested. Gray leaf spot data show that T-phylloplanin was an effective treatment for curing disease in the field plots tested. In curative experiments T-phylloplanin may be preventing secondary infections.

These results are consistent with data obtained in *in vitro* and sprayed-potted-plant experiments which showed that T-phylloplanin was effective in inhibiting the diseases studied here (Shepherd, et al. 2005; Kroumova, et al. 2007, and King, et al., in press). T-phylloplanin concentration dependence (differences between high and medium concentrations) was observed in 6 of the 8 trials performed. Future field testing is needed to examine T-phylloplanin efficacy at additional locations and to investigate utilizing adjuvants or “stickers” to assess T-phylloplanin persistence and durability after application as well as dispersion and leaf blade coverage. We note that in sprayed potted-plant-experiments and field experiments extensive leaf wetting was observed with T-

phylloplanin treatments while it was not with water treatments. This may be due to residual sugar esters in LWWs. Future studies should also include testing phylloplanins on different cultivars of grass to determine differences in efficacy against known resistant or susceptible grass species.

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Chapter 4: Efforts to understand the mechanism(s) underlying T-phylloplanin inhibition of fungal spore germination and hyphal growth.

4.1. Abstract:

In Chapters 2 and 3 we showed that phylloplanins of tobacco, and to some extent sunflower, inhibit spore germination of *Peronospora tabacina, Pyricularia oryzae*, and *Rhizoctonia solani*, and inhibit hyphal growth of *P. oryzae* and *R. solani*. In another project in the laboratory we were conducting *in vitro* experiments to study the mechanism of action of T-phylloplanin by assessing its ability to cause leakage of protons and rubidium from proton and rubidium loaded, sealed membranes prepared from tobacco roots and artificial liposomes. Those studies showed that T-phylloplanin can cause leakage of those ions from membranes, *in vitro* (Korenkov et al., unpublished). Here we determined if T-phylloplanin could cause leakage of endogenous ions from living fungal spores and hyphae.

Phylloplanins and membrane-pore-forming antimicrobial peptides have in common some chemical and physical features (e.g., >40% of AA residues are hydrophobic, net basic charge, α-helical secondary structure) which suggest that like membrane pore-forming antimicrobial peptides (AMPs) of animals and other organisms, phylloplanins may disrupt membranes to cause leakage of ions and micronutrients leakage. Also, many AMPs, like phylloplanins are found at air/tissue interfaces (lungs, skin, intestinal tract). We hypothesized that phylloplanins, like AMPs can disrupt membranes via one of the four model systems developed to explain pore formation by AMPs. This hypothesis was

tested by measuring conductivity change in spore and hyphae suspensions and by profiling constituent ions released.

4.2. Introduction:

Tobacco phylloplanins (T-Phyllo) are glycopeptides formed in short, procumbent, glandular trichomes that protrude from the tobacco leaf surface. These T-phylloplanins are highly glycosylated with ~50% hydrophobic, ~40% hydrophilic, and <10% neutral charge (Shepherd and Wagner 2007). Potential glycosylation's sites are spread out over the entire length of the peptide chain and are assumed to be serine and asparagine based, but specifics of glycosylation are not known. The protein secondary structure has not been determined by x-ray diffraction, but the nucleotide and amino sequences are known (Shepherd and Wagner 2007). Here we modeled T-phylloplanin structure using SWIS-PROT protein modeling analysis (web site). The SWIS-PROT modeling system analyzes the amino acid residue sequence and compares that sequence with proteins in the data base for which the primary and secondary structures have been determined. Based on homology to other similarly structured, characterized proteins SWIS-PROT predicted that the T-phylloplanin protein was 127aa in length and has an α-helix tube shape (137 angstroms in length). These characteristics are shared with several characterized membrane-pore-forming AMPs (Giuliani, Pirri et al. 2007).

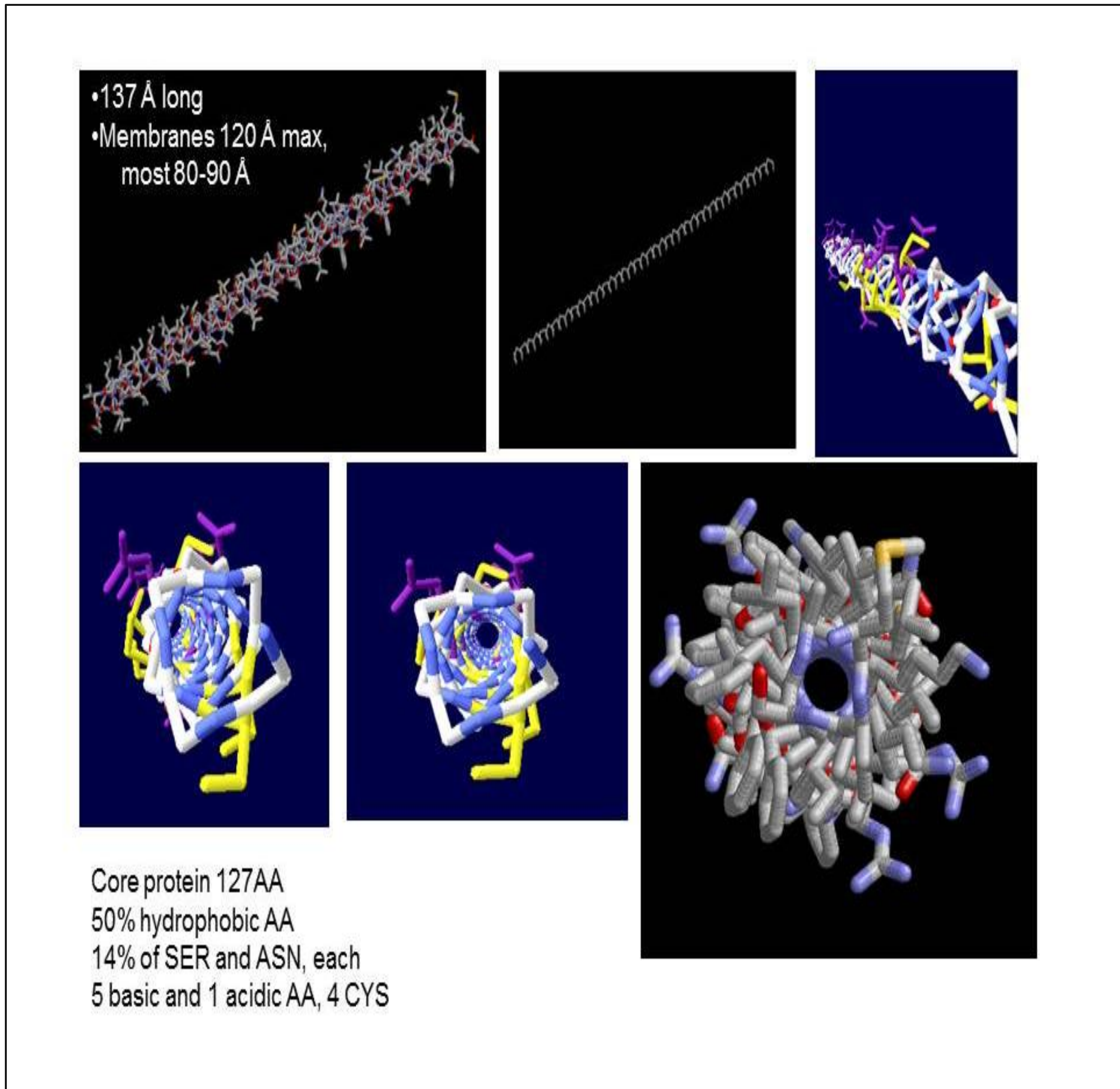


Figure 11: Shows SWIS-PROT structure prediction of Tobacco Phylloplanin (http://expasy.org/sprot/).

AMPs (also called host defense peptides, Hancock and Sahl, 2006) are an evolutionarily conserved constituents of the distinctive immune response system and are found among all classes of organisms. Essential differences exist between prokaryotic and eukaryotic cell membranes that may constitute targets for antimicrobial peptides like; distinctive membrane or cell wall structure, distinctive membrane composition or thickness,

membrane charge and potential (Brogden 2005). These peptides are often potent, broad spectrum antibiotics which demonstrate potential as novel therapeutic agents (Chou, Kuo et al. 2008). Antimicrobial peptides have been shown to kill Gram negative and Gram positive bacteria, mycobacteria (including Mycobacterium tuberculosis), enveloped viruses, fungi and even malformed or cancerous cells (Moore, Devine et al. 1994). Unlike the majority of conventional antibiotics it appears that AMPs may also have the ability to enhance immunity by functioning as immune-modulators (Holt, Hubert et al. 2003). AMPs are a diverse group of molecules, which are divided into subclasses based on their amino acid sequence and arrangement (Brogden 2005). They are generally between 10 and 50 amino acids in length, but can be as large as 150 amino acids (T-phylloplanin has 127), and generally include multiple positively charged residues provided by arginine, lysine (T-phylloplanin has 5) or, in acidic environments, histidine, and a large proportion (generally >40%) of hydrophobic residues (Sitaram and Nagaraj 2002; Papagianni 2003; Durr, Sudheendra et al. 2006). The secondary structures of these molecules follow 4 themes, including i) α-helical, ii) β-strand due to the presence of 2 or more disulfide bonds, iii) β-hairpin or loop due to the presence of a single disulfide bond and/or cyclization of the peptide chain, and iv) extended(Dhople, Krukemeyer et al. 2006). T-phylloplanin is most like the linear, cationic α-helical peptides, but has only 5 basic residues.

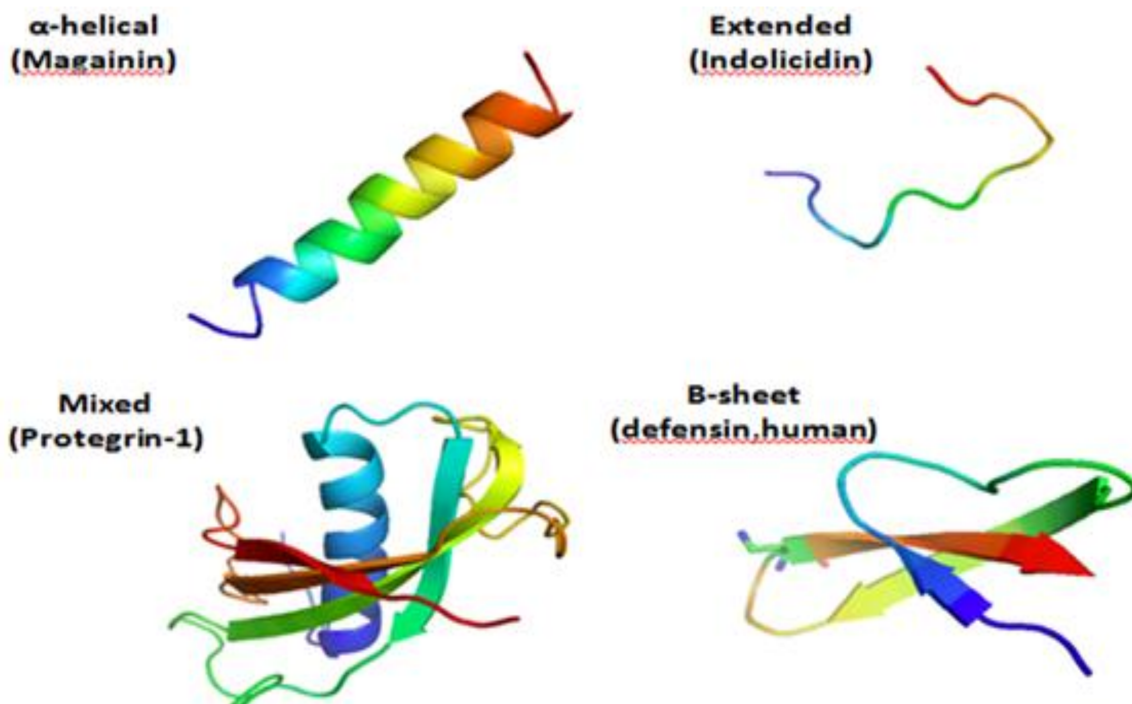


Figure 12 Examples of several morphological variations of antimicrobial peptide structures. Pictures were created using JMOL on PDB protein databank (Zasloff 1997; Rozek 2003; Yang 2003; Lubkowski To be published).

(Zasloff 1997; Rozek 2003; Yang 2003; Lubkowski To be published). Also, T-, and S-phylloplanin are highly glycosylated (potentially 20% of amino acid residues (Shepherd and Wagner, 2007; Kroumova et al., 2007). Glycosylation is not a common feature of AMPs. We only know of the cecropins of bees as being glycosylated. Many of these peptides are formless in neutral solution, and fold into their final conformational shape upon apportioning into biological membranes. In these peptides hydrophilic amino acid residues line up along one surface (inside or outside of the helix) and hydrophobic amino acid residues line up along the opposite surface of a helical molecule (Yeaman and Yount 2003). The "amphipathicity" of such antimicrobial peptides allows them to interact with the membrane lipid bilayer. The ability to associate with membranes is a definitive

feature of antimicrobial peptides (Hancock and Rozek 2002), although membrane destabilization is not always an outcome. The peptides demonstrate an assortment of antimicrobial actions ranging from membrane destabilization to action on a range of cytoplasmic targets.

Type	Characteristic	AMPs
Anionic peptides	High levels of glutamic and aspartic acids	Maximin H5 from amphibians, Dermcidin from humans
Linear cationic α-helical peptides	Lack in cysteine	Cecropins, andropin, moricin, ceratotoxin and melittin from insects, Magainin, dermaseptin, bombinin, brevinin-1,esculentins and buforin II from amphibians, CAP18 from rabbits, LL37 from humans
Catioinic peptide enriched for specific amino acid	High levels of proline, arginine, phenylalanine, glycine, tryptophan	abaecin, apidaecins from honeybees, prophenin from pigs, indolicidin from cattle.
Anionic and cationic peptides that contain cysteine and form disulfide bonds	contain 1-3 di-sulphide bonds	1 bond: brevinins, 2 bonds: protegrin from pig, tachyplesins from horseshoe crabs, 3 bonds: defensins from humans, more than 3:drosomycin in fruit flies

Table 6 Type, characteristics and examples of some well elucidated antimicrobial peptides. Table courtesy of wikipedia (http://en.wikipedia.org/wiki/Magainin)

The modes of action by which AMPs destroy microorganisms is varied and not well understood. Proposed mechanisms include disruption of the outer membrane, disruption of metabolism, and action on cytoplasmic constituents. The initial contact between the peptide and the target organism would likely be electrostatic, as most bacterial surfaces are anionic (Brogden 2005). Their amino acid composition, amphipathicity, cationic charge and size permit them to fasten to and introduce into membrane bilayers to form pores by ‘barrel-stave’, ‘carpet’ or ‘toroidal-pore’ mechanisms. They may also infiltrate

into the cell and to bind intracellular molecules which are crucial to cell viability (Brogden 2005). Intracellular binding models include inhibition of cell wall synthesis, alteration of cytoplasmic membranes, activation of autolysin, inhibition of DNA, RNA, and protein synthesis, and inhibition of certain enzymes. However, in many cases, the exact mechanism of lethality is not known. In contrast to many conventional antibiotics these peptides appear to be bactericidal (bacteria killer) instead of bacteriostatic (bacteria growth inhibitor). In general the antimicrobial activity of AMPs is determined by measuring the minimal inhibitory concentration (MIC), which is the lowest concentration of drug that inhibits bacterial growth (Lorian 1996). Several methods have been used to determine the mechanisms of antimicrobial peptide activity (Brogden 2005). These are summarized in Table 3.2.

Methods	**Applications**
Microscopy	To visualize the effects of antimicrobial peptides on microbial cells
Fluorescent dyes	To measure antimicrobial peptides to permeabilize membrane vesicles.
Ion channel formation	To assess the formation and stability of an antimicrobial-peptide-induced pore.
Circular dichroism and orientated circular dichroism	To measure the orientation and secondary structure of an antimicrobial peptide bound to a lipid bilayer
Solid-state NMR spectroscopy	To measure the secondary structure, orientation and penetration of antimicrobial peptides into lipid bilayers in the biologically relevant LIQUID-CRYSTALLINE STATE
Neutron diffraction	To measure the diffraction patterns of peptide-induced pores within membranes in oriented multilayers or liquids

Table 7 Methods of visualization and quantification of antimicrobial peptides. This list does not include the use of conductivity and ion sampling and quantification by ICP. Table courtesy of wikipedia (http://en.wikipedia.org/wiki/Magainin)

4.2.1. Fungal membranes:

There are several groups of organisms that may be called "fungi". Some of these groups have been transferred out of the Kingdom Fungi, in part because of fundamental biochemical differences in the composition of the cell wall (Alexopoulos, Mims et al. 1996). Most true fungi have a cell wall consisting largely of chitin and other polysaccharides. True fungi do not have cellulose in their cell walls, but some fungi-like organisms do. Not all species of fungi have cell walls but in those that do (true fungi) the plasma membrane is followed by three layers of cell wall material. From inside out these layers are: i) a chitin layer (polymer consisting mainly of unbranched chains of N-acetyl-D-glucosamine), ii) a layer of β-1,3-glucan (zymosan), and iii) a layer of mannoproteins (mannose-containing glycoproteins) which are heavily glycosylated at the outside of the cell (Alexopoulos, Mims et al. 1996). Fungi that have these characteristics are called true fungi and are represented by *Pyricularia oryzae* Cavara (Ascomycete) and *Rhizoctonia solani* Khun (Basidiomycete). The group oomycete, also known as water molds, are saprotrophic plant pathogens and are represented by *Pythium* spp., *Peronospora hyoscyami* f.sp. *tabacina* and *Phytophthora parasitica* (Dast.) var. *nicotianae* (B. de Haan) Tucker. Until recently they were widely believed to be fungi, but structural and molecular evidence has led to their reclassification as heterokonts, related to autotrophic brown algae and diatoms. Unlike fungi, oomycetes typically possess cell walls of cellulose and glucans rather than chitin, although some genera (such as Achlya and Saprolegnia) do have chitin in their walls. The fraction of cellulose in the walls is no more than 4 to 20%, far less than the fraction comprised by glucans. Oomycete cell walls also contain the amino acid hydroxyproline, which is not found in fungal cell walls.

