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Induction of Resistance in Potato Tuber Tissue to Control Fusarium Sambucinum

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INDUCTION OF RESISTANCE IN POTATO TUBER TISSUE TO CONTROL FUSARIUM SAMBUCINUM

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ABSTRACT

Induction of Resistance in Potato Tuber
Tissue to Control *Fusarium sambucinum*

By

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Fusarium sambucinum is one of the causal agents of Fusarium dry rot in potato tubers. Although thiabendazole has been the primary control of Fusarium dry rot, development of resistance to this fungicide has required that new control strategies be developed. Induced resistance may be one new approach to controlling dry rot.

Potato tuber tissue was treated with several materials known to induce resistance in other plants. These included: salicylic acid, salicylic acid analogues, non-protein amino acids, chitosan, fatty acids and homogenate of *Phytophthora infestans* mycelium. Only treatments of potato tuber tissue with chitosan and homogenate of *Phytophthora infestans* mycelium (MH) reduced infection by *F. sambucinum*. However, the tuber tissue response was visually different between the MH and chitosan treatments and chitosan was slightly more effective at reducing infection.

Protein analysis revealed both chitosan and MH induced a number of putative plant defense mechanisms. The pathogenesis-related protein chitinase was locally and systemically induced in tubers in response to both treatments. β-1,3-glucanase was locally induced by both treatments and systemically induced in response to MH. Treatment with MH elicited a greater increase in chitinase and

β-1,3-glucanase activity than treatment with chitosan. Local induction of the oxidative enzymes peroxidase and polyphenol oxidase (PPO) was also observed in response to both treatments. Steroid glycoalkaloid accumulation, which has been shown to inhibit *F. sambucinum* growth and spore germination, was suppressed by both chitosan and MH treatments.

Cytological examination of of chitosan-treated tissues with autofluorescence and histochemical staining revealed accumulation of non-soluble phenolic compounds in the cell wall areas of tuber tissue, suggesting cell wall modifications had occurred in response to chitosan treatment. Additionally, lacmoid staining revealed that chitosan treated tissue developed a barrier of callose two to three layers from the treated surface.

Induction of resistance by chitosan and *P. infestans* mycelium homogenate is probably a result of multiple plant defense mechanisms (pathogenesis-related proteins, oxidative enzymes, accumulation of phenols, cell wall changes and callose deposition) working simultaneously to stop the ingression of *F. sambucinum*. Induced resistance is therefore a promising alternative to fungicides for the control of Fusarium dry rot and because it controls *F. sambucinum* using multiple defense mechanisms it may be a more stable form of resistance than fungicides.

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INTRODUCTION

Fusarium sambucinum the Causal Agent of Potato Dry Rot

Fusarium dry rot is one of the most devastating post-harvest diseases of potato. It is estimated that an average of 6-10% of the potato crop is lost to dry rot annually, with reports of losses as high as 60% (Carnegie *et al*, 1990; Theron, 1991). The major causal agent of Fusarium dry rot is *Fusarium sambucinum* (teleomorph *Gibberella pulicaris)* (USDA, 1978). In addition to causing dry rot of tubers *F. sambucinum* can also cause seed decay and foliage wilt (Hooker, 1981).

F. sambucinum produces chlamydospores, microspores, and macrospores. The microspores and macrospores provide continual airborne inoculum, while the chlamydospores can persist in the soil for years (Hooker, 1981). The telomorph, *G. pulicaris*, produces ascospores contained in a perithecium.

F. sambucinum can not penetrate the periderm of potato tubers, consequently, infection of potato tubers can only occur through wounds or breaks in the periderm (Hooker, 1981). Potato tubers, therefore, are not infected in the soil, but are only infected post-harvest through wounds or cracks caused by the harvesting process. Tuber infection begins by the growth of hyphae intercellularly and eventually spreads intracellularly subsequent to cell death. As infection progresses hollow cavities form inside the tuber, which cause the periderm to become sunken and wrinkled (Hooker, 1981).

A limited number of potato cultivars and breeding clones exhibit resistance to *F. sambucinum* and none of the cultivars grown in the United States are resistant

(Leach & Webb, 1981). *F. sambucinum* has been controlled by cultural practices, such as, limiting wounding during harvest, transit, and storage; and storing tubers in conditions optimal for wound healing (Secor and Gudmestad, 1999). The disease has been controlled primarily by post-harvest application of the fungicide thiabendazole. However, *F. sambucinum* resistance to thiabendazole is now wide spread (Desjardins, 1995 and Desjardins *et al*, 1993). Since thiabendazole is the only fungicide approved for post-harvest application on potato tubers (Powelson *et al*, 1993) finding an alternative method of controlling this disease is of the utmost importance. Induced resistance may offer an alternative.

Induced or Acquired Resistance

Induced or acquired resistance is a form of resistance in which a susceptible plant becomes resistant after treatment with an inducing agent (Hammerschmidt and Becker, 1997; Hammerschmidt and Kuc, 1995). Acquired resistance is characterized by: a) the induced tissue responding quicker to pathogen attack through the rapid induction of plant defenses; b) increased resistance to wide range of pathogens; c) a long lasting effect of the inducing agent; often lasting weeks or months; d) a delay between the treatment with the inducing agent and full expression of resistance; e) a local and systemic accumulation of pathogenesis-related (PR) proteins; f) typically induced by agents or pathogens that cause necrotic lesions (Hammerschmidt, 1999; Lucas, 1999).

The response may be localized or systemic (Hammerschmidt and Dann, 1997; Hammerschmidt and Becker, 1997). Local acquired resistance (LAR) is confined to a few cells around the area exposed to the inducing agent. Systemic acquired resistance (SAR) increases resistance throughout the plant and appears to be mediated by salicylic acid (Hammerschmidt and Smith-Becker, 1999; Sticher *et al*, 1997).

Acquired resistance can be induced in response to infection or chemical treatment. Activation of acquired resistance by means of infection is generally induced by necrosis caused by pathogenesis (Hammerschmidt, 1997). Induction of acquired resistance has also been associated with the hypersensitive response (HR). The HR is a localized rapid cell death and subsequent necrosis caused by an avirulent pathogen or elicitor. Cell death is coupled with the disruption of the host cell membranes and accumulation of oxidized phenolic compounds. The HR has been correlated with the induction of putative defense mechanisms associated with acquired resistance (Goodman and Novacky, 1994).

Acquired Resistance in Potato Tuber Tissue

Acquired resistance has been previously demonstrated in potato tuber tissue. Treatment of tuber tissue with an incompatible race of *Phytophthora infestans* protected the tissue against subsequent infection by a compatible race of *P. infestans* and *Fusarium caerulem* (Müller and Borger, 1940). A lipoglycoprotein complex (LGP-complex) extracted from *P. infestans* was shown to induce a

resistance response against *P. infestans* (Chalova *et al*, 1977). The LGP-complex induced local resistance at high concentrations (100 μg/ml) and systemic resistance at low concentrations (5-10 μl/ml) (Ozeretskovskaya, 1995). Arachidonic acid (AA) and eicosapentaenoic acid (EPA), components of the LGP-complex, were shown to be the primary agents responsible for induction of resistance against *P. infestans*. AA and EPA ability to induce resistance was enhanced by glucans extracted from *P. infestans*, which on their own could not induce resistance (Chalova *et al*, 1989; Preisig and Kuc, 1985). Extracts from the nonpathogenic fungus *Fusarium culmorum* Sacc, chitosan, and fucosyl-containing oligosaccharides were also able to induce resistance against *P. infestans* (Yurganova *et al*, 1989; Vasyukova *et al*, 2000; Il'inskaya *et al*, 1997). Induced resistance has also been demonstrated against bacterial pathogens in potato. Treatment with oligogalacturonides and *Phytophthora sojae* cell wall hydrolysate decreased infection by *Erwinia carotovora* spp. *atroseptica* (Dutton *et al*, 1997). The ability to induce disease resistance in potato tuber tissue suggests that acquired resistance may be used in place of or in combination with fungicides to control diseases such as *F. sambucinum*.

Defense Mechanisms

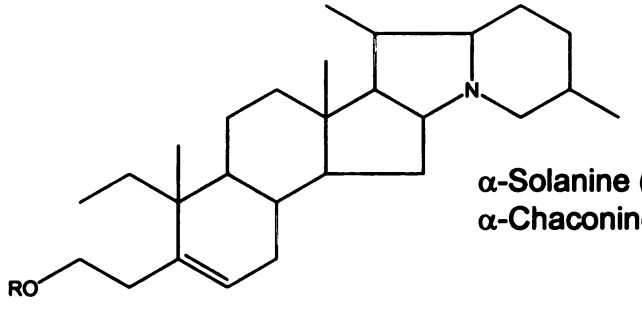
It is believed that acquired resistance involves the induction of or the ability to rapidly induce upon infection a number of putative defense mechanisms. These mechanisms may include phytoalexins, cell wall alterations, activated oxygen, oxidative enzymes, and PR proteins.

Phytoalexins

Phytoalexins are low molecular weight, antimicrobial compounds that are synthesized and accumulate in the plant after exposure to microorganisms (Paxton, 1981). Phytoalexins are believed to contribute to defense because their accumulation has long been correlated with cessation of pathogen development. In addition, fungal detoxification of phytoalexins has been correlated with an increase in virulence, mutants expressing increased levels of phytoalexins have been demonstrated to be more resistant to pathogens, and chemical inhibitors of phytoalexins have been shown to increase susceptibility (Hammerschmidt, 1999).

Phytoalexins present in potato tuber tissue include steroid glycoalkaloids and sesquiterpenes (Kuc, 1982). Steroid glycoalkaloids (SGA) are antimicrobial compounds produced by many species in the Solanaceae family, including potato (Kuc, 1982). The major SGA found in potato tuber tissue are α-solanine and α-chaconine (Figure 1). SGA are highly toxic to humans and can cause potatoes to have a bitter taste (Valkonen *et al*, 1996). Therefore, the concentration of SGA in new potato cultivars has been limited to a maximum level of 20 mg per 100 g fresh weight (Sinden and Webb, 1972). SGA are found in high concentration in potato peel and at wound sites of tuber tissue. SGA biosynthesis is induced by wounding and is inhibited by a number of pathogens and non-pathogens, as well as, preparations of *P. infestans* cell walls, AA and EPA (Ishizaka and Tomiyama, 1972; Kuc, 1982; Tjamos and Kuc, 1982). SGA are believed to have a role in plant defense against pathogens. SGA have been

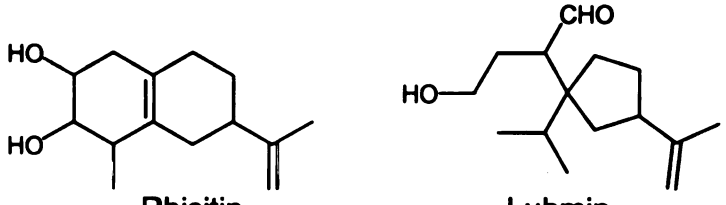
demonstrated to have antifungal activity that synergistically increases when SGA are combined (Kuc, 1968; Fewell and Roddick, 1997; Allen and Fewell *et al*, 1994; Fewell and Roddick, 1993). Additionally, light induced increases of SGA in tuber tissue were correlated with resistance to the tuber rot pathogens *Fusarium sulphureum* and *Fusarium solani* var. *coeruleum* (Percival *et al*, 1998). SGA has been shown to inhibit the growth and spore germination of *F. sambucinum* in vitro (Zeng, 1993). Despite the fact that SGA accumulation is inhibited by *F. sambucinum* infection, (Zeng, 1993) it is believed that SGA may play a role in defense against *F. sambucinum*. Inhibition of SGA accumulation has been reported to be lower in potatoes resistant to *F. sambucinum* than susceptible potatoes (Corsini and Pavek, 1980). Furthermore, increased SGA accumulation, which occurs during wounding, may initially play a role in slowing infection by *F. sambucinum*.



α-Solanine (R = B-solatriosyl)
α-Chaconine (R = β-chacotriosyl)

Figure 1: Major steroid glycoalkaloids found in potato tuber tissue.

The major potato sesquiterpenoid phytoalexins are lubmin and rhisitin (Figure 2). Sesquiterpenes accumulate in response to infection, cell wall preparations of *P. infestans*, AA, and EPA (Lisker and Kuc, 1977; Henfling *et al*, 1980; Bostock *et al*, 1981). However, *F. sambucinum* can tolerate high levels of rhisitin and can detoxify lubmin (Desjardins and Gardner, 1989; Desjardins *et al*, 1989). Therefore, here sesquiterpenes are probably not involved in tuber defense against *F. sambucinum*.

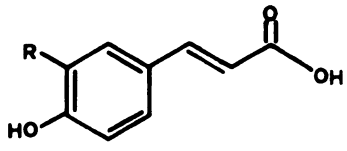


Rhisitin Lubmin

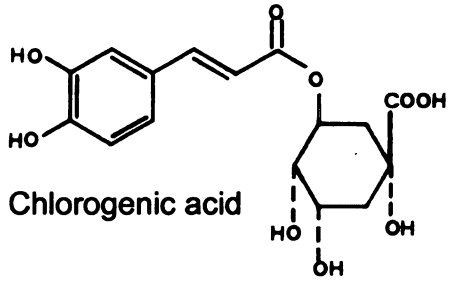
Figure 2: The major potato sesquiterpenoid phytoalexins.

Phenolic compounds (Figure 3) rapidly accumulate upon infection and are thought to slow or halt infection through direct antimicrobial activity, oxidation into toxic quinones, or esterification to cell wall materials (Nicholson and Hammerschmidt, 1992). The phenolic compound, chlorogenic acid has also been reported to accumulate upon wounding and has been correlated with resistance to potato tuber scab. However, chlorogenic acid is a relatively non-toxic compound therefore, resistance is believed to be due to the oxidation of chlorogenic acid to a more toxic compound(s) upon infection (Johnson and

Schaal, 1952 and 1959). Chlorogenic acid has also been shown to accumulate in potato tuber tissue after infection by *P. infestans* (Smith and Rubery, 1981; Friend *et al*, 1973). However, the accumulation of chlorogenic acid has been reported to be more rapid in some susceptible cultivars than in resistant cultivars (Henderson and Friend, 1979). The accumulation of chlorogenic acid in susceptible cultivars may represent activation of the phenylpropanoid pathway and an increase in the biosynthesis of phenolic compounds due to tissue damage rather than a specific response to infection. The slower accumulation of chlorogenic acid in resistant cultivars may indicate that phenols are being shunted from chlorogenic acid biosynthesis to the production of phenols that are more important in plant defense.



Caffeic acid (R = OH)
Ferulic acid (R = OCH_3)
ρ-Coumaric acid (R = H)



Chlorogenic acid

Figure 3: Common phenolic compounds found in potato.

Cell Wall Modifications

The first barrier encountered by pathogens is the cell wall and most pathogens can easily penetrate the cell wall (Schäfer, 1994). Thus, rapid modifications in the wall may prevent the pathogen from further invading the plant tissue. Cell wall modifications may prevent infection by: a) increasing the

mechanical strength of the wall preventing penetration by the pathogen; b) decreasing the susceptibility of the wall to cell wall degrading enzymes; c) providing a barrier to nutrient flow, thus starving the pathogen and d) providing a barrier to toxins or enzymes produced by the pathogen (Ride, 1978). In many cases cell wall modifications act as an initial defense blocking the pathogen until other defenses are induced. In other cases lignin deposition may actually contain the pathogen by the lignification of the pathogen cell wall surrounding the pathogen or by the deposition of lignin around the penetrating pathogen (Ride, 1978).

In potato tuber tissue, the periderm protects tuber tissue against invading pathogens, and wounding of the periderm allows infection by a number of pathogens to occur (O'Brien and Leach, 1983; Hooker,1981). As the wounded tissue ages, wound periderm is deposited. The deposition of wound periderm correlates with a decrease in susceptibility of infection, demonstrating the importance of the periderm in tuber tissue defense (Nolte *et al*, 1987; Hooker, 1981; Müller, 1957; Smith and Smart, 1955).

The cell walls of the periderm consist partly of suberin, which is composed of a phenolic and lipid matrix with waxes embedded into the matrix (Kolattukudy, 1984). Lulai and Corsini (1998) showed that deposition of both the phenolic and lipid component of the suberin was necessary for resistance to *F. sambucinum* during potato tuber wound healing. The waxes in the periderm provide the major barrier of water vapor diffusion from the host (Soliday *et al*,1979). It has been demonstrated that an increase in water vapor diffusion across this layer

increases susceptibility to *F. sambucinum*, implicating the suberin-associated waxes with plant defense (Vander Zaag *et al*, 1984). In addition to suberin, the periderm contains a lignin-like material, which also appears to be important in plant defense. Deposition of the lignin-like material occurred more rapidly in response to incompatible race of *P. infestans* than to susceptible strain (Hammerschmidt, 1984). Furthermore, tuber tissue treated with α-aminooxyacetic acid, an inhibitor of phenylalanine ammonia lyase, showed a decrease in lignin deposition and was then susceptible to the non-pathogen *Cladosporuim cucmerinum* (Hammerschmidt, 1984).

Deposition of lignin, hydroxyproline-rich glycoproteins, and callose may also contribute to the restriction of pathogens attempting to invade potato tuber tissue.

Activated Oxygen

Production of the activated oxygen species (hydrogen peroxide, hydroxyl radicals and superoxide) are associated with the plant's resistance response (Wojtaszek, 1997). In particular, increases in activated oxygen species are associated with the HR (Goodman and Novacki, 1994). Active oxygen species are believed to function in resistance by crosslinking cell wall polymers, via antimicrobial activity, and as a local signal for induction of defense related genes (Hammerschmidt and Schultz, 1996). Superoxide has been shown to increases in potato tuber tissue in response to incompatible races of *P. infestans*, but not in response to compatible races (Doke, 1983). Hyphal cell wall components of *P. infestans*, including AA, were also shown to increase superoxide in potato tuber tissue (Il'inskaya *et al*,1999; Doke, 1983). Diffusates from AA treated tuber

tissue inhibited *P. infestans* growth. If superoxide dismutase, which inhibits generation of superoxide, was added to the diffusates *P. infestans* growth was not inhibited (Il'inskaya *et al*, 1999). Superoxide, therefore, appears to have a role in resistance.

Oxidative Enzymes

Induction of resistance is often accompanied by activation or synthesis of the oxidative enzymes peroxidase, polyphenoloxidase, and lipoxygenase (Goodman and Novacky, 1994). Peroxidase may have a role in defense by producing toxic levels of hydrogen peroxide and phenolic free radicals (Peng and Kuc, 1992; Nicholson and Hammerschmidt, 1992). Peroxidase is also involved in the polymerization of phenolic precursors producing lignin, suberin, and hydroxyproline-rich glycoprotein crossing linking of the cell wall (Nicholson and Hammerschmidt, 1992; Espelie and Kolattukudy, 1985; Espelie *et al*, 1986). Polyphenoloxidase may be involved in defense by interacting with phenolic compounds producing toxic quinones from phenolic compounds (Appel, 1993). Lipoxygenase (LOX) may contribute to plant defense by forming toxic lipid oxidation products and by the formation of lipid signal molecules (Ricker and Bostock, 1994; Croft *et al*, 1993; Farmer, 1994). AA has been reported to increase levels of LOX in potato tuber tissue (Il'inskaya *et al*, 2000; Bostock *et al*, 1992). AA induced increases in LOX activity correlated with an increase in *P. infestans* resistance. If AA treated tuber tissue was treated with salicylhydroxamic acid, an inhibitor of LOX, resistance to *P. infestans* was

inhibited, suggesting that LOX plays a role in tuber tissue resistance response (Il'inskaya *et al*, 2000).

Pathogenesis-related Proteins

Pathogenesis-related (PR) proteins are defined as proteins coded for by the host plant and induced by pathogen, nematode, insect and herbivore attack or comparable condition including wounding and chemical agents that mimic the effect of pathogen infection or induce the host response (van Loon, 1999).

PR proteins are divided into 11 families, named PR1 through PR11. PR1 proteins are the most abundant and therefore, are often used as markers of acquired resistance. The function of PR1 proteins is not known, however, they have been implicated in virus and fungal resistance (Buchel and Linthorst, 1999). Constitutive high-level expression of PR1a reduced infection by *Peronospora tabacina* and *Phytophthora parasitica* (Alexander *et al*, 1993). Additionally, PR1 proteins isolated from tomato infected with *P. infestans* or virus infected tobacco have been shown to have fungicidal activity against *P. infestans* in both *in vitro* and *in vivo* assays (Niderman *et al*, 1995).

PR2 proteins are β-1,3-glucanases that cleave 1,3-β-D-glucosidic linkages in β-1,3-glucans (Leubner-Metzger and Meins, 1999). Many fungal cell walls are partly composed of β-1,3-glucan (Gooday, 1994). It has been proposed that β-1,3-glucanases may be directly involved in fungal resistance by inhibiting fungal growth through the degradation of fungal cell walls. The direct action of β-1,3-glucanases has been supported by *in vitro* studies that have shown β-1,3-glucanases to have antifungal activity against a number of fungal pathogens. β-

1,3-glucanases may also be involved indirectly in fungal resistance by producing β-glucan by fungal cell wall degradation. β-glucans have been reported to elicit resistance against fungal and viral pathogens by inducing plant defenses (Koop *et al*, 1989 and Bhandal and Paxton, 1991). Further evidence for the role of β-1,3-glucanase in resistance has been provided by transgenic expression of PR2 proteins. Expression of β-1,3-glucanases regulated by a constitutive promoter has been reported to reduce infection by fungal pathogens in numerous hosts (Leubner-Metzger and Meins, 1999).

PR3, PR4, PR8, and PR11 proteins are chitinases. Chitinases are able to hydrolyze β-1,4-linkages between N-acetylglucosamine residues of chitin (Neuhaus, 1999). Many fungal cell walls are composed of chitin (Gooday, 1994). It has been proposed that chitinases may be involved directly in defense by breaking down fungal cell walls and indirectly involved by producing elicitors that induce other plant defenses. The role of chitinases in resistance has been supported by their antifungal activity *in vitro* and by reports of increased resistance to a number of pathogens in transgenic plants constitutively expressing chitinases (Neuhaus, 1999).