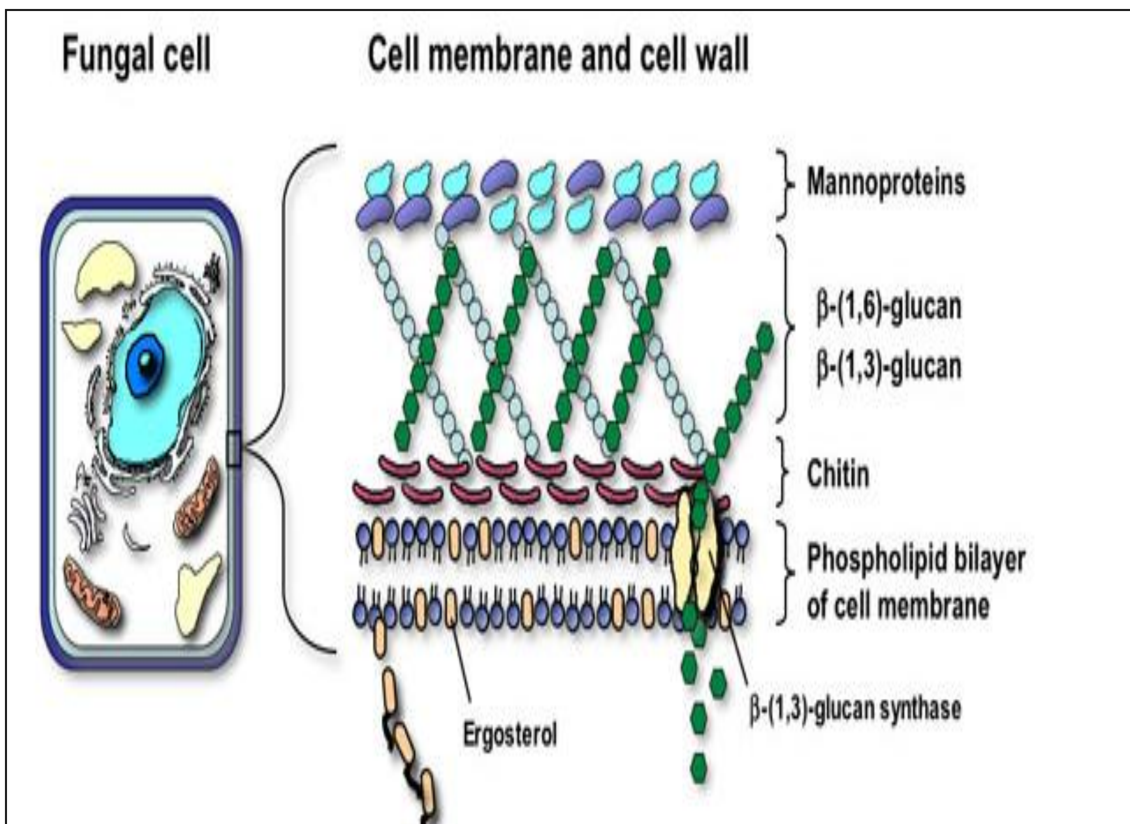


Figure 13 Detail drawing of fungal cell wall constituents**.**

4.3. Materials and Methods:

4.3.1. Fungi:

Living single spore isolates of *Peronospora hyoscyami* f.sp. *tabacina* were obtained from growing *Nicotiana tabacum* L. cultivated in a growth chamber kept at 75^{0}F, 12/12 light cycle and 50% relative humidity. Spores were collected by gently washing sporulating blue mold lesions to remove spores from the infected leaves. Both *P. oryzae* and *P. tabacina* spores were diluted to 100 spores per µl for the purposes of the conductivity and ion analysis assays described here.

Living single spore isolates (hyphal tip method) of *Pyricularia oryzae* Cavara were obtained from Dr. Mark Farman at the University Of Kentucky, Plant Pathology

Department. These cultures were grown aseptically on oatmeal agar which was made by boiling 30 g of plain oatmeal (no additives) in 500 ml of deionized water. After boiling for 20 minutes, the oatmeal solution was drained through cheese cloth and brought to 1 L volume with deionized water. Fifteen grams of agar were added and the whole solution was autoclaved for 30 minutes. Resulting oatmeal agar was poured into 95 X 15 Petri plates in a sterile hood and allowed to cool. One centimeter squares of actively growing *P. oryzae* colonized agar were then placed in the center of a plate and left to grow for 10-14 days, or until active sporulation was evident from ascus formation. Spores were collected using the same method as described in Chapter 2 for use in the potted spray experiments.

P. *oryzae* hyphae were also used to test conductivity and ion profiling. Hyphal cultures were generated as previously described in (Talbot, Ebbole et al. 1993) with one exception. The liquid growth media used was 2XYT, as described in the Molecular Cloning Manual (Maniatis, Fritsch et al. 1982). Mycelium was left to grow until a 1 to 2g spheres of hyphae was formed. The mycelium were removed from liquid culture and washed in sterile (non-deionized) water three times. Hyphae were then left to dry on paper towels for 10 minutes or until most excess water was absorbed. Mycelium was then weighed into 1.5 g experimental units.

4.3.2. T-phylloplanin Preparation and Conductivity Instrument:

A T-phylloplanin stock solution (112 mg/g LWW, dry wt; 4.5 mg/ml protein) was as used for on plant assays referred to in Chapter 2. But, to remove excess ions from LWW containing T-phylloplanin solutions were dialyzed 3 times, one hour per water change, using Spectrapore™ 3.5 KDa MWCO dialysis tubing (Spectrumlabs Broadwick St., Rancho Dominguez, CA 90220). Oakton 300 and 600 conductivity meters (OAKTON Instruments P.O. Box 5136, Vernon Hills, IL USA 60061) were used exclusively for monitoring conductivity. Calibration of the meters was performed monthly to ensure nominal results.

Oakton 300

Sample Id (μS)	Volume (mL)	Cond (μS) A	Cond (μS) B	Accuracy A	Accuracy B	Percision
0.1	10	0.1	0.1	1	1	0
100	10	101.1	101.2	1.011	1.012	-0.098863075
401	10	401.8	401.9	1.001995012	1.002244389	-0.024884907
822	10	822.6	822.7	1.000729927	1.000851582	-0.012155838
1413	10	1413.1	1413.2	1.000070771	1.000141543	-0.00707639
Average		547.74	547.82	1.002759142	1.003047503	-0.028596042
Std Dev		579.890354	579.913965	0.00467583	0.005082965	0.040318537

Oakton 600

Sample Id (μS)	Volume (mL)	Cond (μS) A	Cond (μS) B	Accuracy A	Accuracy B	Percision
0.1	10	0.1	0.1	1	1	0
100	10	99.98	99.99	0.9998	0.9999	-0.0100015
401	10	399.8	399.9	0.997007481	0.997256858	-0.025009379
822	10	821.7	821.8	0.999635036	0.999756691	-0.012169151
1413	10	1412.7	1412.9	0.999787686	0.999929229	-0.014156285
Average		546.856	546.938	0.999246041	0.999368555	-0.012267263
Std Dev		579.976466	580.054694	0.001258091	0.001183789	0.008966033

Table 8: Conductivity standard solutions for use in calibration of the Oakton meters and the accuracy and precision calculations based upon those standards. Both meters had a high degree of accuracy calculated from two separate conductivity measurements. Precision was zero for both meters and the difference between the two meters was < .01%.

4.3.3. Ion profiling:

ICP analysis of all samples was made using a Varian Vista Pro ICP-OES analyzer (ERTL laboratory, UK, samples analyzed by Tricia Coakley). For both methods of ion sampling (aliquot method and one-tube-per-time-point) 1 ml aliquots were sampled for ICP analysis. Aliquots were stored at 4^0C unto analysis by ICP. All aliquots were prepared for ICP analysis using a modified EPA 3050B sample digestion protocol. This protocol uses a combination of the following: nitric acid (HNO_3), hydrochloric acid (HCL), sulfuric acid (H_2SO_4), and hydrogen peroxide (H_2O_2) to release all ions from organic material. All the acids used were highest grade (Ultrapure). For analysis of spores or hyphal materials after ion leak time courses insoluble materials were placed in acid-pre-rinsed polypropylene tubes, and dried. To each sample, acid mixture was added and the sample was digested in a hot-block at 90^0C for approximately two hours. When no further color change was seen and sample particulates are no longer visible, samples were removed from the hot block and allowed to cool to room temperature. The digests were diluted to a final acid concentration that was equal to that of the calibration standards prior to ICP analysis.

4.3.4. Conductivity experiments:

P. *oryzae* and P. *tabacina* spores were collected as described earlier. Spores were diluted to 100 spores per µL and 10ml (1 X10^6 spores) of spore suspension was added to a 50 ml glass centrifuge tube which was then filled to 50 ml with sterile de-ionized water. The tubes were spun at ~8000 x g to gently sediment spores. These experiments were carried out on a stable vibration free surface at 24^0C. After centrifugation, the tubes were left to sit for a 10 minutes on the stable bench. The conductivity meter probe was slowly lowered into the tube to directly above but not touching the spore mass and conductivity

measurements were recorded. Readings were recorded every minute for the first 10 minutes and then every 10 minutes after that point until 200 minutes were reached. From 200 minutes to 215 minutes data were collected every minute to assure the final conductivity level. An overnight reading was done the following morning, ~15 hours later. Then spores or hyphae were completely ground in water using a glass tissue grinder, and total conductivity in the soluble phase was measured. There were four treatments: 1) water, 2) water plus spores, 3) water plus spores plus T-phylloplanin, 4) water plus spores plus T-phylloplanin that had been treated with ProteinaseK. All individual experiments are shown to convey the qualitative similarities among them, despite variation that was observed in the starting conductivity value, and the presence or absence of a second increase in conductivity after the first (usually at ~20 minutes).

P. *oryzae* hyphae were analyzed in much the same way. Mycelium was prepared from liquid culture as described. Mycelium was placed into 50 ml glass tubes and sterile, de-ionized water was added to a volume of 50 ml. The tubes were spun at ~8000 x g to sediment all hyphae. After centrifugation, the tubes were left to sit for a 10 minutes after transfer to the stable bench. Data was collected by lowering the conductivity meter probe into the tube and measuring the conductivity in μS directly above, but not touching, the hyphal mass at the bottom of the tube. Recordings of conductivity were taken every minute for the first 10 minutes then every 10 minutes after that point until 200 minutes were reached. From 200 minutes to 215 minutes data were collected every minute. An overnight reading was recorded the following morning, ~15 hours later. Then hyphae were completely ground in water using a glass tissue grinder and total conductivity was measured. There were four treatments: 1) water, 2) water plus spores, 3) water plus

spores plus T-phylloplanin, 4) water plus spores plus T-phylloplanin that had been treated with ProteianseK. All individual experiments are shown to convey the qualitative similarities among them, despite variation that was observed in the starting conductivity value, and the presence or absence of a second increase in conductivity after the first (the first usually at ~20 minutes).

4.3.5. Ion Data Collection:

Two methods of ion analysis/profiling were used. The first method (aliquot method) was to analyze treatment tubes by removing aliquots at given time points. Ion data was collected using the same 50 ml centrifuge tube arrangement and procedure as described above for conductivity experiments. No conductivity meter probe was inserted. Rather, a BDL-4647 transpet (Becton/Dickinson 1 Becton Drive, Franklin Lakes, NJ USA 07417) was used to remove 1 ml aliquots from the area directly above the spore or hyphal mass at specific time points of 1, 4, 9, 30, 60, 100, 170, 215 minutes as well as overnight. The following morning a total grind of spores or hyphae was prepared, and the supernatant sampled. A T-phylloplanin control was also prepared to assess its conductivity. These parameters were used regardless of the spore or hyphae tested. The second method (one-tube-per-time-point) was performed as above with the exception that every tube was its own time point. For example there was a 1 minute tube, a 4 minute tube, etc (see figures 14, 19, 24) . This allowed for a direct assessment of the ion concentration without removal of samples during the experimental period, and less disturbance of the spore or hyphal mass.

4.4. Results:

Our hypothesis was that T-phylloplanin would pass through the spore or hyphal wall, accumulate at the plasma membrane surface, aggregate, and then infiltrate the membrane to cause formation of a pore through which endogenous ions began to leak. The lag in initiation of leakage could be the time required to penetrate the wall, time required for T-phylloplanin to aggregate, time required to infiltrate, or all of these. A first bump in conductivity might represent the point when the plasma membrane was breeched. During some experiments, two bumps in conductivity were suggested. The hypothesis was that such a second bump might represent infiltration of T-phylloplanin from the cytosolic space (after the plasma membrane was breeched) into the vacuolar membrane, which is thought to be another store of ions, in addition to the cytosol.

4.4.1 P. *tabacina* spores:

Suspensions containing *P. tabicina* spores showed increases in conductivity as compared with the water or water plus spores control alone suspensions (see Figure 14 A-D). Conductivity changes often occur between 20 and 70 minutes for P. *tabicina* spores and always produced at least one bump, or relatively sudden rise in conductivity where intact T-phylloplanin was present. Figure 14 C seems to show a second bump in conductivity at the ~200 minute mark. As shown in Figure 14 D, pre-treatment of T-phylloplanin (destroys T-phylloplanin protein) resulted in no bump in ion efflux. A ProtK in water control showed no effect (not shown). Differences in the conductivity of the water plus spores plus T-phylloplanin experiments are thought to be due to the variability inherent in dialysis treatment of phylloplanins and other factors. When experiments were conducted to profile the ions leaked (the aliquot method of ion analysis as described above, results

showed that the first bump of ion leakage (seen previously as increased conductivity) was due to release of primarily K, Ca, Na, P, Mg; see figures 15-17). Figures 15 through 17 each show two graphs of the same data and differ only in the scale on the Y axis. In each case graph B was prepared to extenuate the bump in ions released after ~20 minutes. . This corroborates the fact that conductivity is a measure of leakage and that ions are at least part of the materials being leaked into solution upon T-phylloplanin treatment. Figure 18 shows ion data collected using the one-tube-per-time-point method. In this case, each time point: 1 , 4, 9, 20, 30, 60, 100, 170, 215 minutes, overnight, and total grind) was obtained using separate tubes which were aliquoted at their respective times and the aliquots frozen. This method was an attempt to refine data collection. However, results remained consistent with those obtained using the aliquot method of sampling (Figure 15 to 17 data).

4.4.2. P. oryzae spores:

P. oryzae spores also showed increases to conductivity in solution compared with the water and water plus spores control alone (see figure 19 A-C). Conductivity changes (bump) occur between 7 and 10 minutes for *P. oryzae* spores and also produced at least one bump in the overall conductivity when intact T-phylloplanin was present. We speculate that the earlier time of appearance of the conductivity bump compared to that with *P. tabacina* spores may reflect differences in the cell wall composition. ProtK was not used as a control in this group of experiments. Ion profile data was consistent with results of conductivity experiments, and ion profiles for *P. oryzae* indicated the leakage of mainly K, Ca, Na, P, Mg (Figures 20 to 22). As found for *P. tabacina* spores, results of conductivity and ion analysis indicate that leakage of ions is at least partially responsible for conductivity changes observed and there is a drop in conductivity which

is noticed in most of the ion profile graphs around 100-215 minutes (e.g., see Figure 20B and 21 B). This may be due in part with the removal of solution from the reaction vessel for the purpose of ICP testing, but it was also observed when individual tubes were used for each time point (Figures 18, 23 and 27). Figure 23 shows ion data collected using the one-tube-per-time-point method. Here, each time point (1, 4, 9, 20, 30, 60, 100, 170, 215, overnight, and total) were separate tubes which were collected at their respective times and then analyzed. As found for P. *tabacina* spores the aliquot method and the one-tube-per-time-point method gave similar results.

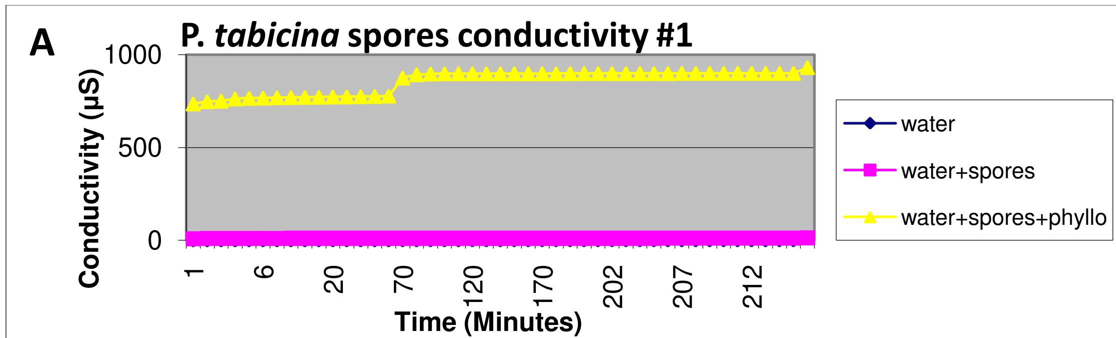
P. oryzae hyphae treated with T-phylloplanin showed a bump in conductivity compared with the water and water plus hyphae controls (see Figure 24 A-C). Conductivity changes occurred between 7 and 30 minutes for *P. oryzae* hyphae. This is different from spores of both *P. tabacina* and *P. oryzae* which tended to show conductivity jumps at later time periods (>20 minutes). ProtK pretreatment of T-phylloplanin resulted in loss of the T-phylloplanin leak effect (see Figure 24 D). Conductivity data and ion profile data were consistent and indicated leakage of primarily K, Ca, Na, P, Mg from *P. oryzae* hyphae upon treatment with intact T-phylloplanin (see Figures 25-27). Figure 27 shows results of the one-tube-per-time-point method experiment with *P. oryzae* hyphae. Here, each time point (1, 4, 9, 20, 30, 60, 100, 170, 215, overnight, and total) were separate tubes which were from at their respective times and then discarded. As in the *P. tabacina* and *P. oryzae* experiments results of experiments using the aliquoting method or one-tube-per-time-point method were similar.

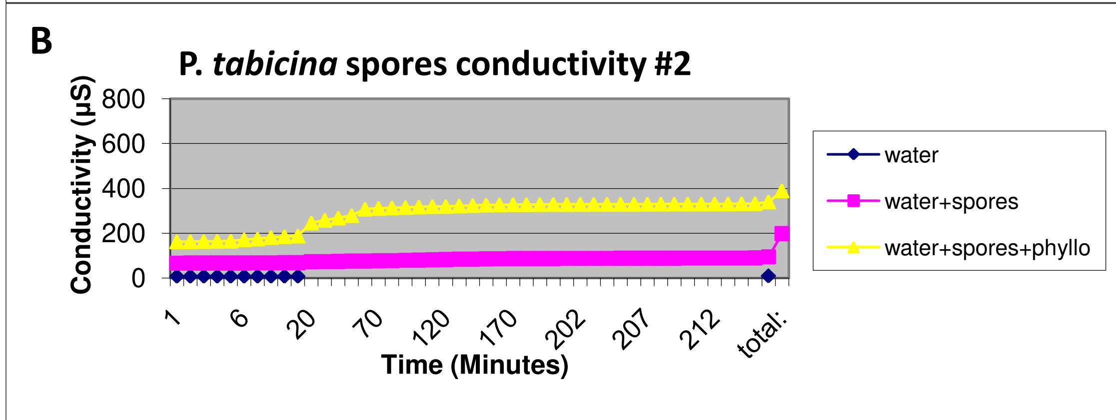
Regarding the observations that the rise in conductivity occurring after ~20 minutes in T-phylloplanin treated spore and hyphae experiments is maintained after its occurrence

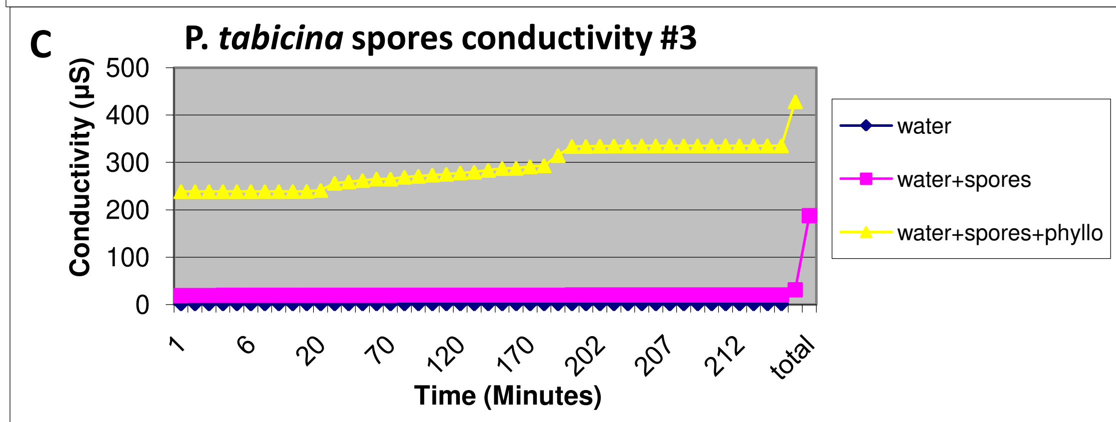
(e.g., Figures 14 A-D), we speculate that while ion leakage measurements show that leakage of ions increases after ~20 minutes then decreases (e.g., Figure 15A)may be due to proton leakage from spore and hyphae vacuoles during and after ion (K, P, etc.) leakage from cytosol. We note that protons contribute more to conductivity (3 to 4 times) than do other ions (e.g., K, P, Ca). Thus, protons may maintain conductivity after released ions are reabsorbed by breached spores or hyphae (eg. To cell wall ligands). Current experiments involve simultaneously measuring conductivity and pH, and will possibly include the use of a potassium-selective electrode, as well.

4.5. Conclusions:

In summary, data in this chapter show directly that intact T-phylloplanin causes leakage of ions from spores of *P. tabacina* (oomycete), *P. oryzae* (Basidiomycete), and hyphae of *P. oryzae*. These data from experiments testing the target organisms directly correlate with data using model membranes isolated from tobacco roots (biological membrane model) and artificial phospholipid vesicles(Korenkov et al., unpublished) to indicate that intact T-phylloplanin causes membrane disruption which results of depletion of endogenous ions (and perhaps other metabolites) to result in death of target spores or hyphae. Together these results suggest that T-phylloplanin is a pore-forming/membrane disrupting antifungal agent. We note that recent experiments in the lab (unpublished) show that artificial membrane vesicles designed to have a composition closer to fungal membranes (>20% ergosterol) are more sensitive to disruption than artificial vesicles with mammalian plasmalemma membrane like composition (~10% cholesterol), or plant plasmalemma composition.


A
P. tabicina spores conductivity #1
Conductivity (µS)
1000
500
0
1
6
20
70
120
170
202
207
212
Time (Minutes)
water
water+spores
water+spores+phyllo


B
P. tabicina spores conductivity #2
Conductivity (µS)
800
600
400
200
0
1
6
20
70
120
170
202
207
212
total:
Time (Minutes)
water
water+spores
water+spores+phyllo


C
P. tabicina spores conductivity #3
Conductivity (µS)
500
400
300
200
100
0
1
6
20
70
120
170
202
207
212
total
Time (Minutes)
water
water+spores
water+spores+phyllo

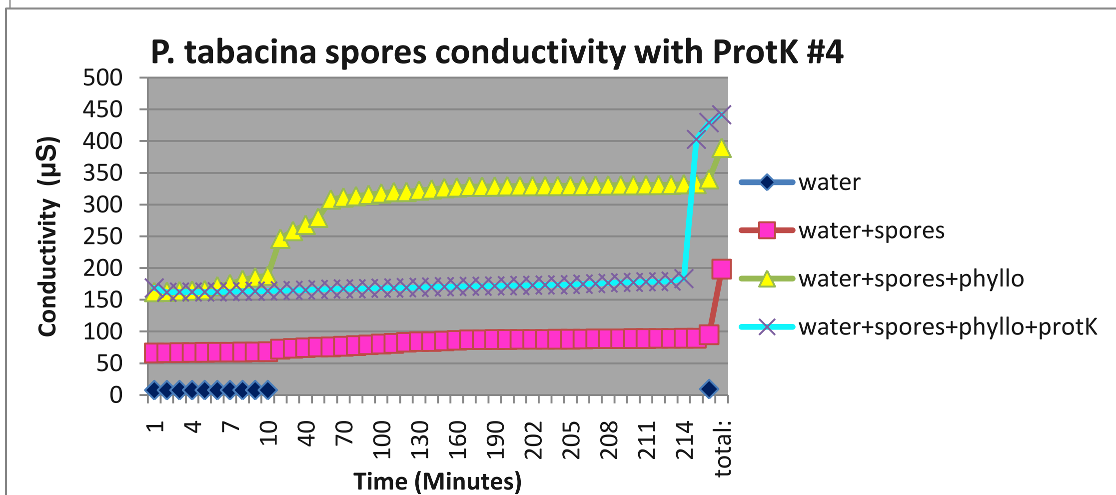

P. tabacina spores conductivity with ProtK #4
Conductivity (µS)
500
450
400
350
300
250
200
150
100
50
0
1
4
7
10
40
70
100
130
160
190
202
205
208
211
214
total:
Time (Minutes)
water
water+spores
water+spores+phyllo
water+spores+phyllo+protK

Figure 14 A-D: Figures A-C show repetition of the conductivity experiment using P. *tabacina* spores and phylloplanin. Observation of one or two conductivity jumps, water control shows no ion leakage, and, Figure 14 D ProtK seems to inhibit phylloplanins from causation of membrane permeabilization.

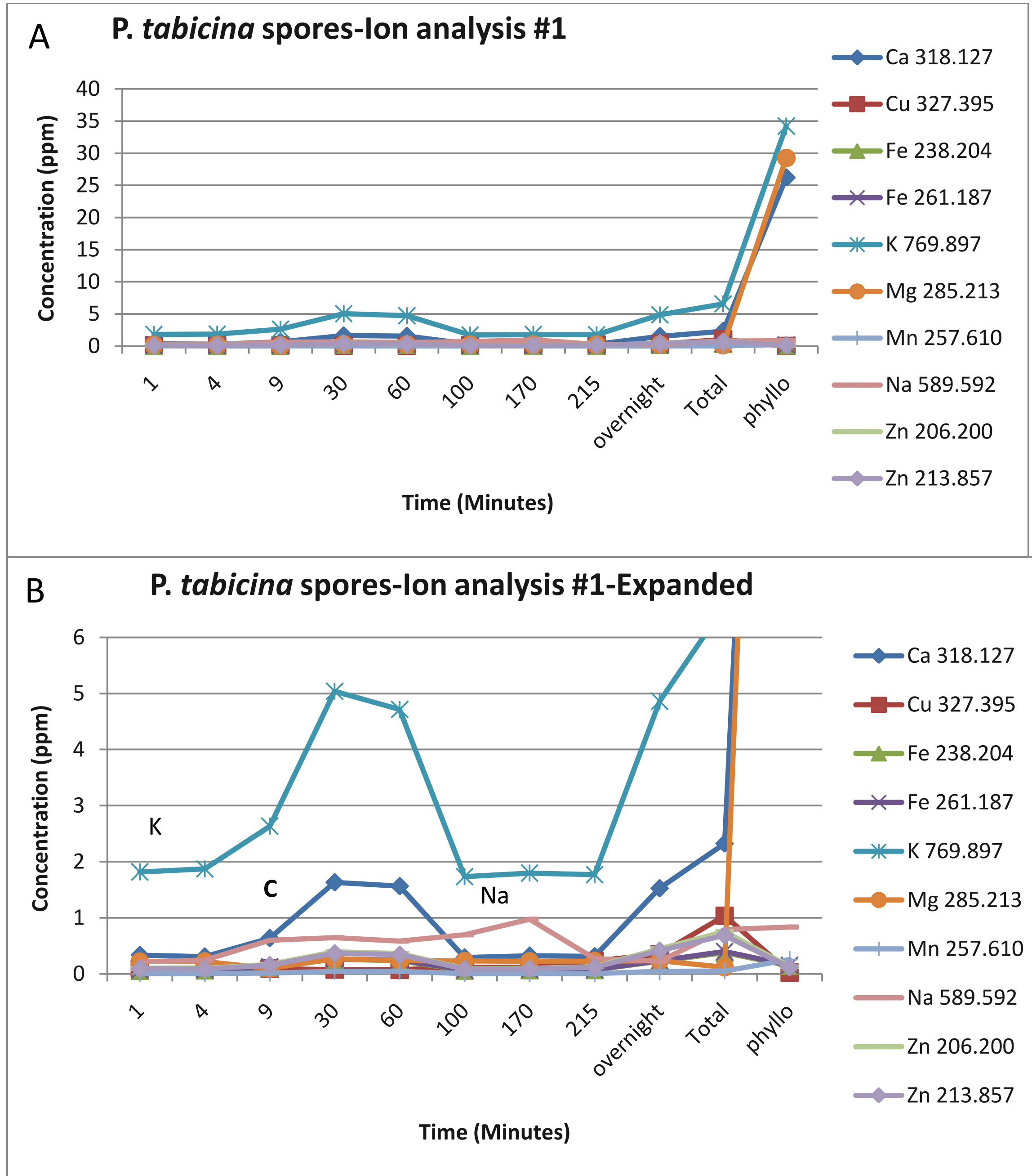


Figure 15 A Shows the first ion profile for P. *tabicina* spores using method 1. 15 B shows the expanded view. 15 B shows K, C, and Na with the highest changing levels

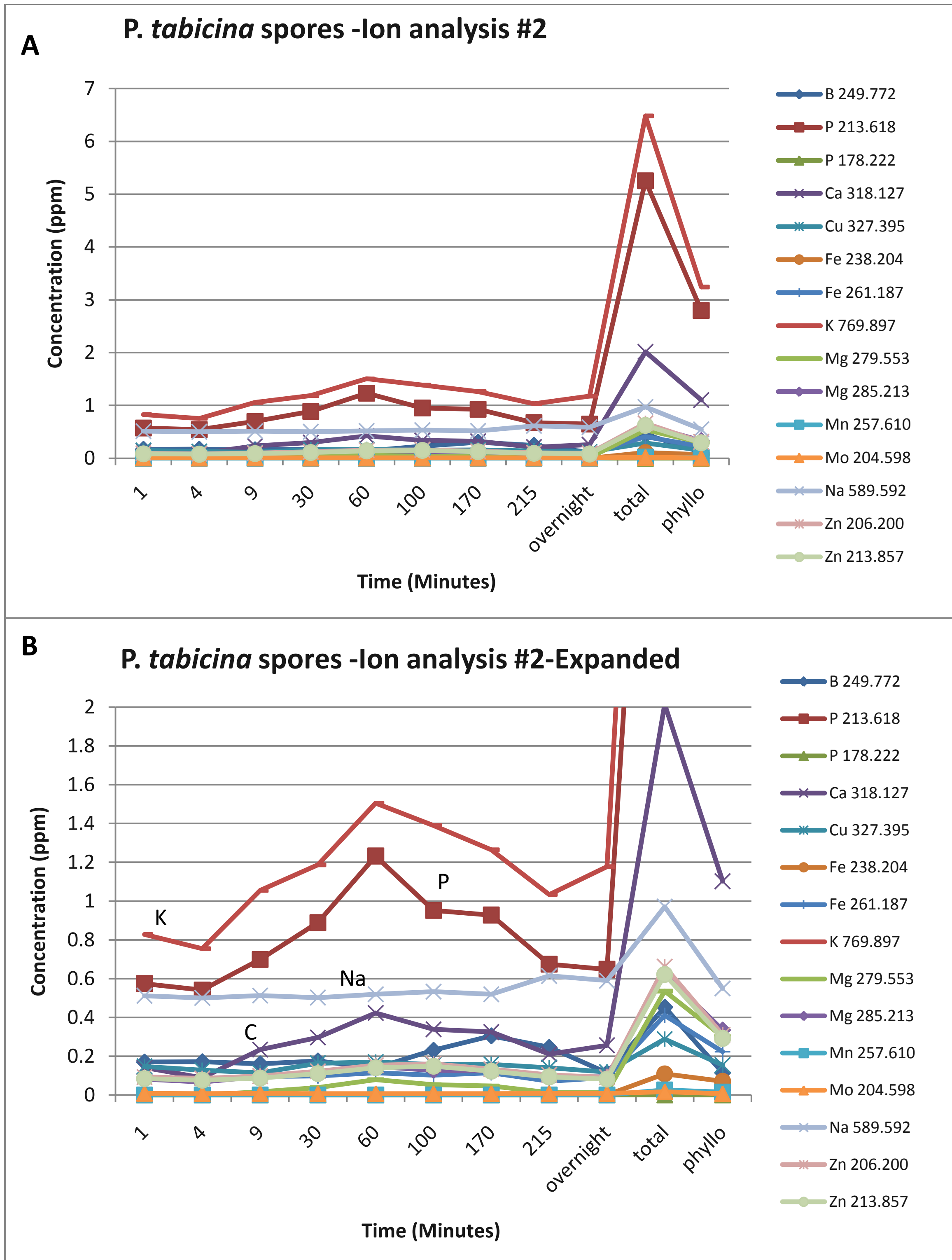


Figure 16 A Shows the second ion profile for P. *tabicina* spores using method 1. 16 B shows the expanded view with K, P, Na and C, the ions with the highest changing levels.

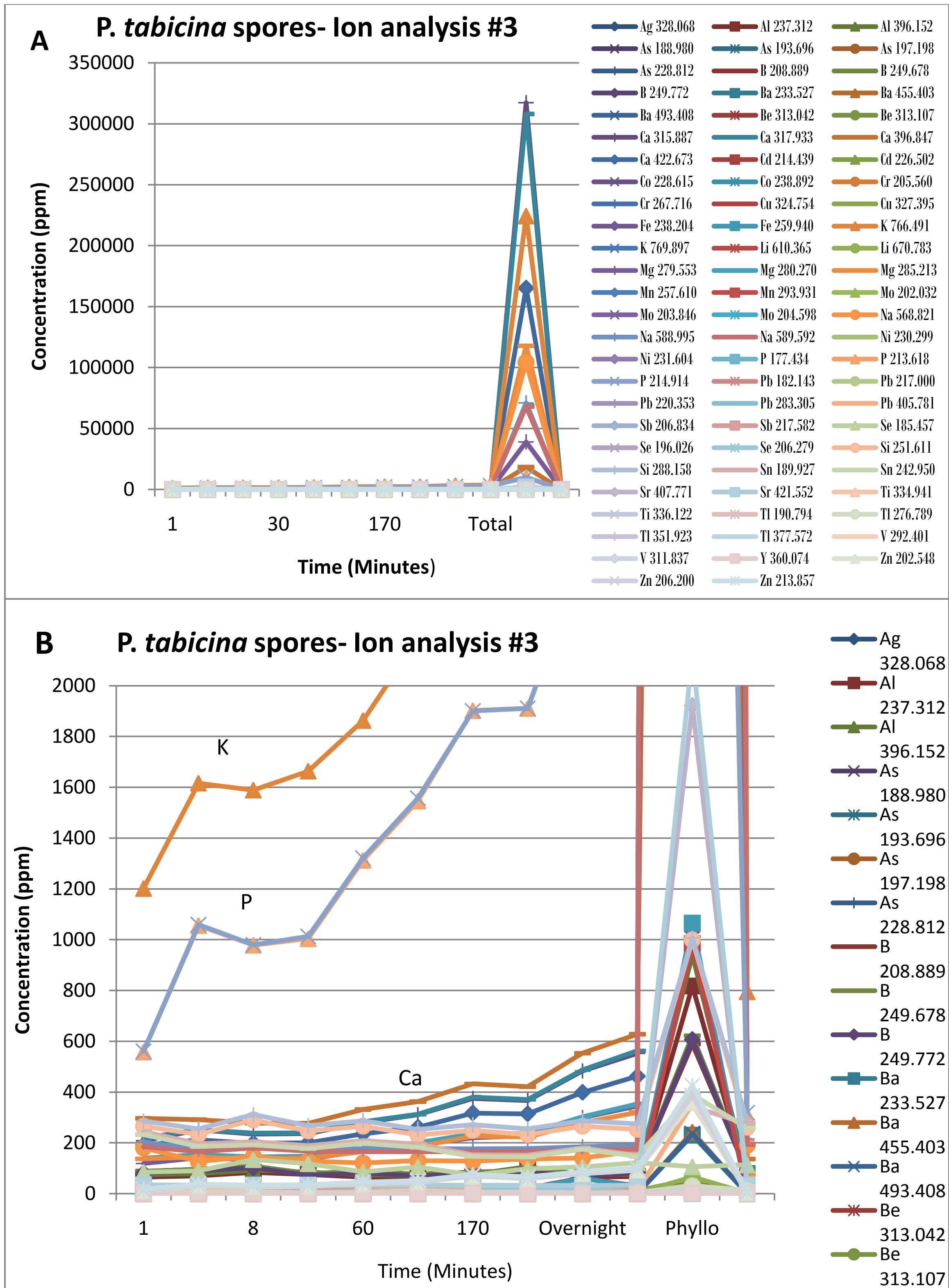


Figure 17 A shows the third ion profile for P. *tabicina* spores using method 1. 17 B shows the expanded view.