Combinations of chitinase and β-1,3-glucanase may increase resistance to pathogens. A combination of chitinase and β-1,3-glucanase synergistically increases antifungal activity *in vitro* compared to their activity alone. Additionally, simultaneous constitutive expression of a β-1,3-glucanase protein and chitinase protein synergistically increased fungal resistance (Leubner-Metzger and Meins, 1999; Neuhaus, 1999).

PR5 proteins are similar to thaumatin in sequence and therefore have been called thaumatin-like proteins. PR5 proteins are believed to be involved in plant defense due to their antifungal activity (Velazhahan *et al*, 1999).

The remaining PR proteins roles in pathogen resistance have been studied less extensively. PR6 proteins are proteinase inhibitors involved in defense against herbivores. There is also some evidence that PR6 proteins are involved in resistance against pathogens (Heitz *et al*, 1999). PR9 proteins are a specific group of peroxidases in tobacco involved in lignin synthesis. PR10 proteins have sequence similarity to ribonucleases (van Loon, 1999).

Elicitors and Plant Resistance Activators

Elicitors are compounds that are able to induce resistance in plants to subsequent infection by a pathogen or induce typical defense responses in a plant (Lyon *et al*, 1995). Elicitors must meet the following criteria to be considered an inducer of plant defense: a) the elicitor must not have antimicrobial activity or metabolites derived from the elicitor must not have antimicrobial activity; b) the elicitor must induce defense mechanisms that resemble the response of the host to an incompatible interaction and c) the elicitor should make the plant resistant to pathogen(s) (Kessmann *et al*, 1994).

There are two types of elicitors, biotic and abiotic elicitors. Biotic elicitors include carbohydrates, phenols, salicylic acid, jasmonic acid, and amino acids. Abiotic elicitors include synthetic chemicals such as 2,6-dichloroisonicotinic acid (INA) and acibenzolar-S-methyl (ASM), which are believed to act as salicylic acid

analogues. They also include inorganic compounds such as phosphate salts, potassium phosphate salts, silicates, and oxalic acid (Lyon *et al*, 1995). Elicitor mediated induction of resistance has potential to control diseases. The use of elicitors to control *F. sambucinum* will be focus of this thesis.

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CHAPTER 1

Evaluation of Potential Inducing Agents of Acquired Resistance in Potato Tuber Tissue Against *Fusarium sambucinum*

INTRODUCTION

Acquired resistance is a type of resistance in which a susceptible plant becomes resistant after treatment with an inducing agent (Hammerschmidt and Becker, 1997; Hammerschmidt and Kuc, 1995). It has been demonstrated in over 25 plant species from at least six different families (Tuzun and Kuc, 1991). Inducing agents include a wide range of biotic elicitors including carbohydrates, phenols, salicylic acid, jasmonic acid, and amino acids; and abiotic elicitors including synthetic chemicals and inorganic compounds (Lyon *et al*, 1995).

Acquired resistance has been proposed as an alternative to pesticides for the control of disease. It has a number of advantages over pesticide use: a) activators of acquired resistance are safer for the environment and animal health b) protection can be systemic c) protection is long lasting d) protection is against a broad spectrum of pathogens including bacteria and viruses to which there are no pesticide controls and e) multiple plant defenses are induced making acquired resistance more stable than pesticides which is directed at a single metabolic function of the pathogen (Kuc, 1995).

F. sambucinum, the causal agent of dry rot in potato tubers, has developed resistance to thiabendazole, (Desjardins, 1995; Desjardins *et al*, 1993) the only approved fungicide for post-harvest application on potato tubers (Powelson *et al*, 1993). Additionally, most potato cultivars and breeding lines have little or no resistance to *F. sambucinum* (Leach and Webb, 1981). Acquired resistance is a promising method of controlling post harvest dry rot of potato tubers. Acquired resistance has been demonstrated in potato tuber tissue. Treatment of tuber

tissue with an incompatible race of *Phytophthora infestans*, glycoprotein extracts from *P. infestans* cell walls, arachidonic acid, eicosapentaenoic acid, extracts from *Fusarium caerulem* and *Fusarium culmorum* Sacc, chitosan, and fucosyl-containing oligosaccharides protected tissue against *P. infestans* (Vasyukova *et al*, 2000; Il'inskaya *et al*, 1997; Ozeretskovskaya, 1995; Chalova *et al*, 1989; Yurganova *et al*, 1989; Preisig and Kuc, 1985; Chalova *et al*, 1977; Müller and Borger, 1940). Additionally, potato tuber tissue was protected against *Erwinia carotovora* spp. *atroseptica* infection when treated with oligogalacturonides and *Phytophthora sojae* cell wall hydrolysate (Dutton *et al*, 1997) and against *Fusarium caerulem* when treated with an incompatible race of *P. infestans* (Müller and Borger, 1940). The goal of this study was to find an activator of acquired resistance in potato tubers that would protect against *F. sambucinum*.

MATERIALS AND METHODS

Preparation of Potato Tuber Disks

Tubers from potato (*Solanum tuberosum* L.) cultivar Snowden, which were stored at 4°C for up to 6 months, were used in all experiments. Procedure for preparation of potato tuber disks was modified from Hammerschmidt (1984). Tubers were allowed to warm to room temperature overnight. They were then washed and surface sterilized by soaking in a 5% solution of Clorox for 5-10 minutes. Just prior to making the tuber disks, tubers were dipped in 95% ethanol and flamed to ensure surface sterilization. The apical and stolen ends of the

tubers were excised and a 2.0 cm core borer was pressed through the cut end producing a cylinder of tuber tissue from the pith medullary tissue. The cylinder was then sliced into 0.5 cm thick slices. The disks were rinsed three times in sterile water and placed in sterile petri plates lined with Whatman #1 filter paper.

Fungal Cultures

Fusarium sambucinum isolate RN1 was grown on potato dextrose agar at 23°C in the dark. The cultures were transferred every 7-10 days.

Preparation of Elicitors

Salicylic Acid

Salicylic acid sodium salt was purchased from Sigma and prepared at concentrations of 1, 5, and 10 mM by dissolving in distilled water.

Non-protein Amino Acids

L-, D-, and DL-α-aminobutyric acid (AABA); β-aminobutyric acid (BABA); and γ-aminobutyric acid (GABA) were purchased from Sigma Chemical Company, St. Louis, MO, USA. The compounds were prepared at concentration of 3000 ppm by dissolving them in distilled water.

Commercial Chemicals

2,6-dichloro-isonicotinic acid (INA, CGA 279202) and acibenzolar-S-methyl (ASM, CGA 245704) were obtained from Novartis Crop Protection, Greensboro, North Carolina, USA. INA was prepared at concentrations 10, 25, and 50 µl/ml by dissolving it in distilled water. ASM was prepared at concentrations between

0.1 and 100 ppm by dissolving it in distilled water. The ASM was formulated with 50% active ingredient; concentrations were prepared based on the concentration of active ingredient.

AXP and GCC were obtained from Auexin Corporation, Lansing, Michigan, USA. The materials were prepared at concentrations between 300 and 3000 ppm by dissolving them in distilled water.

Phytophthora infestans Mycelium Homogenate

Isolate 97-2 of *P. infestans* was obtained from Dr. William Kirk, Michigan State University. It was grown for approximately one month on lima bean agar at 23°C in the dark. Mycelium was peeled from the surface of the lima bean agar, autoclaved, lyophilized, and ground with a pestle and mortar into a fine powder. Mycelium homogenate (MH) were prepared at concentrations of 0.25, 0.5, and 1.0 mg/ml by suspending the powdered mycelium in sterile water by sonication.

Fatty Acids

Arachidonic acid, linolenic acid, and linoleic acid were purchased from Sigma Chemical Company, St. Louis, MO, USA. The fatty acids were prepared at concentrations between $1x10^{-5}$ and 50 µl/ml by homogening the fatty acids in distilled water using a Polytron homogenizer.

Chitosan

Chitosan preparation was based on a procedure from Benhamou and Thériault (1992). Crab-shell chitosan, purchased from Sigma Chemical Company, St. Louis, MO, USA was washed by homogenizing the chitosan in distilled water using a Polytron homogenizer. Centrifuging the homogenate at

10,000 rpm for 10 minutes pelleted the homogenate. The pellet was then air-dried. Twenty grams of dried chitosan were then dissolved in 1000 ml of 0.25 N HCl and the insoluble material was removed by centrifugation. The chitosan was precipitated by adjusting the pH to 9.8 using 2.5 N NaOH. The precipitate was collected by centrifugation, washed three times with distilled water, and lyophilized. The lyophilized chitosan was stored at 4°C.

A stock solution was prepared from the washed chitosan by dissolving it in 0.25 N HCl. The pH was then adjusted to 5.5-6.0 using 2.0 N NaOH. The solution was then adjusted with distilled water to a concentration of 4.0 mg chitosan/ml. The stock solution was sterilized by autoclaving and stored at 4°C. The stock solution was adjusted to the concentrations of 0.5 and 1.0 mg/ml using sterile distilled water just prior to use.

Identification of Elicitors that Induce Resistance to *Fusarium sambucinum*

The surfaces of the tuber slices were treated with 200 µl of sterile water, as a control, or with 200 µl of the elicitor solutions. The treatment was applied within 1 hour of slicing and was spread evenly across the surface with a glass rod. The slices were incubated for 1, 2, or 3 days after treatment at room temperature in the dark. The tuber slices were then challenge with *F. sambucinum* by placing in the center of each tuber slice a 5.0 mm plug of *F. sambucinum*. Plugs were taken from the margin of hyphal growth of 3 or 4 day old cultures. Three or 4 days after challenge each slice was cut in half and the depth and width of infection was measured. Each treatment consisted of 15 slices. Data were

analyzed for significance by one-way analysis of variance, followed by Tukey's significant difference test (α=0.05), using GraphPad InStat software.

Comparison of Cultivars

Potato tuber slices (*Solanum tuberosum* L.), cultivars Snowden and Atlantic, were treated with 1.0 mg chitosan/ml as described in the procedures above. Three days after treatment the slices were challenged with *F. sambucinum* and depth of infection was measured 4 days after challenge.

RESULTS

Effect of Salicylic Acid

Salicylic acid (SA), applied at 1.0, 5.0 and 10.0 mM, did not cause any visible effect on the appearance of treated tuber tissue. SA also did not have an effect on infection by *F. sambucinum* when the treated tuber slices were challenged 2 or 3 days after treatment (Table 1-1).

Effect of Salicylic Acid Functional Analogues

Acibenzolar-S-methyl (ASM, CGA 245704) applied at 100, 50, 25, 0.5, 0.1 ppm had no effect on disease when the treated tuber slices were challenged at 1, 2, or 3 days after treatment (Table 1-2).

Tuber tissue treated with 2,6-dichloro-isonicotinic acid (INA, CGA 41396) applied at 50, 25, and 10 mg/ml, tended to be more susceptible to *F.*

sambucinum when the treated tissue slices were challenged 2 or 3 days after treatment; however, the increase was not significantly over the control (Table 1-3).