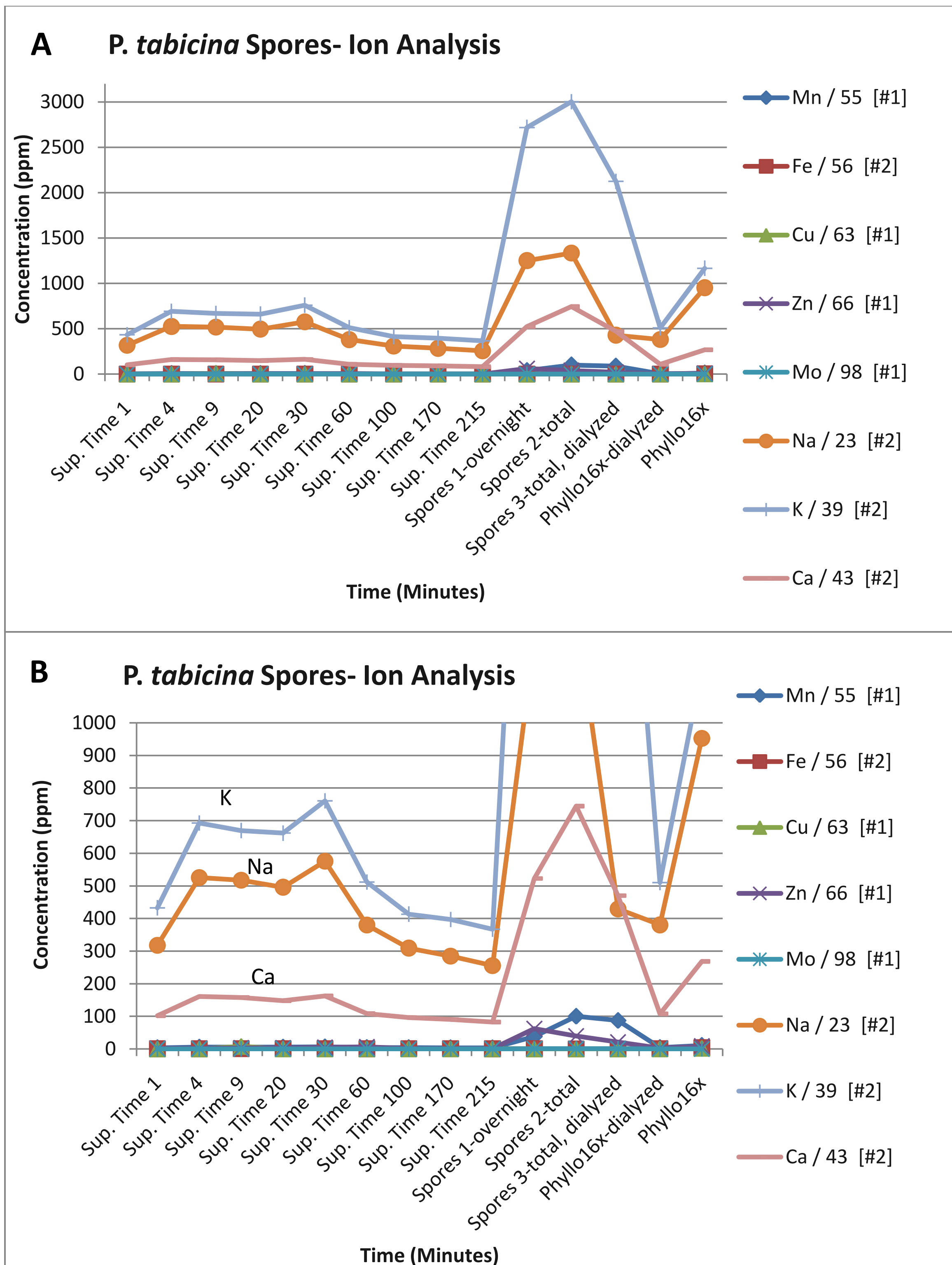


Figure 18 A shows the time course ion profile for P. *tabicina* spores using method 2. 18 B shows the expanded view which shows the K, Na, Ca with highest changing levels.

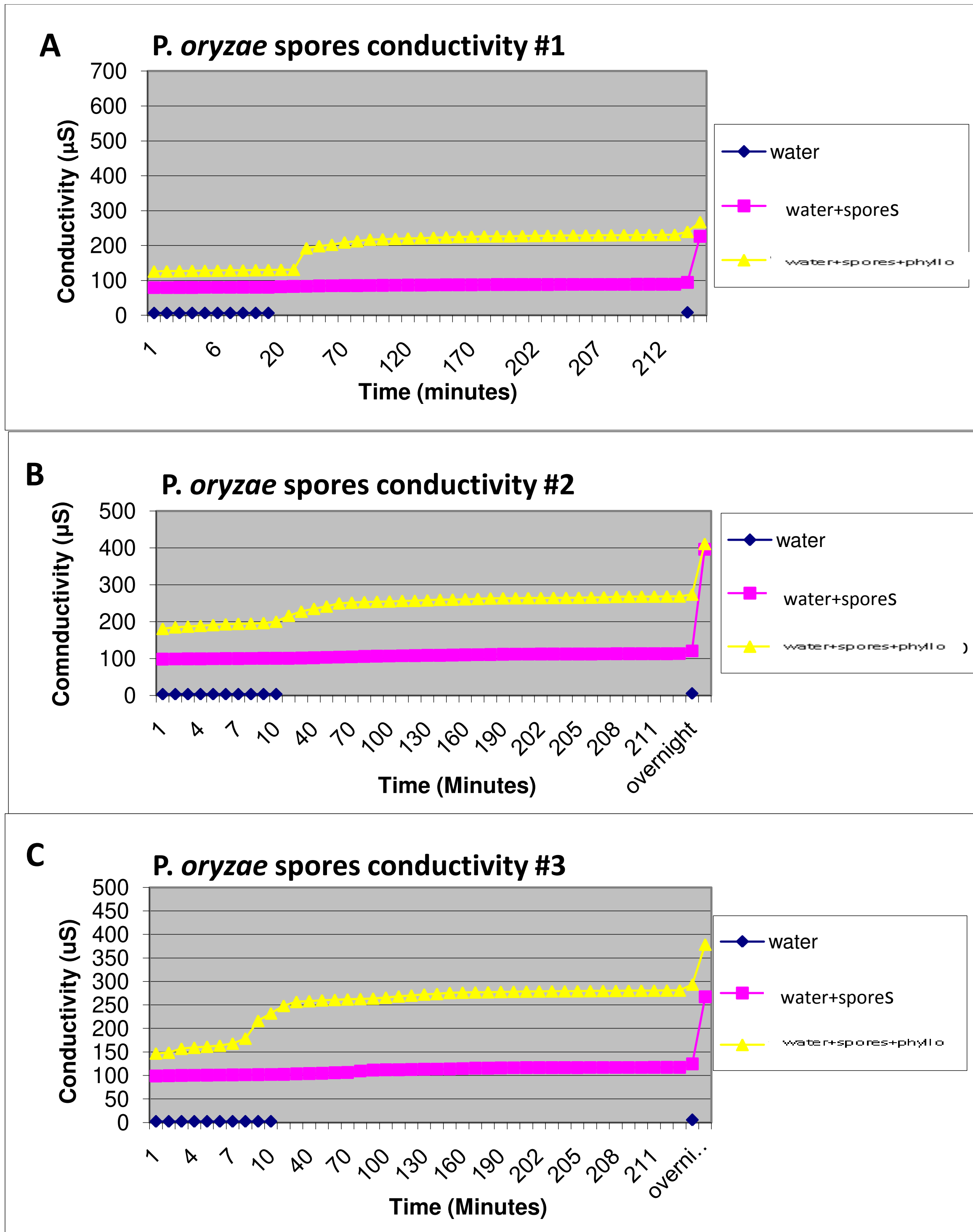


Figure 19 A-C show repetition of the conductivity experiment using P. *oryzae* spores and phylloplanin. Observation of one conductivity jump in phyllo treated spores, water control shows no ion leakage, and water+spores all show no conductivity jump.

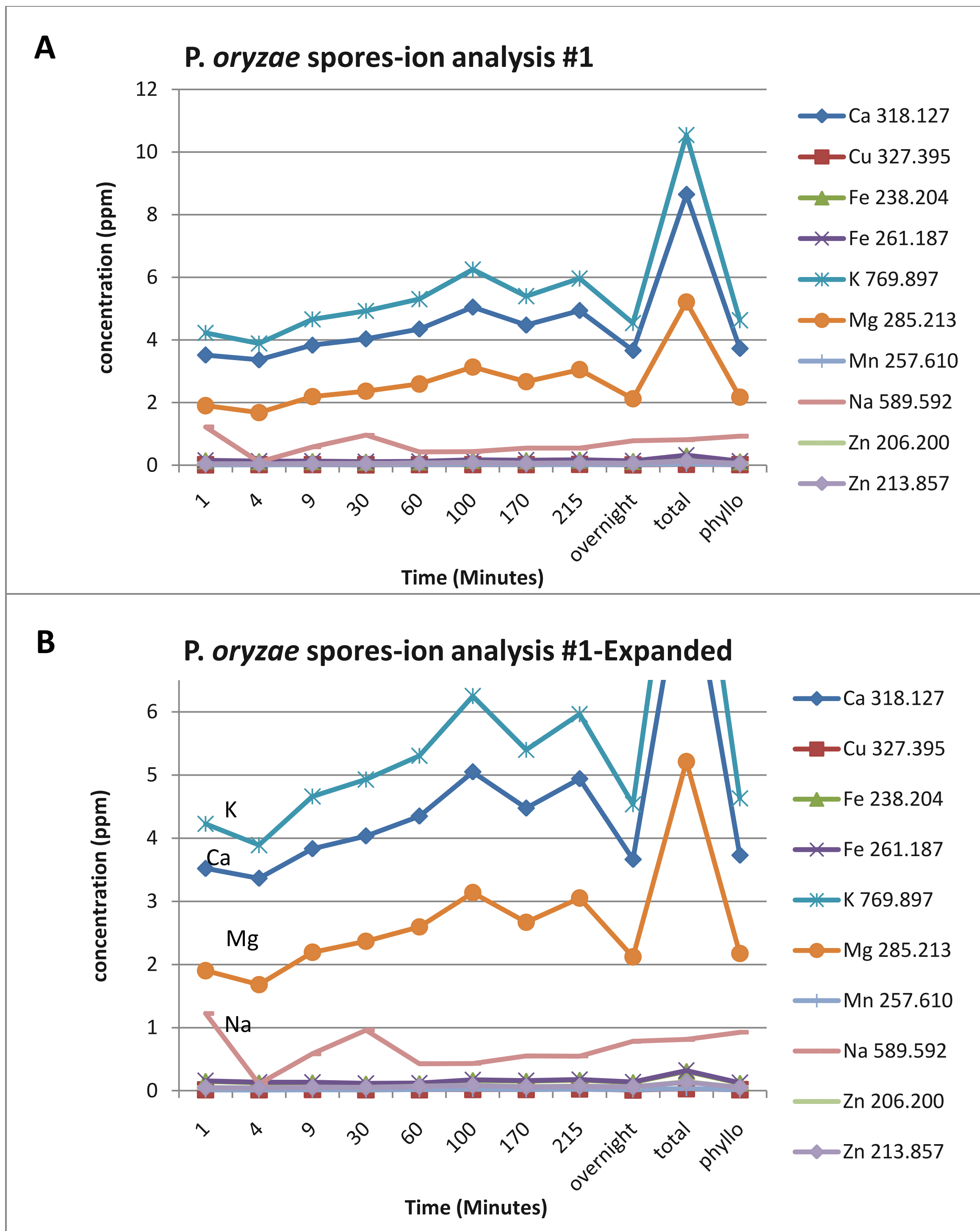


Figure 20 A shows the first ion profile for P. *oryzae* spores using method 1. 20 B shows the expanded view with K, Ca, Mg, and Na with the highest changing levels.

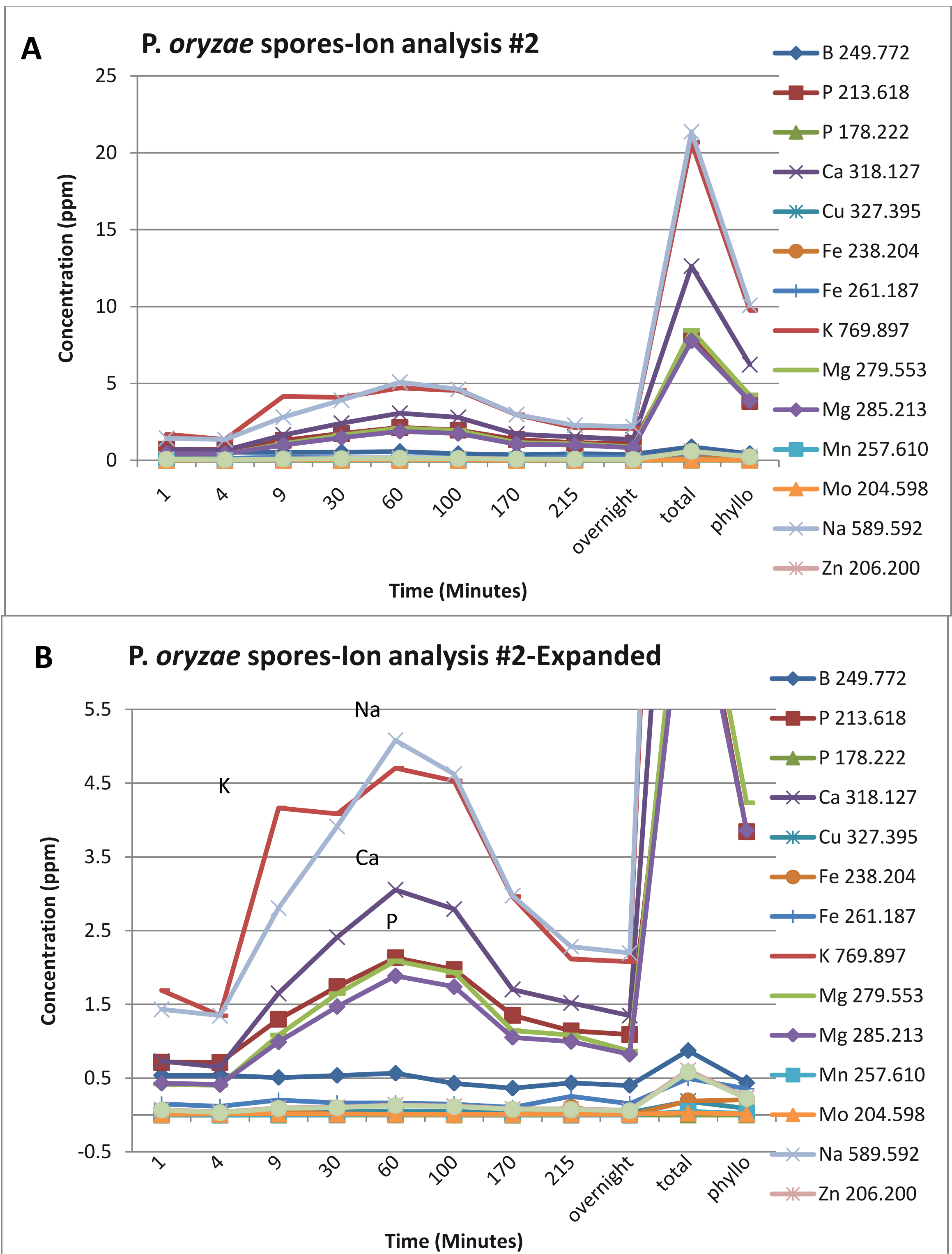


Figure 21 A shows the second ion profile for P. *oryzae* spores using method 1. 21 B shows the expanded view with Na, K, Ca, and P with the highest changing levels.

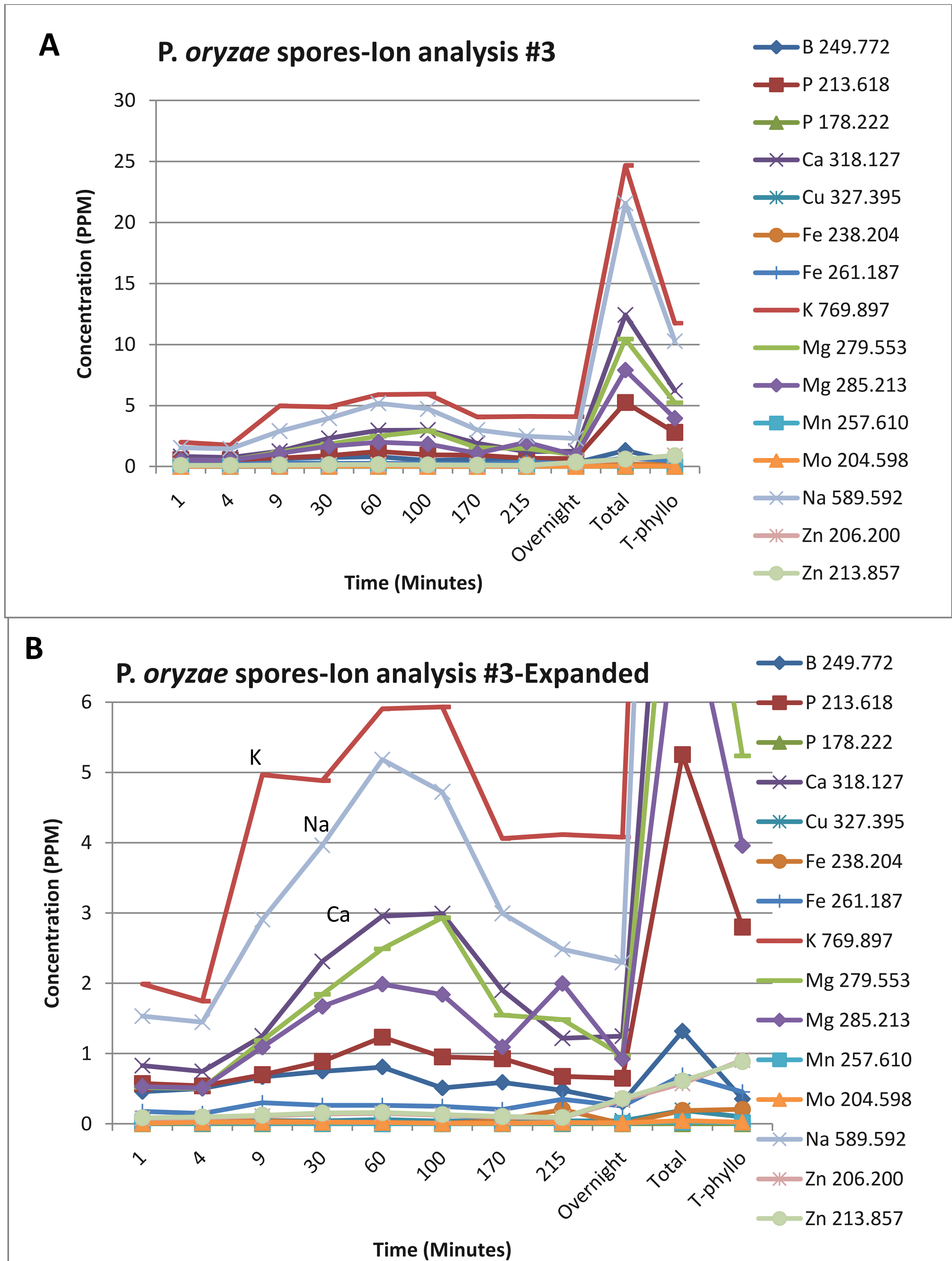


Figure 22 A shows the third ion profile for P. *oryzae* spores using method 1. 22 B shows the expanded view with K, Na, and Ca with the highest changing levels.

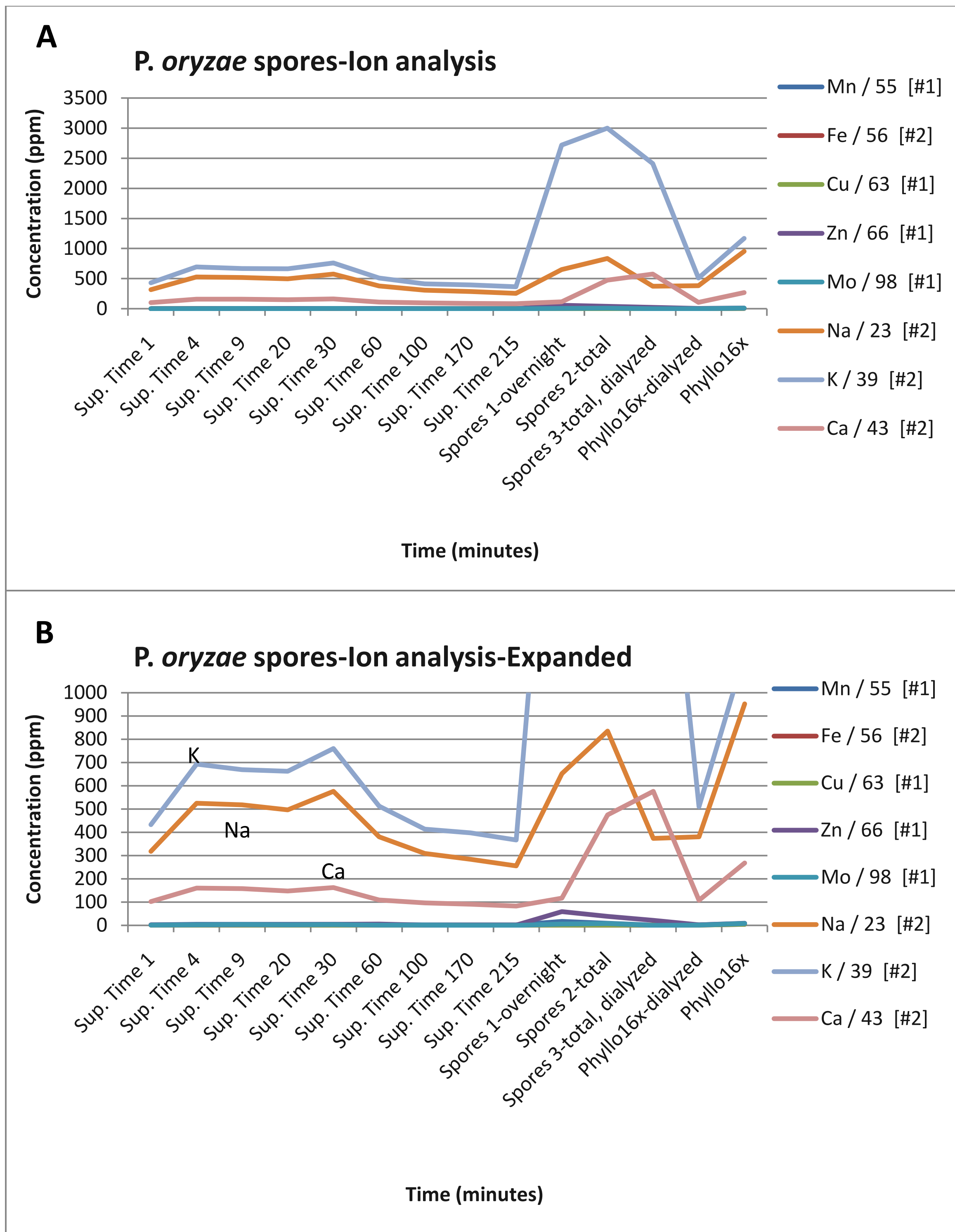
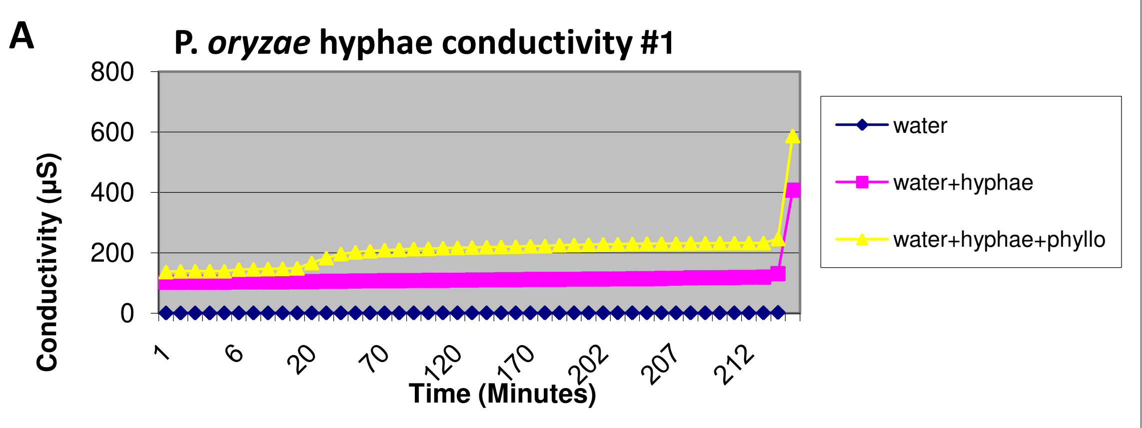
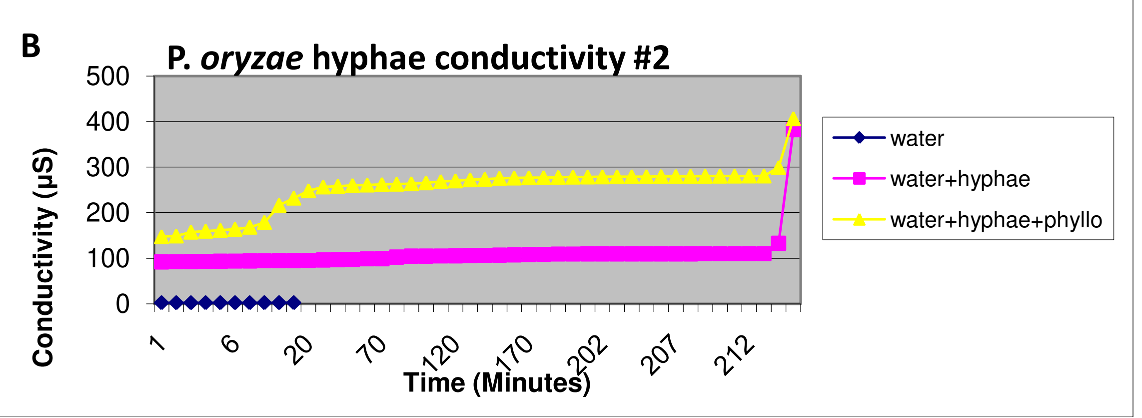


Figure 23 A shows the time course ion profile for P. *oryzae* spores using method 2. 23 B shows the expanded view which shows the K, Na, Ca with highest changing levels.


A
P. *oryzae* hyphae conductivity #1
Conductivity (µS)
800
600
400
200
0
1
6
20
70
120
170
202
207
212
Time (Minutes)
water
water+hyphae
water+hyphae+phyllo


B
P. *oryzae* hyphae conductivity #2
Conductivity (µS)
500
400
300
200
100
0
1
6
20
70
120
170
202
207
212
Time (Minutes)
water
water+hyphae
water+hyphae+phyllo

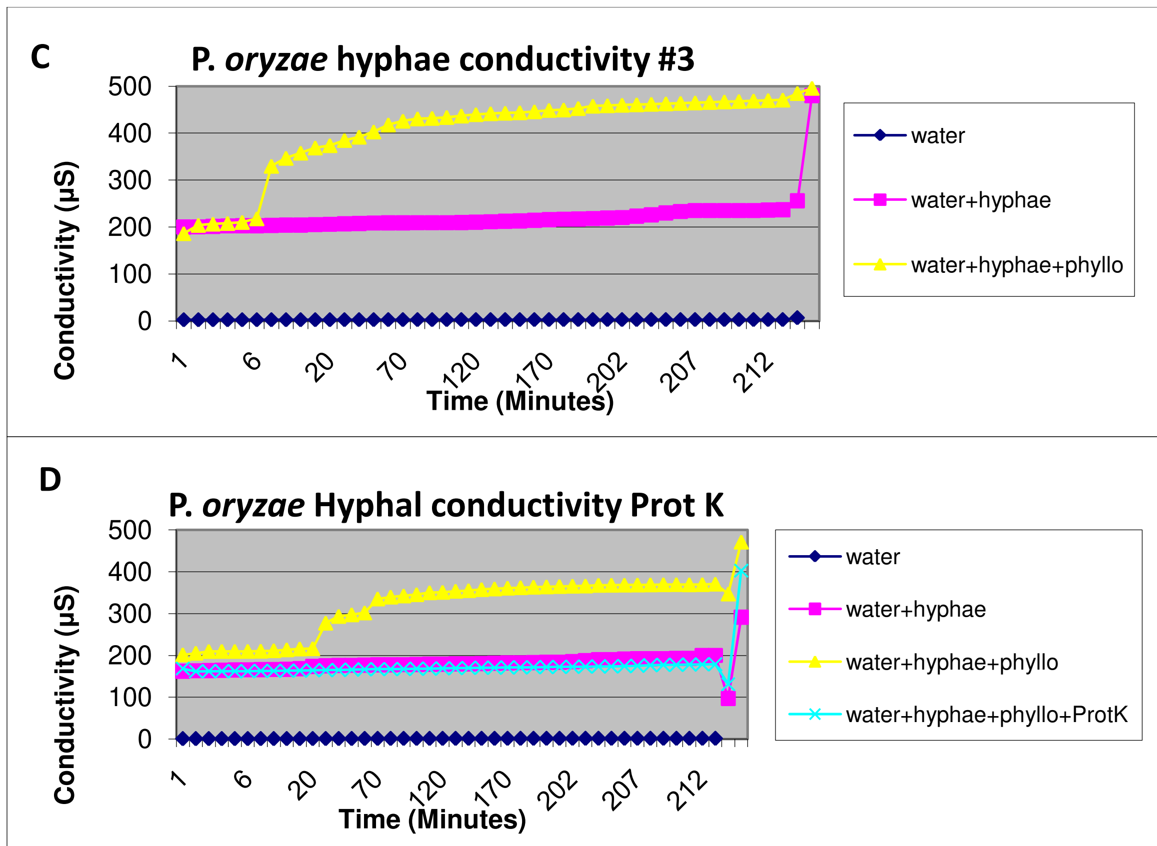


Figure 24 Figures A-C show repetition of the conductivity experiment using P. *oryzae* hyphae and phylloplanin. Observation of one or two conductivity jumps, water control shows no ion leakage, and, Figure 24 D ProtK seems to inhibit phylloplanins from causation of membrane permeabilization.

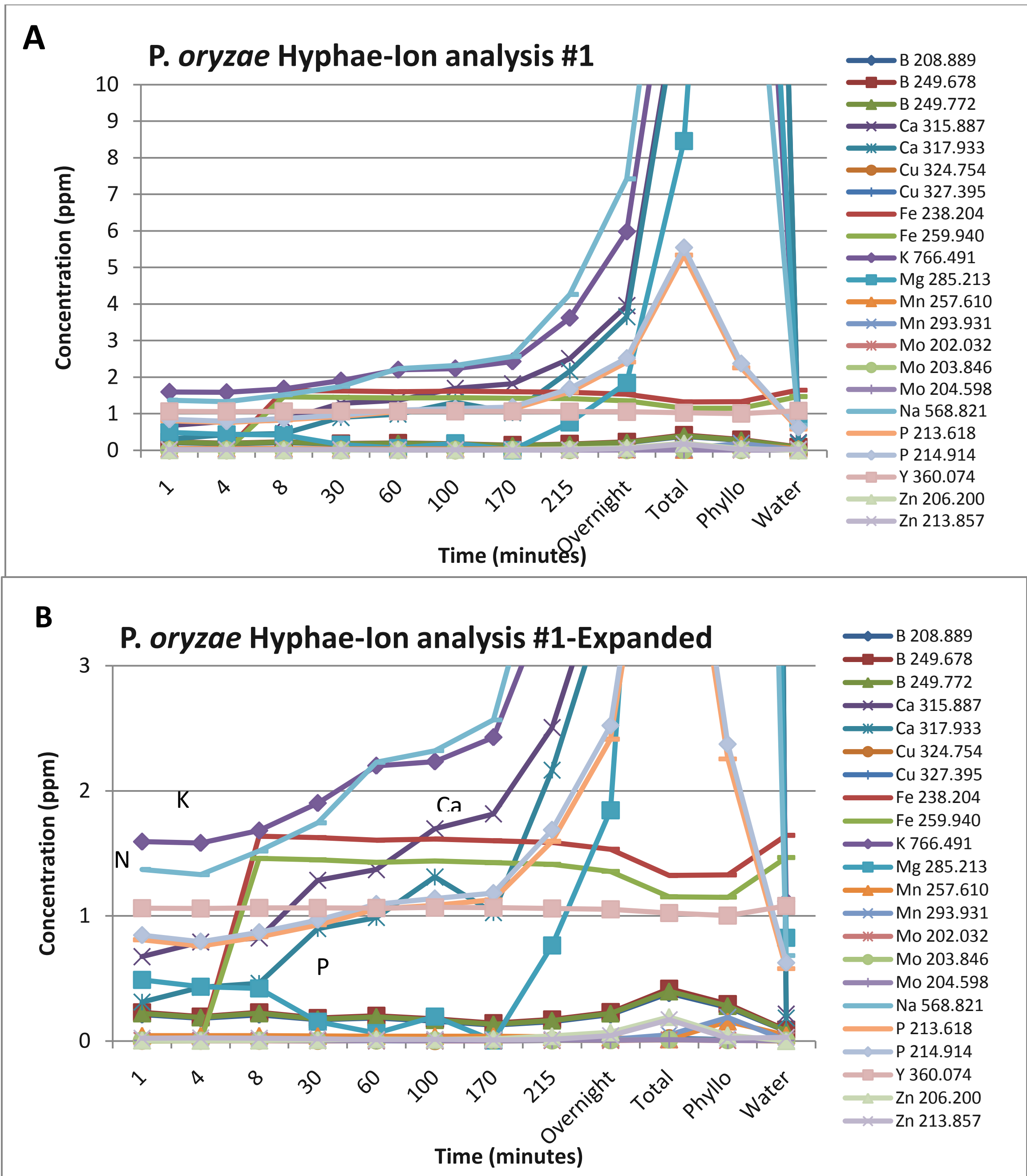


Figure 25 A shows the first ion profile for P. *oryzae* hyphae using method 1. 25 B shows the expanded view.

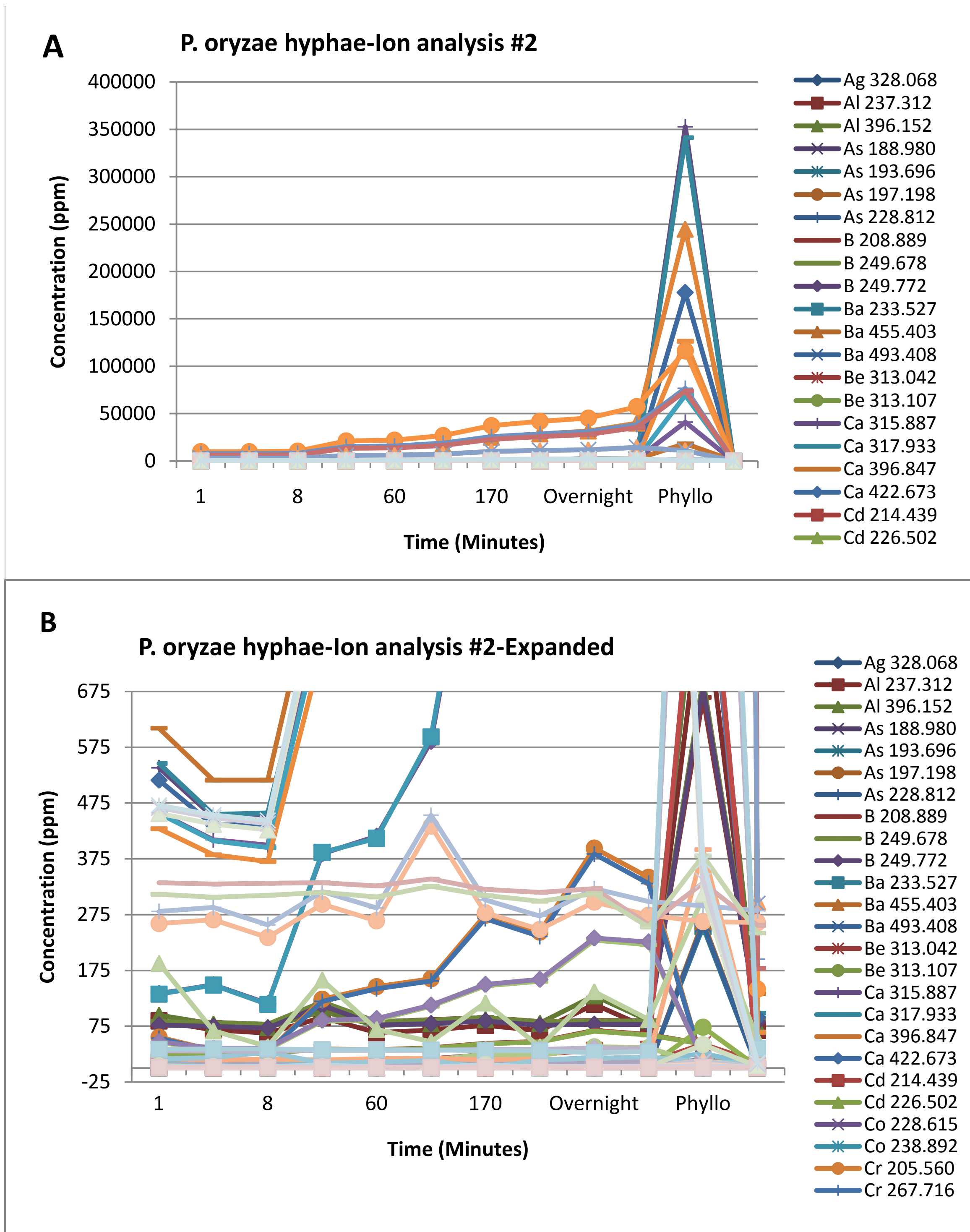


Figure 26 A shows the second ion profile for P. *oryzae* hyphae using method 1. 26 B shows the expanded view.