Effect of Non-Protein Amino Acids

D-, L-, and DL-α-aminobutyric acid (AABA) applied at 3000 ppm, slightly reduced infection in potato tuber tissue challenged 1, 2, or 3 days after treatment. However, the reduction was not significantly different than the control. β-aminobutyric acid (BABA), and γ- aminobutyric acid (GABA) applied at 3000 ppm did not significantly decrease infection by *F. sambucinum*. In fact, BABA appeared to make the tuber tissue more susceptible to infection (Table 1-4).

Effect of Commercial Formulations of Non-Protein Amino Acids

AXP applied at 300 and 3000 ppm did not significantly reduce infection by *F. sambucinum* when challenged 1, 2, or 3 days after treatment. However, AXP did appear to slightly reduce infection. This reduction was greater at 3000 ppm than 300 ppm (Table 1-5).

GCC applied at 1000 ppm did not reduce infection when challenged 1, 2, or 3 days after treatment. However GCC applied at 3000 ppm reduced infection when challenged at all three time points. This reduction was significantly different than the control when challenged 1 and 3 days after infection (Table 1-2).

Effect of *Phytophthora infestans* Mycelium Homogenate

P. infestans mycelium homogenate (MH), applied at 0.25, 0.5 and 1.0 mg/ml, significantly decreased the depth of infection by *F. sambucinum* when treated tissues were challenge inoculated 2 or 3 days after treatment (Table 1-6).

Effect of Fatty Acids

Arachidonic acid (AA), applied at 50, 25, 10, 5, and 1 µl/ml, slightly decreased infection by *F. sambucinum* when treated tuber tissue was challenged at 2 or 3 days after treatment. However, the decrease was not significantly different then controls. AA applied at concentration lower the 1 ppm did not decrease infection and in some cases appeared to make the tuber tissue more susceptible (Table 1-7).

Linolenic acid (LNA), applied at 2.0, 1.0, 0.5, and 0.1 µl/ml, did not decrease infection by *F. sambucinum* when the treated tissue were challenged at 2 or 3 days after treatment (Table 1-8).

Linoleic acid (LA), applied at 10, 5, and 1 µl/ml, significantly decreased infection by *F. sambucinum* when challenged at 2 or 3 days after treatment in some trial. However, other trials showed no reduction in disease. LA applied at concentration less than 1.0 µl/ml consistently had less infection. However, the decrease was not significantly different then controls (Table 1-9).

Tuber tissue treated with AA, LNA and LA exhibited browning and cell death similar to a hypersensitive response.

Effect of Chitosan

Chitosan applied at 1.0 and 0.5 mg/ml significantly decrease both the width and depth of infection by *F. sambucinum* when the tuber slices were challenged at 2 or 3 days after treatment. When challenged 1 day after treatment, infection was reduced, but not significantly compared to the control (Table 1-10).

Effect of Combining Elicitors

ASM at concentrations of 0.5 and 0.1 ppm in combination with 3000 ppm GGC had no significant effect on *F. sambucinum* infection when the treated tuber tissue was challenged 2 or 3 days after treatment (Table 1-11).

Chitosan at concentrations of 1.0 and 0.5 mg/ml in combination with 3000 ppm BABA decreased *F. sambucinum* infection. However, disease reduction was only significantly different then controls when challenged 3 days after treatment with 1.0 mg/ml chitosan and 3000 ppm ASM (Table 1-11).

SA at concentrations of 1.0 and 10.0 mM in combination with 1000 ppm BABA decrease disease, however, it was only significantly decreased when challenged 3 days after treatment with 1.0 mM SA and 1000 ppm BABA (Table 1-11).

TABLE 1-1: Effect of salicylic acid (SA) on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	2 Days		3 Days	
Treatment	Control	Treatment	Control	Treatment
1 mM SA	2.9	2.7	2.7	2.2
5 mM SA	2.9	2.6	2.7	3.0
10 mM SA	2.9	3.0	2.7	3.1

Tuber slices were treated with 1, 5, and 10 mM of SA and challenged with *F. sambucinum* 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. The depth of infection in tuber slices treated with 1, 5, and 10mM SA was not significantly different ($P<0.05$) from controls.

TABLE 1-2: Effect of ASM on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	1 Day		2 Days		3 Days	
Treatment	Control	Treatment	Control	Treatment	Control	Treatment
0.1 ppm ASM	3.5	3.5	3.8	3.6	2.5	2.4
0.5 ppm ASM	3.5	3.7	3.8	3.7	2.5	1.9
25 ppm ASM	2.7	3.4	3.1	3.4	3.5	3.7
50 ppm ASM	2.7	3.1	3.1	3.2	3.5	3.2
100 ppm ASM	2.7	3.6	3.1	3.6	3.5	3.6

Tuber slices were treated with 0.1, 0.5, 25, 50, and 100 mM of ASM and challenged with *F. sambucinum* 1, 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. The depth of infection in tuber slices treated with ASM was not significantly different ($P<0.05$) from controls.

TABLE 1-3: Effect of INA on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	2 Days		3 Days	
Treatment	Control	Treatment	Control	Treatment
10 µl/ml INA	2.3	3.1	2.8	3.2
25 µl/ml INA	2.3	3.1	2.8	2.6
50 µl/ml INA	2.3	2.8	2.8	3.1

Tuber slices were treated with 10, 25, and 50 µl/ml of INA and challenged with *F. sambucinum* 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. The depth of infection in tuber slices treated with INA was not significantly different ($P<0.05$) from controls.

TABLE 1-4: Effect of non-protein amino acids on the ability of *Fusarium sambucinum* to infect potato tuber tissue

Treatment	1 Day		2 Days		3 Days	
(3000 ppm)	Control	Treatment	Control	Treatment	Control	Treatment
L-AABA	4.4	4.2	4.0	3.0	4.1	3.6
D-AABA	4.4	4.0	4.0	3.7	4.1	3.3
DL-AABA	4.4	4.3	4.0	3.4	4.1	3.9
BABA	3.3	3.6	3.0	2.9	2.7	3.1
GABA	3.3	2.8	3.0	2.9	2.7	2.5

Tuber slices were treated with 3000 ppm of L-, D-, and DL-AABA, BABA and GABA and challenged with *F. sambucinum* 1, 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. BABA and GABA were tested separately from the AABA isomers. The depth of infection in tuber slices treated with L-, D-, and DL-AABA; BABA; GABA was not significantly different ($P<0.05$) from controls.

TABLE 1-5: Effect of Auexin Corp. chemical elicitors on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	1 Day		2 Days		3 Days	
Treatment	Control	Treated	Control	Treated	Control	Treated
300 ppm AXP	2.8	2.3	3.1	2.6	2.5	1.9
3000 ppm AXP	2.8	2.5	3.1	2.5	2.5	1.7
1000 ppm GGC	4.7	4.1	3.9	4.1	4.1	4.2
3000 ppm GGC	4.7	3.8*	3.9	3.7	4.1	3.1*

Tuber slices were treated with 300 and 3000 ppm AXP and 1000 and 3000 ppm GGC. The tuber slices were then challenged with *F. sambucinum* 1, 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. AXP and GGC were tested in separate experiments. Asterisks (*) represent a significant difference ($P<0.05$) from controls.

TABLE 1-6: Effect of homogenate of *Phytophthora infestans* mycelium (MH) on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	2 Day		3 Days	
Treatment	Width	Depth	Width	Depth
Control	12.5^{a}	3.9^{a}	11.8^{a}	3.3^{a}
0.25 mg MH/ml	13.6^{a}	2.9^{b}	$13.7^{a,b}$	2.2^{b}
0.5 mg MH/ml	12.2^{a}	2.3^{b}	$9.6^{a,c}$	1.5^{b}
1.0 mg MH/ml	13.2^{a}	2.3^{b}	12.1^{a}	1.5^{b}

Tuber slices were treated with 0.25, 0.5, and 1.0 mg MH/ml and challenged with *F. sambucinum* 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. Superscripts indicate significant difference ($P< 0.05$) between control and treatments.

TABLE 1-7: Effect of Arachadonic acid (AA) on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	2 Days		3 Days	
Treatment	Control	Treatment	Control	Treatment
50 µl/ml AA	2.1	1.6	2.1	1.6
25 µl/ml AA	2.1	1.5	2.1	1.5
10 µl/ml AA	2.1	1.6	2.1	1.5
5.0 µl/ml AA	2.1	1.6	2.1	1.6
1.0 µl/ml AA	2.1	1.8	2.1	1.8
0.1 µl/ml AA	2.4	3.3	2.5	2.4
0.03 µl/ml AA	2.4	3.3	2.5	2.3
0.01 µl/ml AA	2.4	3.3	2.5	2.4
0.003 µl/ml AA	2.4	3.0	2.5	2.2
0.001 µl/ml AA	2.4	2.6	2.5	2.3
0.0003 µl/ml AA	3.7	3.5	3.2	3.2
0.0001 µl/ml AA	3.7	4.1	3.2	3.3
0.00003 µl/ml AA	3.7	3.6	3.2	3.1
0.00001 µl/ml AA	3.7	3.7	3.2	3.3

Tuber slices were treated with AA at concentrations ranging from 0.00001 µl/ml to 50 µl/ml. The tuber slices were challenged with *F. sambucinum* 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. The depth of infection in tuber slices treated with AA was not significantly different ($P<0.05$) from controls.

TABLE 1-8: Effect of Linolenic acid (LNA) on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	2 Days		3 Days	
Treatment	Control	Treatment	Control	Treatment
2.0 µl/ml LNA	2.2	2.2	2.1	3.0
1.0 µl/ml LNA	2.2	2.2	2.1	3.2
0.5 µl/ml LNA	2.2	1.9	2.1	2.4
0.1 µl/ml LNA	2.2	1.9	2.1	1.8

Tuber slices were treated with 2.0, 1.0, 0.5, and 0.1 µl/ml LNA and challenged with *F. sambucinum* 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. The depth of infection in tuber slices treated with LNA was not significantly different ($P<0.05$) from controls.

TABLE 1-9: Effect of Linoleic acid (LA) on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	2 Days		3 Days	
Treatment	Control	Treatment	Control	Treatment
10 µl/ml LA	2.8	2.7	2.6	3.4
10 µl/ml LA	3.3	0.6*	3.8	1.0*
10 µl/ml LA	3.4	1.2*	2.6	0.5*
5.0 µl/ml LA	2.8	3.0	2.6	3.7
1.0 µl/ml LA	2.8	3.6	2.6	2.5
1.0 µl/ml LA	3.3	0.4*	3.8	0.1*
1.0 µl/ml LA	3.4	2.6	2.6	2.7
0.8 µl/ml LA	3.1	2.5	3.8	3.2
0.6 µl/ml LA	3.1	2.7	3.8	3.7
0.4 µl/ml LA	3.1	2.7	3.8	3.4
0.2 µl/ml LA	3.1	2.8	3.8	3.3
0.1 µl/ml LA	3.3	2.4	3.8	2.1*
0.1 µl/ml LA	3.4	3.0	2.6	2.7

Tuber slices were treated with LA at concentrations ranging from 0.1 µl/ml to 10 µl/ml. The tuber slices were challenged with *F. sambucinum* 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. Asterisks (*) represent significant difference ($P < 0.05$) from controls.