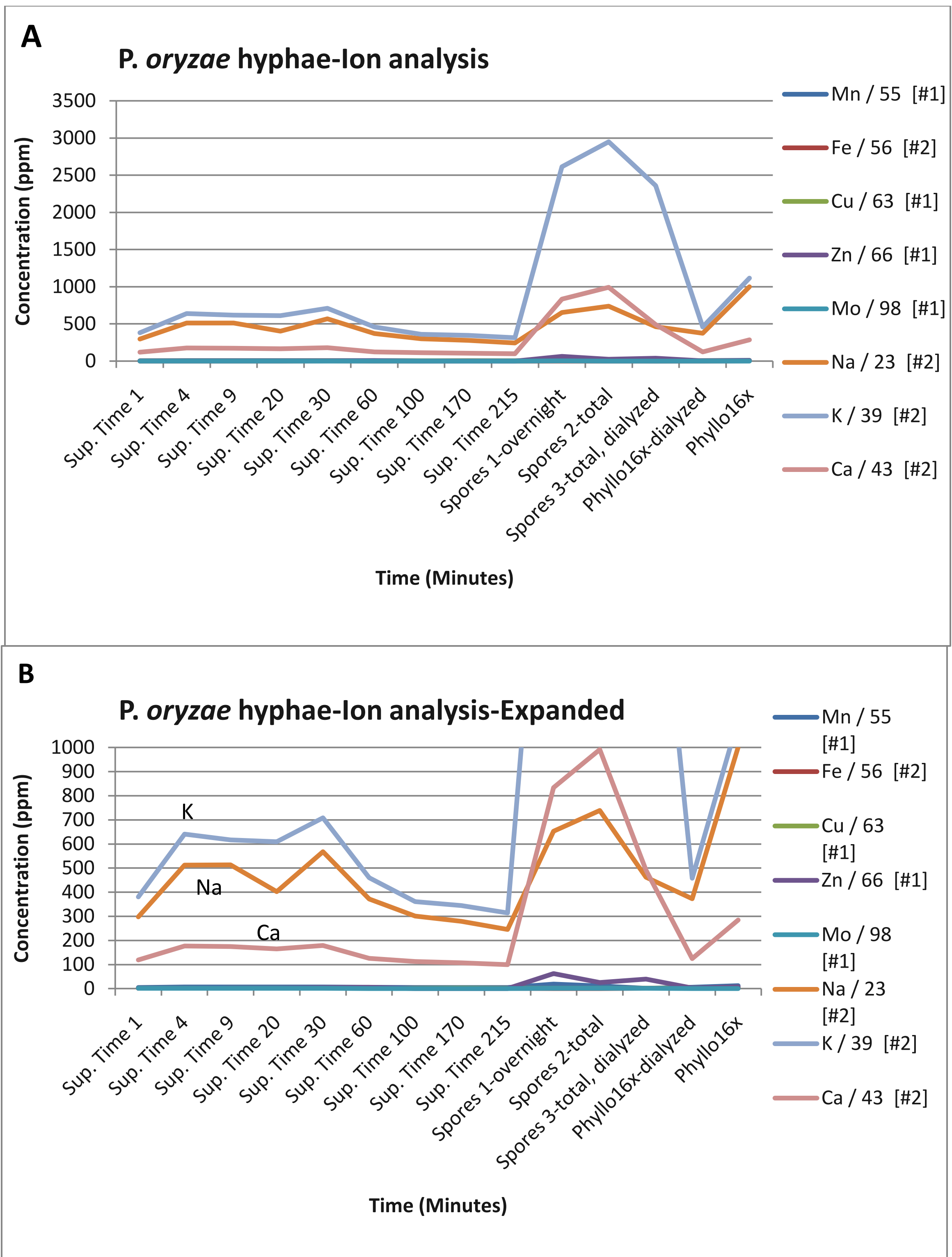


Figure 27 A shows the time course ion profile for P. *oryzae* hyphae using method 2. 27 B shows the expanded view which shows the K, Na, Ca with highest changing levels.

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Chapter 5: Phylloplanin and *cis*-abienol Inhibit *Pythium* and *Phytophthora parasitica* var. *nicotiana* Parasitica at the Zoospore Stage

5.1. Abstract:

Pythium is arguably the most important fungal disease of crop plants, in general (Birch and Cooke 2004). In contrast, *Phytophthora parasitica* which causes black shank disease is specific to tobacco, but is one of the most important pathogens of tobacco (Chacón, Hernández et al. 2009). What these pathogens have in common is that they are both oomycetes (fungi-like pathogens, aquatic filamentous protists, Kingdom Chromalveolata) with a motile zoospore life cycle stage. Preliminary experiments showed that T-phylloplanin did not inhibit hyphal growth of these organisms in Petri plate, hyphal extension assays such as those described in Chapter 2. This led to the question of whether their zoospore stage might be vulnerable. It has been shown that the tobacco trichome secretes diterpene sclareol which inhibits black shank disease caused by *Peronospora parasitica (Bailey, Vincent et al. 1974; Cutler, Reid et al. 1977).* The structure of sclareol and *cis*-abienol are similar, and because at the time of our investigation of the fungicidal activities of phylloplanins we were working with *cis*-abienol in another project, we decided to test T-phylloplanin and *cis*-abienol effects on *Pythium* and *P. parasitica* zoospores, in parallel. Our results show that T-phylloplanin, a glycopeptide formed and secreted to aerial surfaces of tobacco by short, procumbent trichomes of tobacco; and *cis*-abienol, formed in glands of tall trichomes of tobacco and secreted to aerial surfaces inhibit both *Pythium* and black shank diseases at the zoospore

stage in the test systems used. Thus, both may represent a first line of defense against these pathogens of tobacco.

5.2. Introduction:

5.2.1. *Pythium* Species:

Commercial tobacco production increasingly relies on the generation of transplants in a greenhouse float bed system. Float bed based transplant production increased from 50% in 1991 to 78% in 1999 in Kentucky (Stull 2009). Due to the propagation environment of the float system, *Pythium* root rot has become one of the most important diseases encountered during production of tobacco seedlings (Booker, Bedmutha et al. 2010). This is due to the ability of *Pythium spp.* to spread based on spore motility (Fajardo, Parker et al. 2001). *Pythium* zoospores utilize front steering flagella and rear "motor-like" flagella, mechanisms that can propel them through water towards light, or in some species towards chemo-attractants given off by tobacco roots and roots of other plants (Seebold and Johnson 2009). Swimming zoospores can spread rapidly through the float bed and Pythium disease can reach epidemic levels in a short time. Seedlings that survive Pythium in the float bed and are transplanted into the field have reduced vigor and poor performance(Seebold and Johnson 2009). There are several key symptoms of *Pythium* Root rot disease in tobacco. The telltale signs are usually observed when seedlings are 20 days or older, after roots have begun to extend down through the float tray into the float-bed solution (Booker, Bedmutha et al. 2010). The first symptoms appear as round, yellowed groups of plants in a tray. Rapid expansion of disease usually

occurs in a few days to rapidly cover the entire tray and float bed section in the greenhouse. After lower leaves turn yellow, plants start to decline and wilt, and roots become tan to gray colored. In 3-5 days roots become brown to dark gray and feel greasy to touch. Infected roots then rot completely and slough off. Without a viable root system seedlings quickly die. In some cases, white roots re-grow, only to be killed later. *Pythium* is very adapted to living in water, where this organism produces sporangia, or fruiting bodies. Sporangia develop to form zoospores which will swim to roots, infect, and colonize them, to repeat the life cycle.

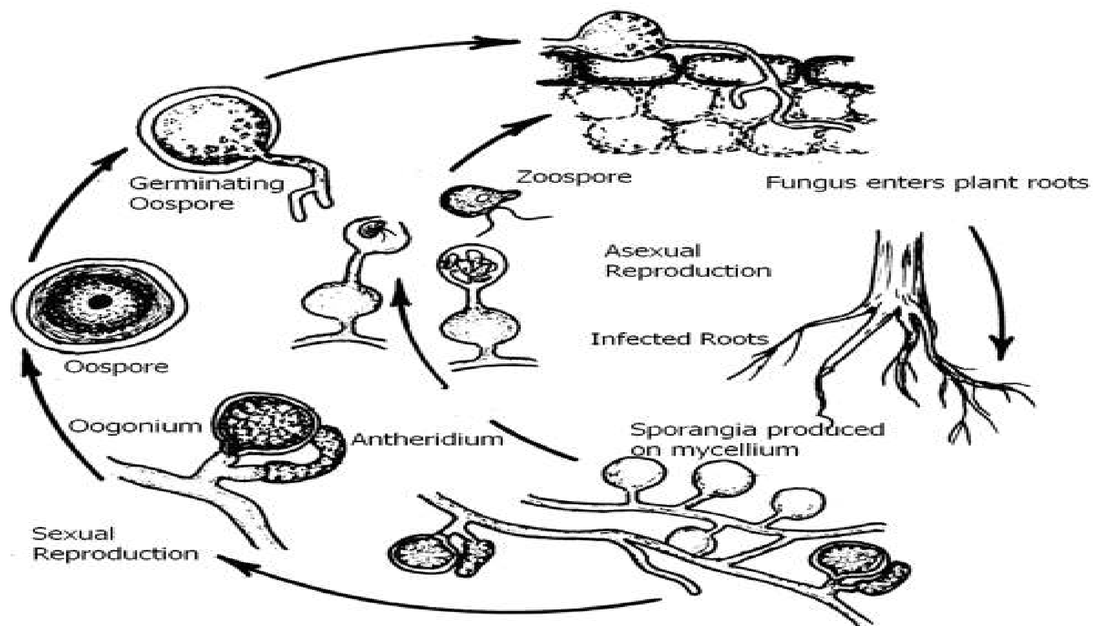

Figure 28 Life Cycle of *Pythium*
http://www.agf.gov.bc.ca/cropprot/images/Pythium_cycle.gif

Zoospores are the main means of dissemination of this pathogen in the float bed system. *Pythium* will also form resting structures (chlamydospores and oospores) that allow the

organism to survive for long periods in soil, or under the float tray system in greenhouses where it can survive in fragments of infected roots trapped in the gaps of styrofoam float trays, etc. Five species of *Pythium* have been identified as possible pathogens affecting tobacco transplants. *Pythium myriotylum*, *P. volutum, P. dissotocum*, *P. irregulare*, and *P. spinosum* in greenhouses. The first two are the most aggressive species which effect tobacco seedlings at water temperatures above 72° F. No above ground symptoms have been observed with the other *Pythium* species at 72° F, but seedlings exposed to these species do have root rot and a poor root system development at transplanting time. But, aggressive isolates of these species can kill seedlings in a few days. The severity of *pythium* on tobacco seedlings will depend on the growth stage of plants when the disease begins. Without controlling the disease plants that get infected as late as 45 days after seeding will not reach the adequate size and vigor required for survival in the field. Conditions for disease spread include high planting density, over fertilization, extreme humidity, and water temperatures above 78°F.

Control recommendations include the use of new float trays or tray sanitation, which are costly or time consuming. Used trays must be thoroughly washed, and allowed to dry completely before being reused. Often bleach or quaternary ammonium compounds are used, but these are not completely effective. Washing trays in any solution is not considered a 100% effective means to kill fungal pathogens, especially those with persistent oospores. Another, more costly but effective method is to fumigate with methyl bromide at 3 lb/1000 cubic feet. Preventative chemical control agents such as Aliette™, Subdue™, Terrazole™ and Turban™ are used, but these are not highly effective curative agents.

5.2.2 *Phytophthora parasitica* var. *nicotiana* Parasitica:

Black shank is the common term used to describe with the pathogen, *Phytophthora parasitica* var. *nicotiana* Parasitica. Black shank disease is one of the most serious and prevalent diseases of tobacco in the southern United States (Parkunan, Johnson et al. 2010). Black shank first appeared in the United States in 1915 in southern Georgia (Shew and Lucas 1991). Currently, black shank can be found in almost every tobacco field in the southern United States and has been estimated to cause damage in approximately 2 to 5.5 % (`18,000,000 dollars) of all tobacco fields each year as reported in the North Carolina Extension service Flue-cured Tobacco disease Report for 2010. Flue-cured tobacco is very susceptible to black shank disease. This disease is found in every Kentucky County where tobacco is grown. *P. parasitica* has its highest infection rates in fields that are poorly-drained, or where infected tobacco was planted the previous year. *P. parasitica* thrives at temperatures between 84 to 90° F (Parkunan, Johnson et al. 2010). Soils which contain black shank inocula cannot be remediated and must be treated for the disease every year, on an ongoing basis. Symptoms of the disease include swift chlorosis and wilting followed by degeneration of the vascular system of the plant. A dark brown to black, somewhat sunken lesion usually appears on the stalk at or near ground level. This lesion often extends up the stalk of the plant causing it to turn black. Stalks, when split, usually reveal the blackened pith separated into discrete disks. This feature is of diagnostic value only when used in conjunction with other observations (chlorosis, wilting) because stem disking may occur due to other factors. Infected plants may be scattered or uniformly distributed in a given field. Roots and crowns are usually decayed

in the later stages of this disease. Only root and crown symptoms may be observed in very dry years, or on partially resistant varieties. *P. parasitica* lives and is harbored in the soil. As already noted, this pathogen belongs to a group of fungi that occurs commonly in areas of high soil moisture. The fungus produces microscopic zoospores that swim in water surrounding roots and/or soil particles. Swimming spores are attracted to tobacco their only natural host, by root exudates produced primarily at growing points and wounds. While wounds are not required for penetration, they do favor more rapid disease build-up within the host.

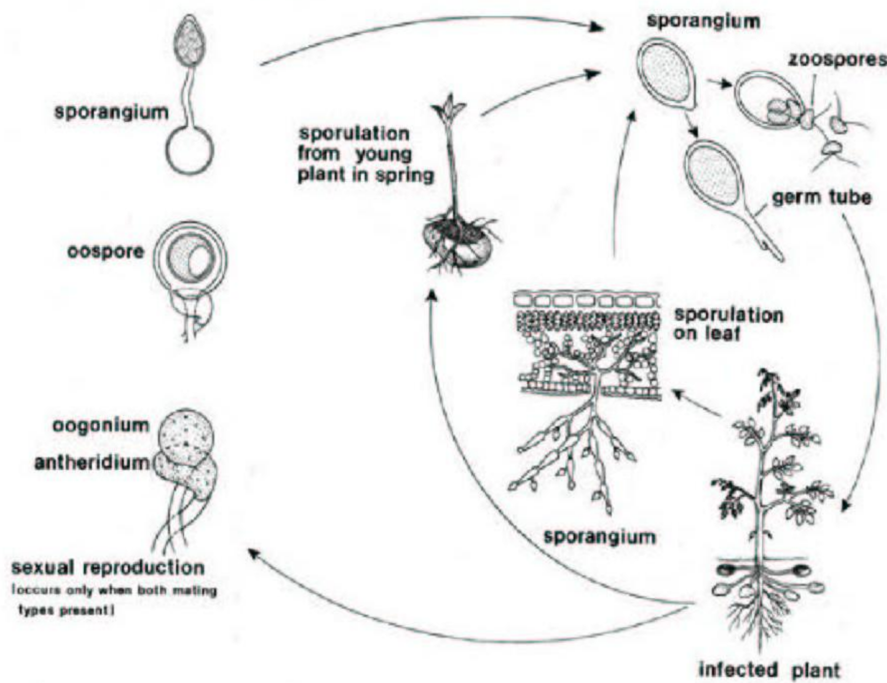

Figure 29 Life cycle of *P. parasitica*
http://www.agf.gov.bc.ca/cropprot/images/Pythium_cycle.gif

When the soil environment is not suitable for survival of the motile spores, the fungus forms thick-walled, resistant spores some of which may survive for years during conditions unfavorable to the fungal proliferation. Once conditions become favorable the resistant spores germinate and motile spores are produced. During favorable conditions, a new cycle of infection and generation of motile spores is produced every 72 hours. The black shank fungus is spread when infested soil is moved from one place to another. Contaminated irrigation or runoff water may also aid in its movement within a field or from one field to another. Although the root is the most commonly affected plant part, occasionally the fungus infects leaves and forms circular, yellowish-to-brown lesions up to 3 inches in diameter. Leaf infection may occur as a result of zoospores in splashing water or by contact with infested soil.

Common methods for control of black shank include crop rotation, which, should be the foundation of every black shank management program because the fungus attacks only tobacco. Leaving the field out of tobacco for one or more years will reduce, but not eliminate this fungus. Any crop can be grown between tobacco crops to reduce the level of the pathogen. Resistant varieties possessing various levels of resistance to black shank are available; however, they should be used as part of an integrated approach including crop rotation and other appropriate cultural practices. Finally, chemical controls can be used which include several soil applied chemicals that are labeled for black shank control. Currently these include the multi-purpose fumigants Telone C-17™, Chlor-O-Pic 100™, and Terr-O-Gas 67™. However, the systemic fungicides Ridomil Gold™ or Ultraflourish™ are the most effective materials for black shank control. All synthetic

chemicals are expensive and moderately effective when properly used and included in an integrated pest management system.

Because of the importance of black shank and pythium, and their ability to disseminate in tobacco fields and float systems, and since they are in the same Class as another previously tested oomycete (*Peronospora tabacina*) it was logical that we should investigate the possible impact of tobacco phylloplanin on these fungus-like organisms Here we describe results of experiments designed to test the effectiveness of T-phylloplanin and the tobacco trichome secreted compound *cis*-abienol as means for fungicidal or fungistatic control of these pathogens. As noted above, preliminary, Petri plate hyphal extension assays were made to test T-phylloplanin effectiveness against the vegetative growth stage of both these pathogens. No hyphal inhibition was observed. We then hypothesized that if these pathogens might be vulnerable at a different life cycle stage, the zoospore stage.

5.3. Methods and Materials:

Two types of experiments were used to test the hypothesis that T-phylloplanin and *cis*-abienol can inhibit *Pythium* and *P. parasitica* at the zoospore stage. These were: 1) an assay consisting of tobacco seedlings (variety, T.I. 1068) floating on a solution above an agar piece producing zoospores – placed at the bottom of the tube. This float-bed-mimic experimental system is hereafter referred to as the "float tube assay", and 2) a "soil drench assay" where a solution of *cis-abienol* in 100% ethanol is applied to the mostly dry soil surface around the stem of a potted seedling. Because of time constraints, T-phylloplanin was only tested in the float tube assay.

5.3.1. Pathogens:

Both species of pathogens were obtained as a pure, single spore cultures from Ed Dixion, University of Kentucky, Plant Pathology Department. Pythium was sub-cultured by taking a 1 cm square plug of actively growing mycelia and inoculating a V8 agar plate as described in Chapter 2. The V8 media was prepared by dissolving 3g $CaCO_3$ into 200mL V8 Vegetable Juice™ (Cambell Soup Company, Camden, NJ 08103-1701) followed by adding, to solution, 15g agar (BP1423-500, Fisher Scientific, Fair Lawn, New Jersey 07410), then bringing the volume to 1 liter. After adjustment of the pH to 6.3 with HCl, the medium was sterilized for 30 min at 121°C, cooled and plates were poured. The V8 agar plates were used for both oomycetes tested. This particular media was chosen to give prolific mycelial growth as well as sporulation. After 24 hours of growth under constant Vitalux™ grow florescent light at 23^0 C, plates were overgrown by the pathogens. At this point, mature sporangia were formed in water to yield swimming zoospores. Experiments with both oomycetes were run in parallel. For root drench experiments 4 different *Nicotiana tabacum* species: T.I.1068, 'Red Russian', and the dark tobacco type's msD2602R (black shank resistant), and NL Madole LC (highly black shank sensitive) were selected. Seeds were sown in Promix™ and covered with humidity domes. After 5 days germinated seedlings were transplanted into individual pots. For float tube assays, the seedlings were grown for an additional 10-12 days or until the seedlings had reached a diameter of ~3cm. For the soil drench assays, plants were transplanted using ProMix™ into 6 inch pots and left to grow for several weeks in the

greenhouse (29^0 C, 50% RH) with normal watering, after an initial fertilization with 25 ml per pot of Peters™ 20-20-20 (3g per gallon).

5.3.2. Float Tube Assays with *Pythium* and *P. parasitica*, T-phylloplanin and *cis*-abienol Treatments:

For the float tube experiments, I cm blocks of agar were cut from the edge of an actively-growing Petri plate culture of *Pythium* or *P. parasitica* and dropped into a round bottomed, 50ml, Pyrex, centrifuge tube or a 50 ml beaker (*Pythium* /T-phylloplanin experiment only), and the vessel was filled with 40ml of sterile deionized water. Host plant seedlings were selected for equal size (~3 cm diameter) and roots were gently washed to remove ProMix™. One seedling was floated on the surface of the water, or water containing treatment in each tube, with roots submerged in the water solution.

Treatments for T-phylloplanin tests with *Pythium* (as in Figure 30) were: 1) T-phylloplanin plus spores (1ml of 112 mg/ml T-LWW); 2) Water control (untreated, no spores); 3) T-phylloplanin control (no spores); 4) Infection control (water with spores). In addition to the experiment shown in Figure 30, three separate T-phylloplanin/*Pythium* experiments were made in 50 ml tubes (pictures not shown) included: a) an infection control [infection control (untreated)] in Table 9); b) 1ml of 112 mg/ml T-phyllo added to the top of a tube (1 ml compound in Table 9); c) seedling roots dipped in T-phylloplanin (112mg/ml) and left to partially dry before placing the seedling onto the top of a tube (1 root dip in Table 9): d) seedling roots dipped two times, left to dry between each dip, and then placed on top of a tube (2 root dip in Table 9); e) seedling roots dipped three times, left to dry between each dip, and then placed on top of the tube (3 root dip in Table 9).

For treatments a and b, seedlings were floated on the top of tubes. The tubes with plants were placed under 24 hour florescent lightning at 23^{0}C and were visually inspected each day until hyphae were visible on roots of the water control. The hyphae appeared as a cotton-like covering around roots. The days before hyphae appeared on roots was recorded, as was the extent of hyphal growth on roots (on a +/- scale).

Similarly three experiments were made to test the impact of *cis*-abienol on *pythium* infection (pictures not shown - data are shown in Table 9). *Cis*-abienol treatments were as follows: 1) roots dipped in cis-abienol 3 times; 2) roots dipped 2 times; 3) roots dipped 1 time; 4) one ml of *cis*-abienol in 100% ethanol (concentration proprietary) solution added to the top of the tube, 5) water only infection control After treatments 1, 2, 3 were added seedlings were floated on the top of tubes. Similar experiments were also made to test the impact of T-phylloplanin and *cis*-abienol on black shank infection data are shown in Table 9. Pictures of one *cis*-abienol/*P. parasitica* experiment is shown in Figure 31)

5.3.3. Soil Drench Assays Were Made with *P. parasitica* Only, and *cis*-abienol Only:

For soil drench assays, *P. parasitica* inoculum was prepared by placing five, 1 cm blocks of agar cut from the edge (the youngest tissue) of a Petri plate culture of *P. parasitica* and dropped into a sterile 95 X 15cm plastic Petri plate filled with 25 ml of sterile de-ionized water. After 24 hours the Petri plates were examined using microscopy to visually check for motile spores. The spore solution was diluted to ~1000 spores per ml (hemocytometer). A drench solution containing *cis*-abienol in 100% ethanol was prepared. To each pot containing a host plant, 2.5 ml of drench was applied evenly around the base of the plant stem followed by addition of 1ml of spores, where used).

Treatments were as follows: 1) water control (no spores), 2) 100% ethanol alone control (no spores), 3) water plus spores (infection control), 4) ethanol + spores (infection control), 5) commercial fungicide (Subdue^{TM}) in water at labeled rate (1 oz per 100 gal, 200 ml used per drench) plus spores, and 6) *cis*-abienol plus spores Experiments were done in sets consisting of 1 plant each of msD2602R, and NL Madole LC, or T.I. 1068, msD2602R, and NL Madole LC. The variety Red Russian was tested separately because this species is highly enriched in *cis*-abienol (in leaf trichome exudate). In this case we wanted to test if this tobacco which produces substantial *cis*-abienol possessed endogenous resistance in roots.

5.4. Results:

5.4.1. Float Tube Assays:

Float tube assays showed several different outcomes of interest. Data in Table 9 show that in nearly all experiments, both T-phylloplanin and *cis-abienol* treatments delayed onset of infection, but phylloplanin was less effective than *cis*-abienol. For example in experiment 1, days before infection (DBI) for the 1 time root dip was 4 days for T-phylloplanin, while it was 5 days for *cis*-abienol. This trend was seen with all the root dip treatments over all three experimental rounds. It is also interesting to note that, roots dipped three times with *cis-abienol* never got disease, while the 3 timed dipped T-phylloplanin treated plant always showed symptoms after 8 days. This may be due to the low solubility of *cis*-abienol in water. *Cis*-abienol appeared to be crystallized on roots during drying(s) after dipping(s), while this did not occur with T-phylloplanin. Also, when 1 ml of *cis*-abienol was added to the surface of water in a float tube, it became

cloudy, then later became clear, suggesting its precipitation from solution followed by plating out on the tube wall or on roots. In contrast, when T-phylloplanin was added to the surface of water in a float tube it appeared to dissolve into solution with time. Extremely high disease pressure (due to high spore concentration) may also have been a factor in differences observed between T-phylloplanin and *cis*-abienol treatments. The concentration of the T-phylloplanin stock solution used in these experiments was that used for experiments of Chapters 2 and 3 (112mg/ml of LWW, and 3.73 mg protein/ml). But dilution in these float tube assays (assuming total solution) reduced the concentration in treatments 2 and 3 to 1/40th and 1/20th, respectively. Therefore concentrations were low compared to those used in earlier experiments (Chapters 2 and 3). Results for *P. parasitica* were similar to those found with *Pythium*, however in 2 of the 3 experimental rounds, roots dipped three times also developed low infection after 8 days (see Table 9). Again, we observed that development of disease was always postponed or halted for both diseases with both T-phylloplanin and *cis*-abienol as compared to the water control (infection control) and data indicate that directly treating roots has advantage over adding either T-phylloplanin or *cis*-abienol to the float solution. The results of the float tube assays described clearly indicate that both T-phylloplanin and *cis*-abienol can at least delay the onset of both *pythium* and black shank diseases in a float-tray like environment. However we consider these experiments to be preliminary and recognize that further study is needed to determine if the effects observed can be achieved in a conventional float bed design. Possibilities include imbuing float trays with T-phylloplanin or *cis*-abienol, and inserting dissolvable tablets or substrates containing

these compounds (at appropriate concentrations) in float tray growth medium. We are currently testing the later possibility.

5.4.2. Soil Drench Assays:

Table 10 shows results obtained in soil drench assays to test the impacts on *P. parasitica* of applying a soil drench containing *cis*-abienol in ethanol to several tobaccos. Similar experiments were made for *pythium*, but results were variable (possibly due to host/pathogen non-specificity) and we abandoned these attempts so as to focus on black shank. Also, soil drench experiments with T-phylloplanin were not made.

As shown in Table 10 treatments and ratings for soil drench experiments involving the tobacco, NL Madole (susceptible variety) versus msD2602R (resistant variety) showed that disease was inhibited by application of the *cis*-abienol containing drench (or fungicide application - Subdue™) in all cases - compare the infection control (spores but no *cis*-abienol) with spores plus *cis*-abienol or fungicide treatments. Controls were; no spores or *cis*-abienol (water control), and ethanol control (2.5 ml ethanol only). The later control was to test for possible impacts of ethanol soil drench on plants. An extra control was added to the experiment 2 to control for the possibility that ethanol (carrier solvent for the *cis*-abienol) might have a significant impact on normal infection. We were surprised to find that application of 2.5 ml of 100% ethanol (with or without cis-abienol) around the base of young plants (~20 cm tall) in 6 inch pots containing semi-dry ProMix™ did not appear to effect plant health or growth.

As shown in Table 10, black shank disease developed in the susceptible tobacco NL Madole after 10 days while it did not develop in plants drenched with *cis*-abienol or

Subdue™. As expected, black shank resistant msD2602R did not develop disease in any case. Also shown is the data from an experiment testing Red Russian, a tobacco that produces high levels of *cis*-abienol in aerial-tissue-trichomes on leaves and stems. This experiment was an attempt to determine if roots of Red Russian tobacco might show endogenous resistance. We also tested T.I. 1068, a tobacco that has very high levels of aerial-tissue-trichome-produced diterpenes (other than *cis*-abienol) and sugar esters. Neither Red Russian nor T.I. 1068 appeared to have endogenous resistance to black shank in our experiments.

Figure 32 shows photos of leaves and stems from all treatments in Experiment 1, after the infection control showed visible signs of disease. Figure 32 A, infection control on NL Madole, shows clear signs of vascular rot from infection; B shows no effect on the plant due to water alone; C shows no disease after treatment with *cis*-abienol (no disease or signs of stress due to *cis*-abienol treatment); D shows resistance due to fungicide treatment; and E shows no signs of plant stress due to the presence of ethanol alone, applied in the same amount as used in the *cis*-abienol treatment. Figure 33 shows very similar results, however, since this is the resistant msD2602R (resistant variety) there is no positive infection control associated with experimental system. We note that all experiments were run in tandem utilizing the same pooled inoculum. Every plant was exposed to the same pooled inoculum at the same time.

Figures 34 and 35 show photos of leaves and stems from all treatments in Experiment 2 of Table 10. Results were similar to those of Experiment 1 (figure 36). Results with Red Russian (Figure 36) were similar to those observed with NL Madole (Figure 32 and 34), identifying Red Russian as a black-shank-susceptible tobacco.

5.5. Conclusion:

In summary, both T-phylloplanin and *cis*-abienol were effective in delaying or preventing pythium and black shank diseases in the float tube and soil drench assays described in this chapter. More study is needed to develop a practical approach for infection control and to determine mode(s) of action. The later could be researched by testing the hypothesis that these compounds affect spore motility as opposed to ion leakage. This could be assessed by taking motility counts of spores before and after treatments. The possibility of ion leakage could be tested using vital stains to determine the ratio of living to dead spores at different time points after addition of T-phylloplanin or *cis*-abienol. While many aspects of the experiments reported are considered preliminary, all results are consistent with the conclusion that T-Phylloplanin and *cis*-abienol are inhibitory to *Pythium* and *P. parasitica* spores, and therefore both compounds show promise for conducting additional work to determine if either or both could be used in an IPM strategy to control *Pythium* or black shank diseases in the tobacco float bed or field.

Table 9 Float tube assays tested using T.I.1068 with T-phylloplanin and *cis*-abienol on *Pythium* and P. *parasitica*. Days before infection and disease ratings are shown.

	Pythium				*P. parasitica*			
	Phylloplanin		*cis-abienol*		Phylloplanin		*cis-abienol*	
Treatments	DBI	Ratings	DBI	Ratings	DBI	Ratings	DBI	Ratings
Experiement 1								
a)Untreated	3	+++	3	+++	4	+++	4	+++
b)1ml compound	6	+	5	+++	6	++	4	+++
c)I root dip	4	++	5	+++	4	++	5	+++
d)2 root dip	4	++	7	+	5	++	7	+
e) 3 root dip	8	+	0	–	6	+	0	–
Experiement 2								
a)Untreated	4	+++	3	+++	4	+++	4	+++
b)1ml compound	5	+	4	+++	5	++	3	+++
c)I root dip	3	++	5	+++	4	++	5	+++
d)2 root dip	4	++	7	+	4	++	7	+
e)3 root dip	5	+	0	–	7	+	8	+
Experiement 3								
a)Untreated	2	+++	4	+++	3	+++	4	+++
b)1ml compound	2	+	4	+++	2	++	4	+++
c)I root dip	3	++	6	+++	4	++	6	+++
d)2 root dip	3	++	7	+	4	++	7	+
e)3 root dip	8	+	0	–	7	+	8	+

DBI= Days before infection
*=Plant died before infection by pathogen
- = No infection
+ =Almost no visable signs of pathogen
++ = Some visable signs of pathogen
+++ =Clear signs of pathogen present

T

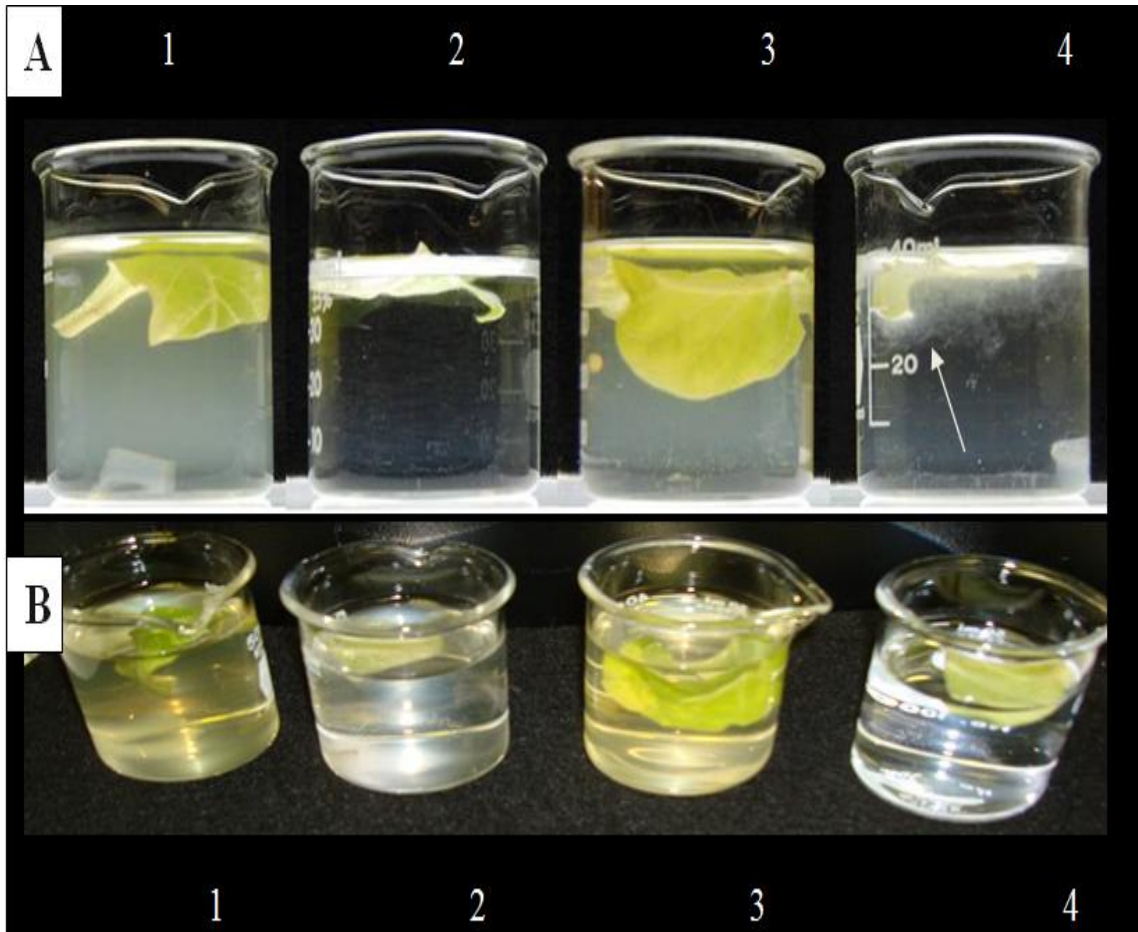

Figure 30 A - Preliminary float tube assay testing the impact of T-phylloplanin on *pythium* disease using T.I. 1068 seedling leaves. Treatments are 1) T-phylloplanin plus spores, 2) water control 3) T-phylloplanin control, 4) Infection control. B - Shows beakers without bottom lightning; arrow denotes presence of hyphae.

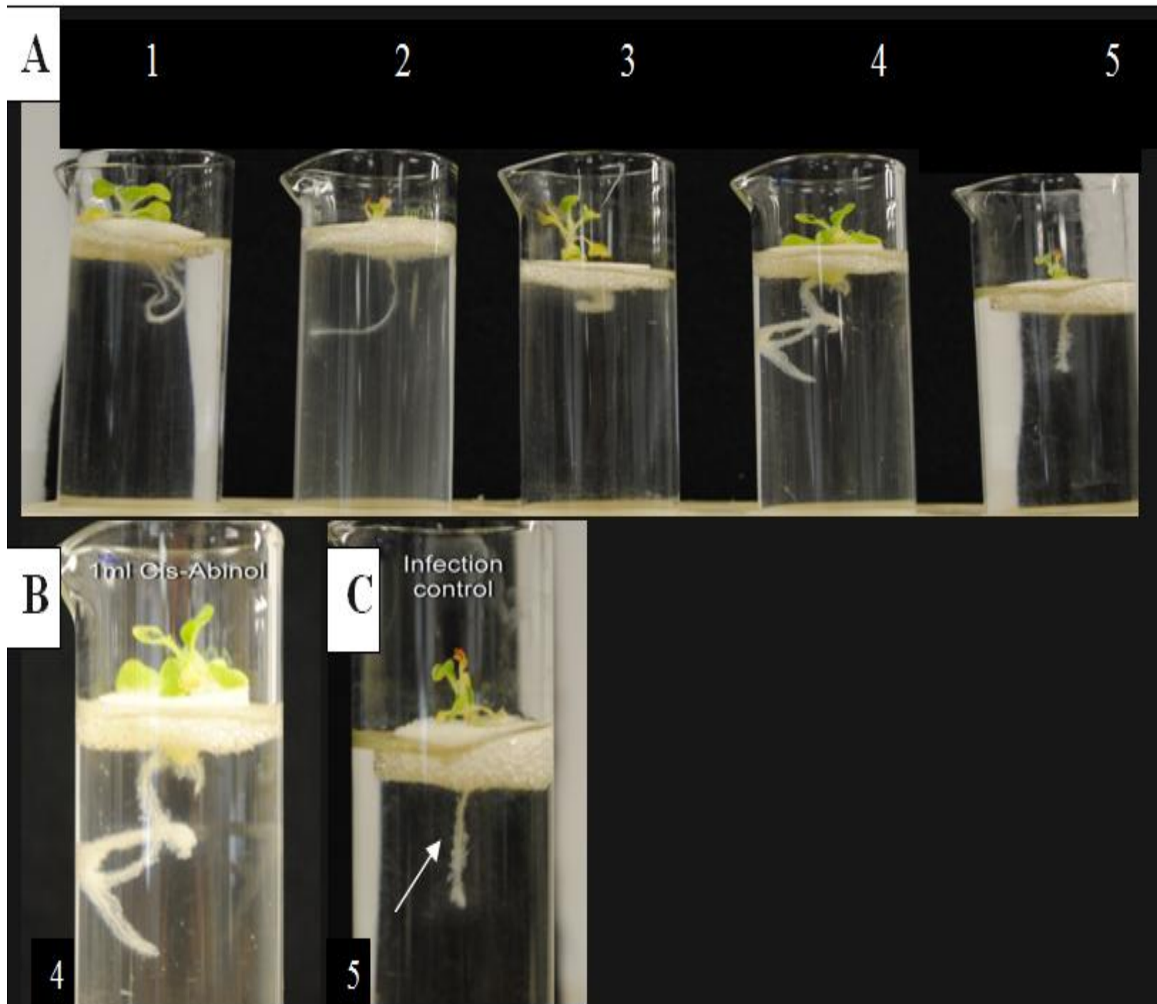

Figure 31 A - Float tube assay testing the impact of *cis*-abienol on *P*. *parasitica* with T.I. 1068 seedlings. Treatments are 1) roots dipped in *cis*-abienol 3 times, 2) roots dipped 2 times, 3) roots dipped 1 time, 4) 1 ml *cis*-abienol added to the top of the tube, 5) Infection control. B - Expanded view of 1 ml *cis*-abienol treatment. C - Expanded view of infection control; arrow denotes presence of hyphae.