TABLE 1-10: Effect of chitosan treatment on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	1 Day		2 Days		3 Days	
Treatment	Width	Depth	Width	Depth	Width	Depth
Control	12.7^{a}	4.3^{a}	12.9^{a}	4.0^{a}	11.5^{a}	4.2^{a}
0.5 mg/ml Chitosan	11.3^{a}	3.5^{a}	6.3^{b}	2.0^{b}	5.6^{b}	2.3^{b}
1.0 mg/ml Chitosan	12.3^{a}	4.0^{a}	7.6^{b}	2.3^{b}	3.6^{b}	1.5^{b}

Tuber slices were treated with 0.5 and 1.0 mg/ml chitosan and challenged with *F. sambucinum* 1, 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. Superscripts represent significant differences ($P<0.05$) between control and treatments.

TABLE 1-11: Effect of combining elicitors on the ability of *Fusarium sambucinum* to infect potato tuber tissue

	2 Days		3 Days	
Treatment	Control	Treated	Control	Treated
0.1 ppm ASM/ 3000ppm GGC	3.3	3.5	3.6	4.0
0.5 ppm ASM/3000 ppm GGC	3.3	3.1	3.6	2.9
0.5 mg/ml Chitosan/3000ppm BABA	2.8	2.9	3.8	3.0
1.0 mg/ml Chitosan/3000ppm BABA	2.8	2.3	3.8	2.7*
1 mM Salicylic acid/1000ppm BABA	2.8	2.4	3.1	2.5
10 mM Salicylic acid/1000ppm BABA	2.8	2.5	3.1	2.0*

Tuber slices were treated with combinations of ASM and GGC, chitosan and BABA, and SA and BABA. The tuber slices were challenged with *F. sambucinum* 2 and 3 days after treatment. Depth of infection (mm) was measured 4 days after challenge. Asterisks (*) represent treatments that were significantly different ($P<0.05$) from the control.

Visual Comparison of Tuber Tissue Response to MH and Chitosan

Tuber tissue treated with MH exhibited browning and cell death similar to a hypersensitive response while tuber tissue treated with chitosan did not display a visual response to treatment (Figure 1-1).

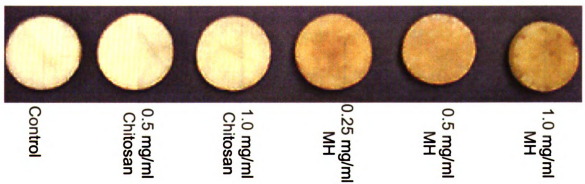

Figure 1-1: Visual comparison of tuber slices 3 days after treatment with 0.25, 0.5, or 1.0 mg/ml MH or 0.5 or 1.0 mg/ml chitosan. Images in this dissertation are presented in color.

Comparison of Cultivars

Tuber slices made from Atlantic tubers appeared to be more susceptible to *F. sambucinum* infection than Snowden tuber slices, however, the difference was not significant (Figure 1-2). Treatment of the Snowden and Atlantic tuber slices with chitosan made them significantly less susceptible to *F. sambucinum* than the controls (Figure 1-2). Chitosan treated Atlantic slices were less susceptible than chitosan treated Snowden slices, however the difference was not significant (Figure 1-2). These results may indicate Atlantic tuber tissue is more inducible than Snowden tuber tissue.

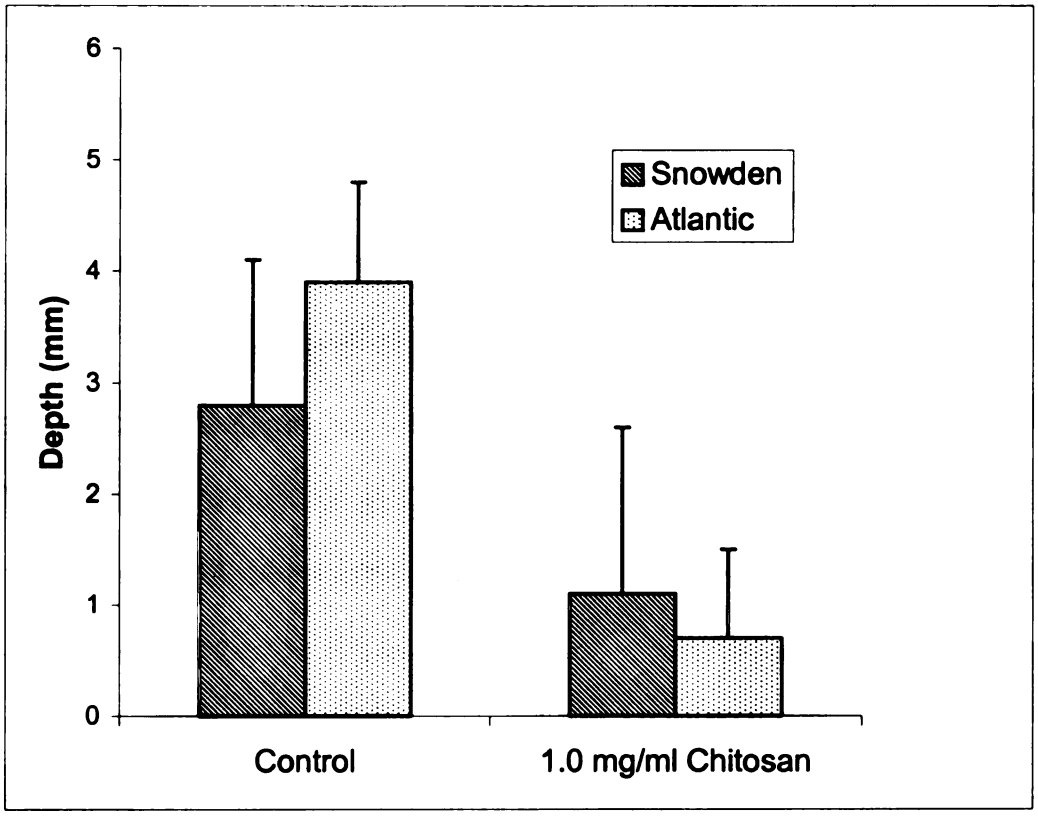

Figure 1-2: Comparison of the effect of chitosan treatment on the depth of infection by *F. sambucinum* in potato tuber slices made from cultivars Snowden and Atlantic. Bars indicate standard deviation.

DISCUSSION

Salicylic acid (SA) appears to have a role in plant resistance by inducing plant defense responses (Hammerschmidt and Becker, 1999). The amount of endogenous SA in potato plants has been correlated with resistance to pathogens (Coquoz *et al*, 1995). However, potato plants transformed with the *nahG* gene, which encodes for salicylate hydroxylase (an enzyme that converts SA to catechol), were not more susceptible to *P. infestans*, indicating that basal levels of SA are not responsible for resistance (Yu *et al*, 1997). Exogenous treatment of potato plants with SA has been reported to cause local induction of PR-1, (Coquoz *et al*, 1995) and to decrease susceptibility to *P. infestans* in cultivars resistant to *P. infestans* (Quintanilla and Brishammar, 1998). SA treatment of seed tubers from cultivars resistant to *P. infestans* has also been shown to increase resistance of the plants to *P. infestans* (Quintanilla and Brishammar, 1998). However, SA also increased susceptibility in cultivars susceptible to *P. infestans* (Quintanilla and Brishammar, 1998).

SA has been implicated in systemic acquired resistance (SAR) based on the observations that SA levels systemically increase at the onset of SAR (Ryals *et al*, 1996). Accumulation of SA throughout the plant is believed to be responsible for the systemic induction of PR proteins observed in SAR (Hammerschmidt and Smith-Becker, 1999; Klessig and Malamy, 1994). Initially it was believed that SA was the systemically translocated signal for SAR. However, it is more likely that systemic increases in SA are induced by a translocated signal that has yet to be

identified (Hammerschmidt and Smith-Becker, 1999) and, to a lesser degree, by the movement of SA through the phloem (Klessig and Malamy, 1994).

Treatment of potato plants with AA induced systemic resistance to *P. infestans* and *Alternaria solani*, even though SA accumulation and PR-1 expression was only at the site of AA treatment (Coquoz *et al*,1994; Coquoz *et al*, 1995). Infection with *P. infestans* also caused only local induction of SA (Coquoz *et al*, 1994), but systemic protection against *P. infestans* (Cohen *et al*, 1991). As a result, SA appears not to have a role in SAR in potato plants when induced by fatty acids. Induction of SAR by fatty acids may instead be due to oxidation of fatty acids leading to production of jasmonic acid (JA), which in turn triggers induction of resistance. However, AA did not induce SAR in potato plants expressing the *nahG* gene, suggesting SA is necessary for SAR expression (Yu *et al*, 1997). It has been suggested that increases in SA are not observed in potato because there is an increase in sensitivity to SA instead of an increase in SA (Yu *et al*, 1997).

There has been very little research done on the accumulation or affect of SA in potato tuber tissue and it is uncertain whether tuber tissue responds in the same manner as the foliage. SA has been shown to stimulate production of ethylene in tuber tissue (Liang *et al*, 1997). Ethylene has been reported to induce some PR proteins and enhance cell wall modifications (Sticher *et al*, 1997). AA and EPA are able to induce resistance to *P. infestans* in potato tuber tissue; however, exogenous application of acetyl salicylic acid has been shown to inhibit induction of phytoalexins and HR cell death caused by AA and EPA

treatment (Lee and Currier, 1996). SA has been reported to inhibit JA biosynthesis (Farmer and Ryan, 1992) and may block a JA induced ISR pathway.

SA's role in potato induced resistance, as well as, its ability to induce resistance to *P. infestans* in some potato cultivars, locally induce PR proteins, and stimulate production of ethylene made it a candidate as an activator of acquired resistance against *F. sambucinum*. However, application of SA to potato tuber tissue did not induce resistance against *F. sambucinum* (Table 1-1). Several problems have previously arisen with the use of SA as an activator of acquired resistance: a) SA is not translocated when applied exogenously, therefore, only treated tissue is resistance; b) SA is not always taken up by the plant tissue; and c) SA can be phytotoxic and the range between SA efficiency as an activator and its phytotoxicity is narrow (Kessmann *et al*, 1994). In this experiment the tuber tissue was evenly treated with SA and there were no apparent symptoms of phytotoxicity. The SA treatment may not have induced resistance because SA was not taken up the tuber tissue, the concentration of SA applied was not suitable to activate a response, or SA is not an activator of acquired resistance in potato tuber tissue.

INA and ASM are functional analogs of SA (Hammerschmidt and Dann, 1997). They are structurally different from SA, but they induce SAR and activate the same plant defenses as SA (Kessmann *et al*, 1994; Tally *et al*, 1999). There is evidence that INA and ASM work down stream of SA in the SAR signal transduction pathway (Kessmann *et al*, 1994; Tally *et al*, 1999). ASM and INA

have both been shown to induce resistance in wide range of crops against viruses, bacteria, and fungi (Tally *et al*, 1999; Kessman *et al*, 1994). However, neither of these compounds induced resistance to potato tuber tissue against *F. sambucinum* (Table 1-2 and Table 1-3). SA, INA, and ASM are not promising elicitors for the control of *F. sambucinum*.