Table 10 Soil drench assays showing days before infection (DBI) and disease ratings for P. parasitica with cis-abienol

	P. parasitica				*P. parasitica*			
	NL Madole		msD2602R		Red Russian		T.I.1068	
Treatments	DBI	Ratings	DBI	Ratings	DBI	Ratings	DBI	Ratings
Experiement 1								
Infection Control	10	+++	*	-	12	+++	8	+++
Water Control	*	-	*	-	*	-	*	-
cis-abienol	*	-	*	-	*	-	*	-
Fungicide Control	*	-	*	-	*	-	*	-
Ethanol Control	*	-	*	-	*	-	*	-
Experiement 2								
Infection Control	10	+++	*	-	8	+++	8	+++
Water Control	*	-	*	-	*	-	*	-
cis-abienol	*	-	*	-	*	-	*	-
Fungicide Control	*	-	*	-	*	-	*	-
Ethanol Control	*	-	*	-	*	-	*	-
Ethanol+Infection control	9	+++	*	-	8	+++	8	+++

DBI= Days before infection

* =Plant was never infected

- = No infection

+ =Almost no visable signs of pathogen

++ = Some visable signs of pathogen

+++ =Clear signs of pathogen present

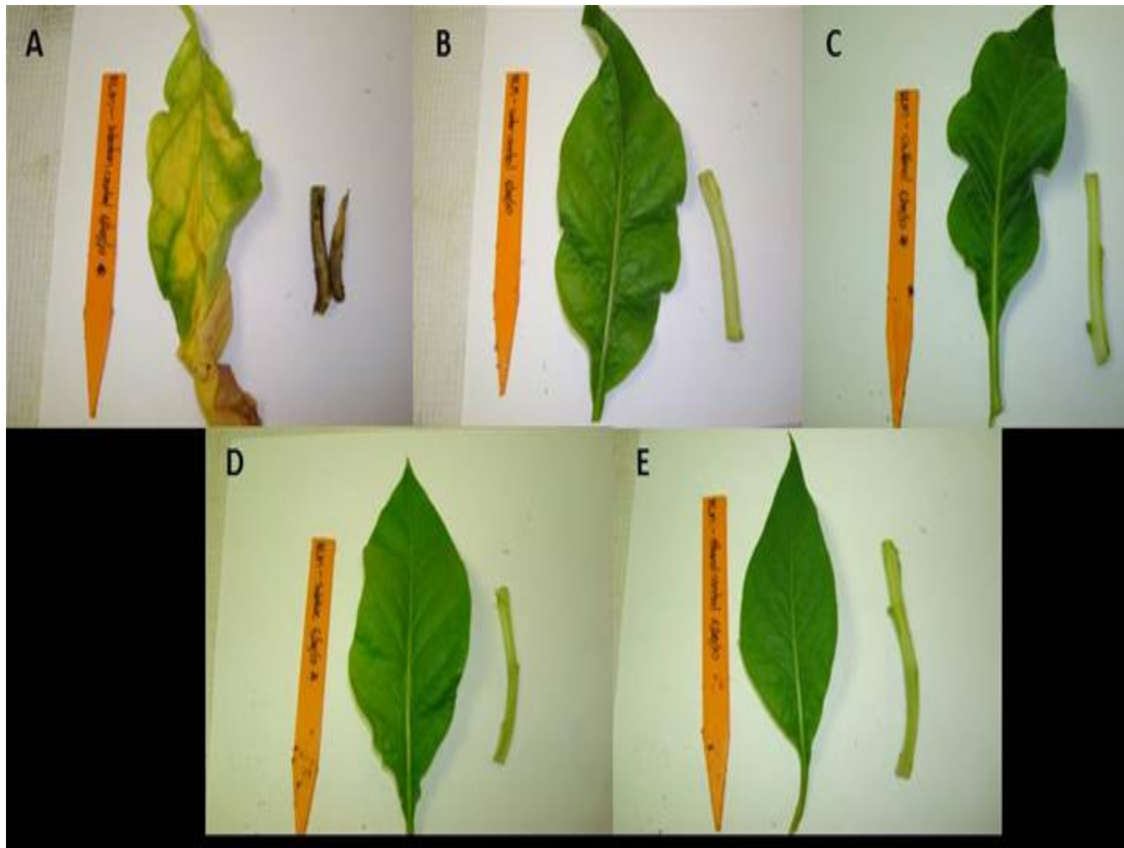

Figure 32: Impacts of *cis*-abienol on *P*. *parasitica* in the black shank susceptible tobacco NL Madole root drench assay. Treatments are A - infection control, B - water control, C - *cis*-abienol control, D - fungicide control, and E - ethanol control.

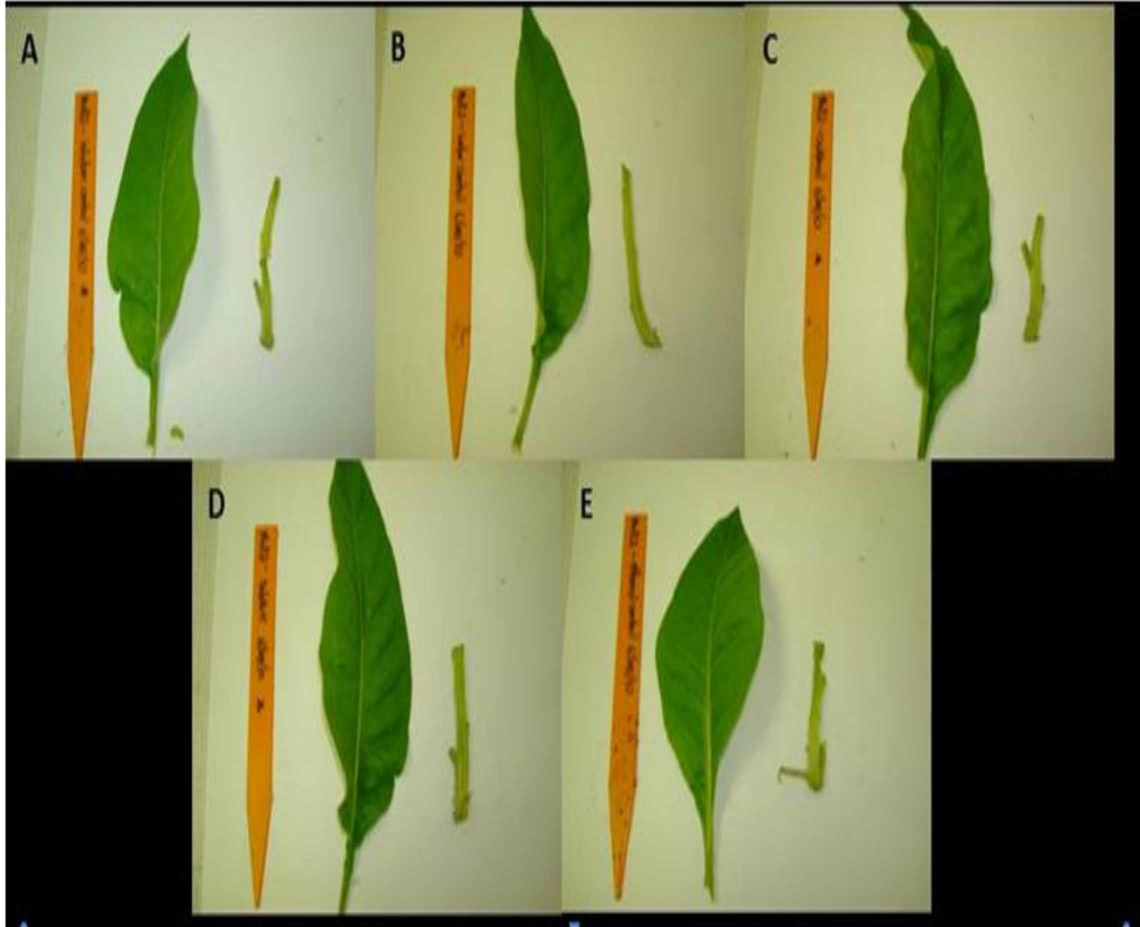

Figure 33: Impacts of *cis*-abienol on *P. parasitica* in the black shank resistant tobacco msD2602R root drench assay. Treatments are A - infection control, B - water control, C - *cis*-abienol control, D - fungicide control, and E - ethanol control.

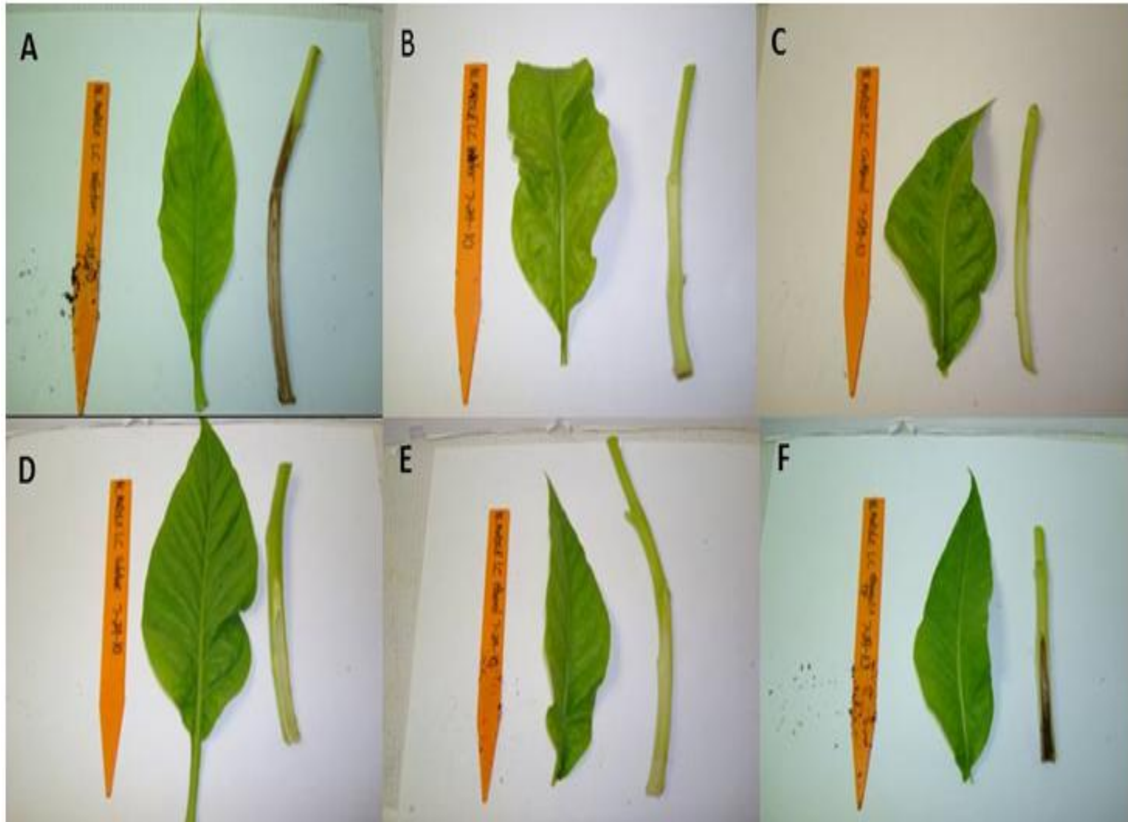

Figure 34 Impacts of *cis*-abienol on *P*. *parasitica* in the black shank susceptible tobacco NL Madole root drench assay, experiment 2. Treatments are A - infection control, B - water control, C - *cis*-abienol control, D - fungicide control, E - ethanol control, and F - ethanol plus infection control.

Figure 35 Impacts of *cis*-abienol on *P. parasitica* in the black shank resistant tobacco msD2602R root drench assay, experiment 2. Treatments were A - infection control, B - water control, C - *cis*-abienol control, D - fungicide control, E - ethanol control, and F - ethanol plus infection control.

Figure 36 Impacts of *cis*-abienol on *P. parasitica* in Red Russian tobacco root drench assay. Treatments are A - infection control, B - water control, C - *cis*-abienol control, D - fungicide control, E - ethanol control, and F - ethanol plus infection control.

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Chapter 6: Conclusion, Proposed Further Studies, and Related Work

This dissertation provides evidence that T-phylloplanin (and to some extent S-phylloplanin) constitute a broad-spectrum, protein-based, surface-disposed, antifungal defense system that provides the plant with first-point-of-contact resistance against various fungal pathogens. Demonstration of the broad-spectrum activity of T-phylloplanin against many fungi and fungi-like pathogens is a key accomplishment of this thesis. Phylloplanins of *Nicotiana tabacum* are now shown to have antifungal activity against at least one member of all 3 classes of true fungi (Ascomycetes, Basidiomycetes, and Zygomycetes), and also towards three species of oomycetes - fungi-like water molds. Other leaf surface compounds of tobacco (i.e. diterpenoids, sesquiterpenes, and sugar esters) have been shown to inhibit fungi. Therefore, we conclude that phylloplanins represent a surface protection strategy that is in addition to, and different from previously known, secondary-product-based strategies that protect surfaces of some sessile plants from microbial pathogens.

Field tests reported here show that spraying of T-phylloplanin on turf grasses in the field can provide protection against several diseases of turf in the natural field setting, with natural disease infection, and indicate that T-phylloplanin can be considered and pursued as a potential component of IPM in turf. Thus, the work of this thesis demonstrates, for the first time, the broad-spectrum, antifungal activity of T-phylloplanin, in both the laboratory and the field. Broad-spectrum effectiveness of sunflower phylloplanin is supported by laboratory experiments as well. Further work is needed to provide some second and third year field trial tests to substantiate findings of the two years of field tests reported here. And, further study is needed to establish optimal concentrations and

optimize other conditions for use of T-phylloplanin and other phylloplanins in IPM control of fungal pathogens of turf. Clearly phylloplanins should be tested as a possible fungicide (also bactericide) for use against pathogens of other crops (vegetables, fruits, etc.). Also, further research is needed to understand the interplay between surface disposed antifungal agents and surface bacteria, both beneficial and potentially pathogenic. We note that use of phylloplanins for microbial control is protected by three patents (issued or pending), one of which lists Brian King as a co-inventor.

Here it is shown, directly and for the first time, that the antifungal activity of T-phylloplanin is due to its ability to disrupt fungal spore and hyphal membranes to cause ion depletion and cell death. Results of previous work only supported this mechanism of action in model membrane systems. Further research is needed to define how phylloplanins associate with a target membrane and then proceed to enter the bilayer to cause formation of a pore or a disturbance that allows soluble ions and other micronutrients to escape. And, further study is needed to determine if phylloplanins can disrupt plant and animal membranes, and to what degree this might occur in comparison to disruption of fungal membranes. Since phylloplanins are water soluble and dispersed on the surface of plants outside the cuticle, they are not likely to contact plant membranes when applied to aerial surfaces as a topical spray. So, even if they are shown to be capable of disrupting plant membranes *in vitro*, this may not limit their use as surface protectants in crop production. Regarding possible mammalian toxicity, recent experiments in our lab suggest that the particular, unique constituents of fungal membranes are likely to make them more susceptible to intrusion by phylloplanins than

animal and plant membranes. However, direct tests of mammalian toxicity must be made if phylloplanins are to receive clearance for use in commercial crop production.

Finally, in the course of the work of this thesis we developed several new methods for testing the activity of natural products having potential as fungicides. In Chapter 5 we describe a float tube assay for testing antifungal activities of both water soluble (phylloplanin) and water insoluble (*cis*-abienol) compounds against motile fungal spores. This assay system was designed to mimic the float tray production system that is used commercially for production of tobacco transplants. This float tube assay system allows for efficient and in-minimal-space testing of effects of various chemicals and conditions in a way that we believe will be scalable to the commercial type float tray system. Also in Chapter 5 we describe the possibility of treating tobacco plants grown in soil (or in a float tray) with a water insoluble compound (*cis*-abienol) by utilizing a soil drench containing the test compound in 100% ethanol. This system should be applicable to the testing of other plant natural products having low water solubility for their potential to protect against plant diseases. The mode of action of *cis-abienol* is till unknown. The potential use of *cis*-abienol for prevention of black shank disease in tobacco is the basis of a patent application that is in preparation (Brian King will be a co-inventor on this application).

Related Work:

Here we briefly describe two other related activities that were carried on in the course of this thesis. These studies are ongoing.

First, we tested the possibility that topical application (painting) of unpasteurized cheese with a solution of T-phylloplanin might prevent of fungal growth on stored cheese.

Either application of this natural product to solid dairy products or its incorporation into packaging might be used as an alternative to antibiotics that are currently used. We found that fungal growth (mostly consisting of mixed Aspergillus species, by morphology) was indeed prevented during storage. A patent describing this discovery and its application have been submitted (G. Wagner, B. King, R. Shepherd, co-inventors). Second, a collaboration was established with Dr. T. Webster, KY State University to determine if T-phylloplanin might be useful for the control of Nosema disease in honey bees. Nosema infection coupled with a viral pathogen has been implicated in colony collapse disorder, a serious problem in the production and maintenance of honey bees for crop pollination and honey production. Preliminary results are promising, and the work continues. When this work is submitted for publication Brian King will be in the authorship.

Epilogue:

A PhD thesis should contain significant novel discoveries, and ideally its results can point the direction for applying these discoveries to useful applications. This thesis does both. It expands our knowledge about the properties of recently discovered phylloplanins in a critically important way, i.e., phylloplanin efficacy against many important plant pathogenic fungi, but importantly, it has extended this aspect to include field testing. If a fungicide is to achieve wide scale use, particularly if it's promise is mainly in use as a non-systemic treatment, it should have activity against a broad array of expected pathogens, and must function well in the real world outside the laboratory.

This thesis also connects earlier work regarding the membrane-disruption mechanism of antifungal action obtained using model systems to the direct demonstration of this

mechanism using living, intact fungal spores and hyphae. Understanding the mechanism of action of an agent can only enhance the information and application values of its discovery. Finally, novel experimental systems were developed in this thesis work and used to extend conventionally-applied tests for assessing antifungal activity (hyphal extension assays, topical spray assays) to the non-conventional testing of the motile spore stage of certain target fungal pathogens. New methods development invariably expands exploitation of knowledge.

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