Amino acids have been considered as a potential method of controlling disease for years, because certain amino acids or metabolites of amino acids have antimicrobial activity, the ability to affect the development and pathogenicity of fungi, and the ability to increase resistance of plant tissue to pathogens (van Andel, 1966). The ability of the non-protein amino acids AABA, BABA, and GABA to induce resistance has been extensively studied. BABA has been shown to increase resistance to viruses, fungal pathogens, and nematods. BABA elicited protection against pathogens in potato (van Andel, 1966), tomato (Cohen, 1993), tobacco (Cohen, 1994), pea (Papavizas, 1964), pepper (Sunwoo *et al*, 1996), and cucurbits (van Andel, 1966). AABA has been shown to increase resistance to fungal pathogens in tobacco (Cohen, 1994), tomato (Cohen, 1993), pepper (Sunwoo *et al*, 1996) pea and apple (van Andel, 1966) and GABA has been shown to increase resistance to *P. infestans* in tomato (Cohen, 1993). BABA appears to have a broader affect than AABA and GABA, and BABA has been shown to be more effective than AABA and GABA at inducing resistance in tomato against *Phytophthora infestans*, pepper against *Phytophthora capsici*, and tobacco against *Peronospore tabacina* (Cohen, 1993; Sunwoo *et al*, 1996 and Cohen, 1994). AABA, BABA, and GABA were shown to induce PR-proteins

(Cohen, 1994; Cohen *et* al,1994; Eyal *et* al, 1992; Lotan and Fluhr, 1990; Asselin *et al*, 1985) and AABA and BABA caused ethylene evolution (Cohen, 1994; Lotan and Fluhr, 1990), which has been reported to induce PR proteins and cause cell wall alterations (Sticher *et al*, 1997). However AABA, BABA, and GABA did not significantly reduce infection by *F. sambucinum* in potato tuber tissue (Table 1-4).

AXP and GGC are commercial formulations of non-protein amino acids. AXP also had no affect on tuber tissue resistance to *F. sambucinum* (Table 1-5). However, GGC significantly decreased *F. sambucinum* infection. Why GGC caused an increase in resistance and not the purified non-protein amino acids is unknown. It may be a result of other substances in the formulation rather than the proteins, a synergistic affect of compounds in the formulation, or the concentration of the amino acids.

BABA was tested in combination with SA. SA has been shown to enhance the inducing activity of some elicitors. BABA in combination with SA significantly reduced infection by *F. sambucinum* after 3 days (Table 1-11), indicating that SA may enhance the activity of BABA. GGC was tested in combination with ASM, however, there was no significant decrease in disease (Table 1-11). Non-protein amino acids do not seem to be promising elicitors of resistance in tuber tissue against *F. sambucinum*. Even though GCC and BABA in combination with SA decreased infection, the level of disease reduction is not great enough for effective control of the disease.

Extracts from *P. infestans* mycelium have long been studied as elicitors of resistance in potato tuber tissue against *P. infestans*. Extracts or homogenates of *P. infestans* mycelium have been reported to increase accumulation of sesquiterpene phytoalexins (Henfling *et al*, 1980) and induce resistance to *P. infestans* (Chalova *et al*, 1977).

P. infestans mycelium homogenate (MH) significantly increased the resistance of tuber tissue to *F. sambucinum* as shown by decreasing the degree of infection by as much as 50% (Table 1-6). It is unlikely that induction of sesquiterpene phytoalexin accumulation is responsible for the increase in resistance since *F. sambucinum* can detoxify lubimin (Desjardins *et* al, 1989) and with stand high concentrations of rishitin (Desjardins and Gardner, 1989). However, MH may induce other putative plant defense mechanisms, which could cause the tuber tissue to be more resistant to *F. sambucinum*.

Further studies demonstrated that the fatty acids arachidonic acid (AA) and eicosapentaenoic acid (EPA) from *P. infestans* mycelium were the most active elicitors of sesquiterpene phytoalexin accumulation (Bostock *et al*, 1981) and resistance to *P. infestans* in potato tuber tissue (Chalova *et al*, 1989). Additionally, the fatty acids AA and EPA, as well as, linoleic acid (LA) and linolenic acid (LNA) from *P. infestans* mycelium were able to protect the foliage of potato plants against *P. infestans* (Cohen *et al*, 1991).

AA and LNA were unable to induce resistance in potato tuber tissue to *F. sambucinum* (Table 1-7 and Table 1-8). LA did increase resistance, however, the results were very inconsistent between replications (Table 1-9). It appears

that component(s) other than these fatty acids are responsible for the tuber tissue resistance induced by MH. It has been reported that a β-1,3-β-1,6 glucan from *P. infestans* mycelium homogenate can also induce sesquiterpene phytoalexin and resistance to *P. infestans* (Chalova *et al*, 1976). Additionally, glucans have been shown to increase the effect of fatty acids (Bostock *et al*, 1982; Maniara *et al*, 1984; Preisig and Kuc, 1985). The increased resistance produced by the MH may be a result of untested fatty acids (EPA and eicosatrienoic acid), glucans or an additive affect between glucans and fatty acids. If the active component(s) of the MH can be determined they may be a successful elicitor of resistance against *F. sambucinum* in potato tuber tissue.

Chitosan, a polymer of β-1,4-linked glucosamine, has also been extensively studied as an elicitor of resistance. Chitosan has been shown to decrease susceptibility in celery (Bell *et al*, 1998), bean (Pospieszny *et al*, 1991), pea (Hadwiger and Beckman, 1980; Pospieszny *et al*, 1991), carrot (Chaeh *et al*, 1997), potato (Vasyukova *et al*, 2000), tomato (Pospieszny *et al*, 1991; Benhamou *et al*, 1992; Benhamou *et al*, 1994), wheat (Reddy *et al*, 1999), peppers (Kim *et al*, 1997), tobacco (Pospieszny *et al*, 1991) and fruit (Ghaouth *et al*, 1992; Du *et* al, 1997; Fajardo *et al*, 1998; Reddy *et al*, 2000) against viruses, bacteria and fungal pathogens. Reduced susceptibility may be due to chitosan's reported antifungal activity (Allen and Hadwiger, 1979; Stössel and Leuba, 1984) or by induction of plant defense mechanisms. Chitosan has been shown to induce a number of putative plant defense mechanisms including accumulation of phytoalexins (Kendra and Hadwiger, 1984; Kneer *et al*, 1999), deposition of

phenolic material (Benhamou *et al*, 1994; Reddy *et al*, 1999; Stange and McDonald, 1999), induction of PR-proteins (Wilson *et al*, 1994; Fajardo *et al*, 1998), production of hydrogen peroxide (Orozco-Cardenas and Ryan, 1999), accumulation of jasmonic acid (Doares *et al*, 1995), and increase phenylalanine ammonia-lyase activity (Peltonen *et al*, 1997).

Treatment of potato tuber slices with chitosan significantly decreased infection by *F. sambucinum* (Table 1-10). Increased resistance did not occur until two days after treatment (Table 1-10) indicating the increase in resistance was not due to antifungal activity of chitosan. Therefore, it appears that chitosan is inducing a response in the potato tuber tissue that is able to decrease infection. Chitosan was also tested in combination with BABA and BABA was shown to reduce the effectiveness of chitosan to increase resistance in the tissue (Table 1-11).

Chitosan and MH were both effective at reducing infection by *F. sambucinum* and may be potential methods of controlling dry rot of potato tubers. However, the responses to the treatments were noticeably different. MH caused cell death and browning while chitosan treated tuber tissue did not exhibit cell death or browning (Figure 1-1). This may be an indication of difference between the plants response to MH and chitosan.

The effectiveness of elicitors to induce resistance has been shown to vary between cultivars (Quintanilla and Brishammar, 1998). Tuber tissue from potato cultivar Atlantic is a more susceptible than Snowden to *F. sambucinum* infection (Figure 1-2). However, when treated with chitosan the Atlantic tuber tissue

becomes more resistant than the Snowden tuber tissue (Figure 1-2). Chitosan is therefore very promising method of controlling *F. sambucinum* in cultivars that are more susceptible to dry rot.

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CHAPTER 2

The Effect of Chitosan and *Phytophthora infestans* Mycelium Homogenate on the Biochemistry of Potato Tuber Tissue

INTRODUCTION

The criterion used to establish if a material is a "resistance inducer" states that the material must not be antimicrobial or be converted by the plant to an antimicrobial compound. In addition, it must be able to activate plant defense mechanisms such as pathogenesis-related proteins, which are often used as a marker for the induction of resistance, as well as oxidative enzymes, and antimicrobial secondary metabolites (Hammerschmidt and Dann, 1997). These changes must alter the ability of the pathogen to infect and colonize the plant.

The goal of this study was to determine if chitosan and *Phytophthora infestans* mycelium homogenate (MH) are "resistance inducers" and to determine which plant defense mechanisms are involved in the resistance response induced by these two treatments. The antifungal activity of chitosan against *F. sambucinum* and its potential role in reducing infection were evaluated. Induction of chitinase, β-1,3-glucanse, peroxidase, and polyphenoloxidase were examined as well as the effect chitosan and MH treatments had on steroid glycoalkaloid accumulation.

MATERIALS AND METHODS

Assessment of Chitosan's Antifungal Activity

Water agar containing 0, 0.5, and 1.0 mg/ml chitosan was prepared in 9 cm polystyrene Petri dishes as previously described a mycelium plug (5.0 mm) of *F. sambucinum* taken from the outer edge of growth was placed in the center of

each plate and incubated in the dark at 23°C. Six plates were used for each chitosan concentration. The diameter of the radial growth of *F. sambucinum* was measured at 2, 4, 6, 8, 10, and 30 days.

Spore Germination

Tuber discs were prepared and treated as described in chapter 1. Seventy-two hours after treatment with 1.0 mg/ml chitosan or water, the tuber discs were inoculated with a 200 µl suspension of *Fusarium sambucinum* spores at a concentration of $1X10^4$. The discs were sliced longitudinally into 2-4 cell layer sections 1, 3, 5, 8, and 12 hours after inoculation using a Hooker microtome. The sections were immersed in a solution of alcoholic lactophenol cotton-blue and heated until boiling. The sections were left in the alcoholic lactophenol blue solution for 48 hours. The sections were then washed with water and destained in a saturated solution of chloral hydrate (Keeling and Banttari, 1975). The sections were mounted in 50% glycerol and examined using light microscopy.

Potato Tuber Tissue Preparation

To study the local response of potato tuber tissue to chitosan and *P. infestans* mycelium homogenate (MH) treatment, potato tuber slices were prepared and treatments were applied as described in chapter 1.

To study the systemic response of tuber tissue to chitosan and MH treatment, potatoes were prepared as described in chapter 1. Three centimeter long cylinders of potato tuber tissue were made from the center of tubers using a 2 cm

cork borer. The cylinders were rinsed with sterile water and stood on end in sterile petri dishes. The top surface of the cylinders were treated with 0.5 ml of 0.5 and 1.0 mg/ml chitosan; 0.25, 0.5, and 1.0 mg/ml MH; or water. After 2 or 3 days the cylinders were sliced into six 0.5 cm slices and changes in the potato tissue was studied for each 0.5 cm depth.

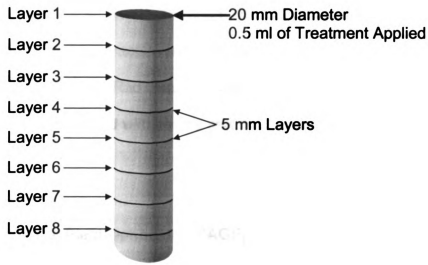


Figure 2-1: Diagram of tuber cylinders used for depth studies. The surface of the cylinder was treated with chitosan or MH. Two to three days after treatment the cylinders were sliced into 0.5 cm layers. Each layer was evaluated for increased resistance to *F. sambucinum* and biochemical changes.

Determination of Systemic Protection Against *Fusarium sambucinum*

Each 0.5 cm slice prepared from the tuber cylinders were inoculated with *F. sambucinum* and disease progression was measured following the protocol described in chapter 1.

Protein Extraction and Quantification

The upper 0.5 mm of tissue from five potato slices was removed using a vegetable peeler. The tissue was quartered and ground with a pestle and mortar in 3.0 ml of 0.2 M borate buffer (pH 8.8) with glass beads and polyvinylpyrrolidone to bind phenolic compounds. The ground tissue was filtered through a double layer of Miracloth. The filtered extract was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was frozen until needed.

Protein concentrations of the extracts were determined using the Bio-Rad Protein Assay, purchased from Bio-Rad Hercules, California. The Bio-Rad standard assay procedure was used with bovine serum albumin as the protein standard.

Native Polyacrylamide Gel Electrophoresis (PAGE)

Polyacrylamide separating gels (7.5%, 1.5mm) were prepared by mixing 4.85 ml deionized water, 2.5 ml 1.5 M Tris-HCl (pH 8.8), 2.5 ml 30% acrlyamide/bis (29:1), 50 µl 10% ammonium persulfate. The solution was degassed for 10 minutes, then 5 µl of TEMED was added and the gel was poured. The stacking gel (4.0 %) was prepared by mixing 6.1 ml of deionized water, 0.5 M Tris-HCl (pH 6.8), 1.33 ml 30% acrylamide/bis, and 50 µl of 10% ammonium persulfate. The solution was degassed for 10 minutes, then 10 µl of TEMED was added and the gel was poured. The upper tank buffer was prepared by mixing 5.16 g Tris base, 3.48 g glycine and 1 liter of water. The lower tank buffer was prepared by mixing 14.5 g Tris base, 60 ml of 1 N HCl, and diluting to 1 liter with water. A Mini-

Protean II or III electrophoresis system from Bio-Rad was used to run the gels. The gels were run at 50 V for approximately 4 hours.

Sample Preparation for PAGE

Protein extracts were mixed at a 1:1 (v/v) ratio with sample buffer. The sample buffer consisted of 5.4 ml of deionized water, 1.0 ml 0.5 M Tris-HCl (pH 6.8), 0.8 ml glycerol, and 0.8 ml 0.05% (w/v) bromophenol blue. An equal concentration of protein was loaded for each sample based on the Bio-Rad protein assay.

PAGE Assay for Peroxidase Activity

PAGE was performed as described above. Peroxidase stain was prepared by dissolving 50 mg of 3-amino-9-ethyl carbazole (AEC) in 2 ml dimethyl foramide. The AEC solution was slowly added to 200 ml 0.2 M sodium acetate buffer (pH 5.0) then 200 µl of 30% hydrogen peroxide was added. The gels were incubated at room temperature in the peroxidase stain until bands developed (Graham *et al*, 1965). The gels were then rinsed in water and photographed.

Spectrophotometric Assay for Peroxidase

Peroxidase activity was determined spectrophotometrically using 0.25% guaiacol and 0.3 % hydrogen peroxide (30%) in 0.1 M sodium phosphate buffer (pH 6) as a substrate. Crude protein extract was added to 1 ml of substrate solution and the absorbance was taken every 30 seconds for 4 minutes at 470

nm using a Cary 50 Bio UV-visible spectrophotometer. The slope of the absorbance verses time was used as a relative measure of peroxidase activity. Activity was based on protein concentration of the crude protein extract and three replicates were done for each sample and averaged.

PAGE Assay for Polyphenoloxidase Activity

PAGE was preformed as described above. Gels were then stained in a solution of L-β-3,4 dihydroxyphenylalanine (L-Dopa) for 1 hour or until brown bands appeared. The L-Dopa solution contained 4 mg of L-Dopa per milliliter of 0.1 M phosphate buffer pH 6.0 (Gomes and Ledward, 1996).

PAGE Assay for Chitinase Activity

PAGE was preformed as described above except 100 μl of a 1% stock solution of glycol chitin was added to the separating gel. Glycol chitin was obtained by acetylation of glycol chitosan (Trudel and Asselin, 1989). Gels were then incubated at 37°C in 1 M sodium acetate buffer (pH 5.0) for 2 hours. Following incubation gels were stained in a solution of 0.02 g fluorescent brightener 28 in 200 ml of 500mM Tris-HCl (pH 8.9) at room temperature for 5 minutes. The gels were removed from the stain and incubated in distilled water over night to destain the gel. Clear zones signifying chitinase activity were visualized under UV light. Activity could be compared by the size of the lytic zone.

Extraction and Quantification of Steroid Glycoalkaloids in Potato Tissue

Steroid glycoalkaloids were extracted according to Allen and Kuc (1968). Steroid glycoalkaloids were extracted from the upper 0.5 mm of 20 tuber slices for each treatment. The tissue was removed using a vegetable peeler, quartered and homogenized for 30 seconds in 100 ml of 10 chloroform: 9 methanol: 1 acetic acid (CAM). The homogenate was stored at room temperature for 24 hours then were filtered through Whatman #1 filter paper. The filtrate was concentrated to near dryness using a rotary-evaporator. 15 ml of 2% acetic acid was added to the concentrated filtrate and it was centrifuged at 12,000 rpm for 10 minutes to remove any insoluble material. The supernatant was washed with chloroform two times by adding 10 ml of chloroform, vortexing, centrifuging at 12,000 for 10 minutes, and discarding the chloroform layer. After washing the aqueous layer was adjusted to pH 10 using ammonium hydroxide. The solution was then heated at 80° C for 30 minutes, cooled to room temperature, and stored overnight at 4° C. The solution was centrifuged at 12,000 rpm for 10 minutes and the supernatant was poured off and the pellet was dried in a desiccator over NaOH pellets for 2-3 days. The pellet was resuspended in 3 ml of 5% acetic acid by sonication.

Concentration of SGA was determined spectrophotometrically (Cadle *et al*,1978). One-half ml of SGA extract was mixed with 1.5 ml of 85% phosphoric acid by vortexing. One ml of p-formaldehyde reagent (0.2 grams of ρ-formaldehyde dissolved in 15 ml of deionized water was combined with 85 ml of 85% phosphoric acid to make ρ-formaldehyde reagent) was added by vortexing

and the mixture was incubated at 60° C for 10 minutes. The mixture was allowed to cool to room temperature and absorbency was read at 600 nm using a Cary 50 Bio UV-visible spectrophotometer. Each sample was done in triplicate and the average absorbency was calculated. The concentration of SGA was determined using a standard curve produced using α-solanine as a SGA standard.

Spectrophotometric Assay for β-1,3-glucanase Activity

β-1,3-glucanase activity was assayed using AZCL-pachyman (obtained from Megazyme) as a substrate. AZCL-pachyman substrate was prepared by adding 0.1 g of AZCL-pachyman to 6 ml water. To assay β-1,3-glucanase activity, 0.3 ml of 10 mM potassium acetate buffer (pH 5.0) was added to 0.1 ml of crude protein extract, spun briefly to mix, and equilibrated in 30° C water bath for 3-4 minutes. Substrate (0.2 ml) was added and allowed to incubate 10 minutes after which 0.7 ml of 20% (w/v) Tris was added and the solution was vortexed to stop the reaction. The mixture was keep at room temperature for 3-4 minutes then spun at 12,400 rpm for 2 minutes to precipitate unreacted substrate. Absorbency of supernatant was determined at 595 nm using a Cary 50 Bio UV-visible spectrophotometer. Each assay was done in triplicate and the average absorbency was used as a relative measure of β-1,3-glucanase activity.

Statistical Analysis

Data was analyzed for significance by one-way analysis of variance, followed by Tukey's significant difference test (α=0.05), using GraphPad InStat software.

RESULTS

Antifungal Activity of Chitosan

At a concentration of 0.5 mg/ml, chitosan significantly reduced radial growth of *F. sambucinum* after 4 days growth (Figure 2-2). Mycelial growth was also more sparse than in water controls (data not shown). At a concentration of 1.0 mg/ml, chitosan completely inhibited *F. sambucinum* growth (Figure 2-2).

Spore Germination

The germination of *F. sambucinum* spores on the surface of chitosan treated tuber discs and water treated tuber discs were compared. Spore germination was observed 3 hours after the tubers were inoculated with the spore suspension. There was no visible difference in the spore germination on the water treated disks verses chitosan treated discs.

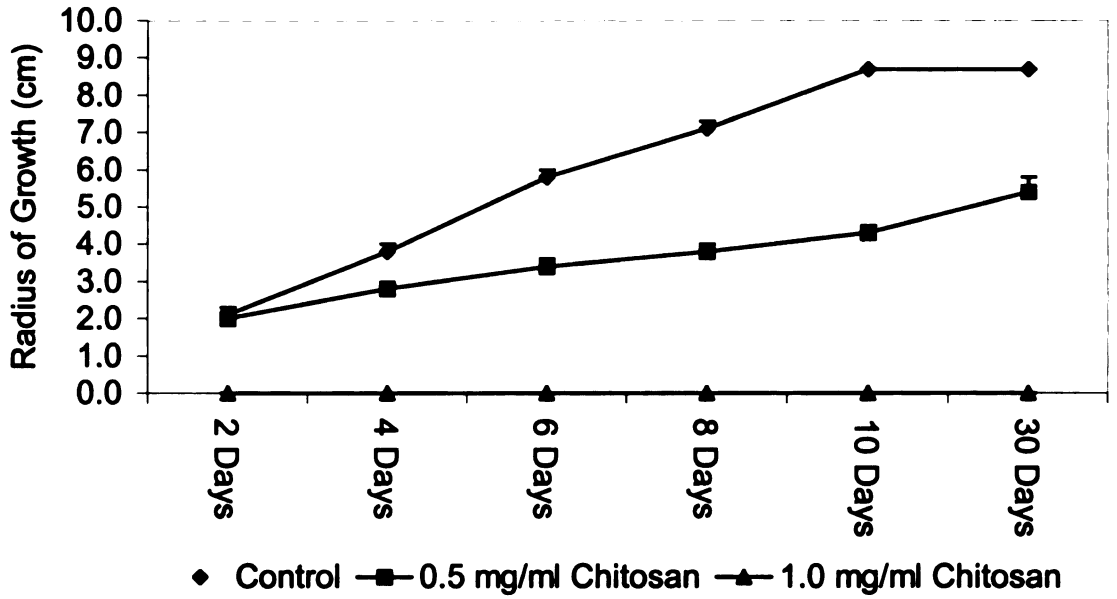


Figure 2-2: Time course of the radial growth of *F. sambucinum* grown in petri plates containing water agar and water agar supplemented with 0.5 or 1.0 mg/ml chitosan.

Local Effect of Chitosan and *P. infestans* Mycelium Homogenate Treatment of Tuber Tissue on the Activity of PR-Proteins

Chitinase activity increased in tuber tissue after *P. infestans* mycelium homogenate (MH) treatment at 0.25, 0.5 and 1.0 mg/ml and chitosan treatment at 0.5 and 1.0 mg/ml (Figure 2-3 and 2-4). There was a marked increase in chitinase activity as the concentration of MH increased (Figure 2-3), however, there was no discernible difference in chitinase activity as the concentration of chitosan was increased (Figure 2-4). MH treatment induced a greater increase in chitinase activity than chitosan at all concentrations and time points tested (Figure 2-5). Chitinase activity occurred lower in the gel as activity increased (Figure 2-3). This appears to be due to the concentration of chitinase and is not due to induction of a different chitinase isozyme.

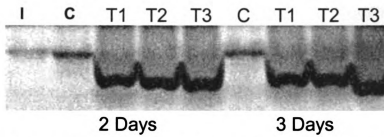


Figure 2-3: Chitinase activity in tuber tissue 2 and 3 days after treatment with water (control) and *Phytophthora infestans* mycelium homogenate (MH). Initial indicates chitinase levels at time of treatment (0 days).

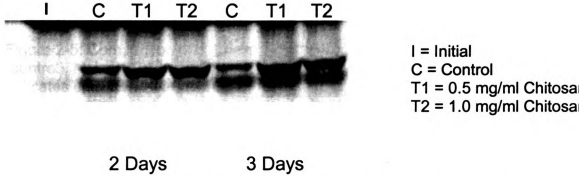


Figure 2-4: Chitinase activity in tuber tissue 2 and 3 days after treatment with water (control) and chitosan. Initial indicates chitinase levels at time of treatment (0 days).

Further examination of the affect of chitosan treatment revealed that chitinase activity increased in response to chitosan within 2 hours of treatment and continued to increase through at least 12 hours and was still elevated at 72 hours after treatment (Figure 2-4 and 2-6).

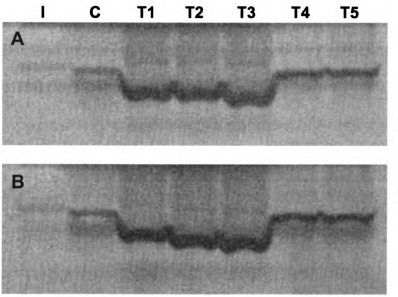


Figure 2-5: Comparison of chitinase activity in tuber tissue treated with water (control), MH, and chitosan 2 days (A) and 3 days (B) after treatment. Initial indicates chitinase levels at time of treatment (0 days).

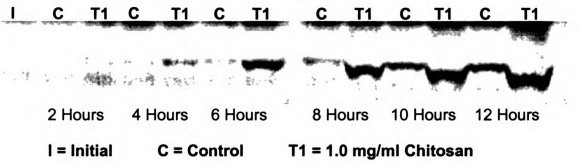


Figure 2-6: Time course of chitinase activity in tuber tissue treated with water (control) and 1.0 mg/ml chitosan. Initial indicates chitinase levels at time of treatment (0 days).

Glucanase activity increased in tuber tissue treated with 0.25, 0.5, and 1.0 mg/ml MH and 0.5 and 1.0 mg/ml chitosan (Figure 2-7). Increases in glucanase activity were observed 2 and 3 days after treatment with MH. Three day levels were slightly higher than 2 day levels (Figure 2-7). Chitosan treatments did not

significantly increase glucanase activity until 3 days after treatment (Figure 2-7). MH treatments increased glucanase activity significantly more than chitosan at 2 and 3 days after treatment (Figure 2-7).

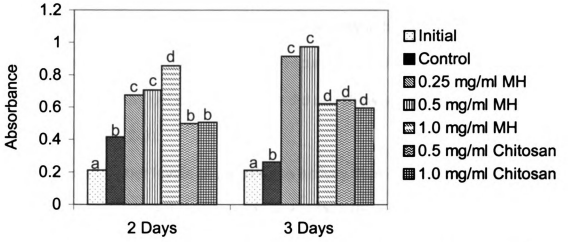


Figure 2-7: Glucanase activity in tuber tissue 2 and 3 days after treatment with water (control), MH, and chitosan. Initial indicates glucanase levels at time of treatment (0 days). The letters indicate significant differences ($P < 0.05$) between treatments.

Local Effect of Chitosan and *P. infestans* Mycelium Homogenate Treatment of Tuber Tissue on the Activity of Oxidative Enzymes

Peroxidase activity was increased by treatment with 0.25, 0.5, and 1.0 mg/ml MH and 0.5 and 1.0 mg/ml chitosan (Figure 2-8 and 2-9). Increased peroxidase activity was observed 2 and 3 days after treatment with MH (Figure 2-8 and 2-9). Chitosan treatment induced peroxidase activity within 12 hours of treatment and peroxidase activity continued to increase for at least 7 days after treatment (Figure 2-10 and 2-11).

There was no detectable difference in induction of peroxidase activity as the concentration of MH increased (Figure 2-8 and 2-9). At two days after treatment there was no detectible difference in the level of peroxidase activity in tissue treated with 0.5 mg/ml and 1.0 mg/ml chitosan (Figure 2-8 and 2-12). At three days after treatment, spectrophotometric analysis of total peroxidase activity indicated there was slightly more peroxidase activity in tissue treated with 0.5 mg/ml chitosan than 1.0 mg/ml chitosan (Figure 2-12). However, analysis of peroxidase activity by gel electrophoresis did not show a difference between the treatments (Figure 2-9).

MH induced higher levels of peroxidase activity than chitosan, and MH and chitosan induced different isozymes of peroxidase (Figure 2-8 and 2-9). Chitosan induced an isozyme pattern similar to that induced by wounding (Figure 2-8, 2-9, 2-10, and 2-11). MH induced some isozymes that were not induced by wounding and inhibited induction of other isozymes normally induced by wounding (Figure 2-8 and 2-9).

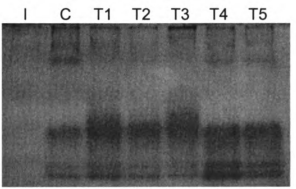
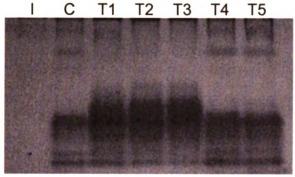
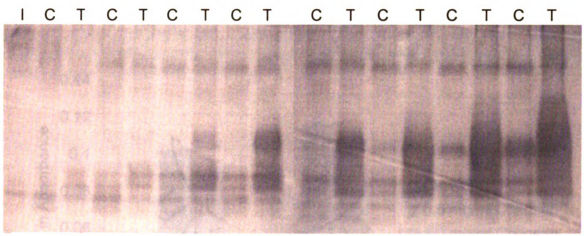


Figure 2-8: Peroxidase activity in tuber tissue 2 days after treatment with water (control), MH, and chitosan. Initial indicates peroxidase levels at time of treatment (0 days).



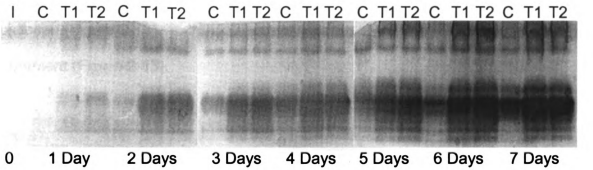
I = Initial
C = Control
T1= 0.25 mg/ml MH
T2 = 0.5 mg/ml MH
T3 = 1.0 mg/ml MH
T4 = 0.5 mg/ml Chitosan
T5 = 1.0 mg/ml Chitosan

Figure 2-9: Peroxidase activity in tuber tissue 3 days after treatment with water (control), MH, and chitosan. Initial indicates peroxidase levels at time of treatment (0 days).



I = Initial C = Control T = 1.0 mg/ml Chitosan

Figure 2-10: Time course of peroxidase activity in tuber tissue treated with water (control) and chitosan. Initial indicates peroxidase levels at time of treatment (0 days).



I = Initial C = Control T1 = 0.5 mg/ml Chitosan T2 = 1.0 mg/ml Chitosan

Figure 2-11: Seven-day time course of peroxidase activity in tuber tissue after water (control) and chitosan treatment. Initial indicates peroxidase levels at time of treatment (0 days).

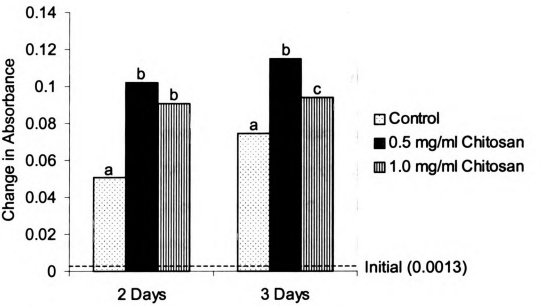


Figure 2-12: Spectrophotometric analysis of peroxidase activity in tuber tissue after water (control) and chitosan treatment. Initial indicates peroxidase levels at time of treatment (0 days). The letters indicate significant differences ($P < 0.05$) between treatments.

Polyphenoloxidase activity increased after treatment with 0.5 and 1.0 mg/ml chitosan (Figure 2-13). Increased activity was observed 2 and 3 days after treatment (Figure 2-13).

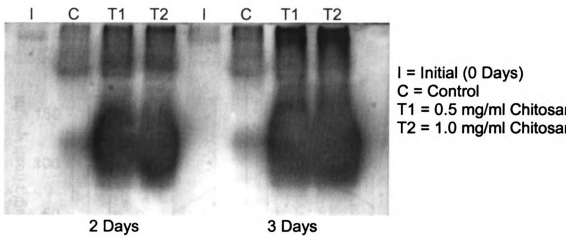


Figure 2-13: Polyphenoloxidase activity in tuber tissue 2 and 3 days after with treatment with water (control) and chitosan. Initial indicates polyphenoloxidase levels at time of treatment (0 days).

Local Effect of Chitosan and *P. infestans* Mycelium Homogenate Treatment of Tuber Tissue on Steroid Glycoalkaloid Levels

Wound induced steroid glycoalkaloid (SGA) accumulation was suppressed in response to treatment with MH at concentrations of 0.25, 0.5 and 1.0 mg/ml and by treatment with chitosan at concentrations of 0.5 and 1.0 mg/ml (Figure 2-14). When tuber discs are prepared wounding occurs causing an increase in SGAs over the initial level (Figure 2-14). However, this increase was suppressed by MH and chitosan treatment (Figure 2-14). Levels were still above initial SGA levels, however significantly less than controls (Figure 2-14). Decreases were observed at 2 and 3 days after treatment and were greater at 3 days (Figure 2-

14). MH reduced SGA accumulation more than treatment with chitosan at all tested concentrations and both time points (Figure 2-14).

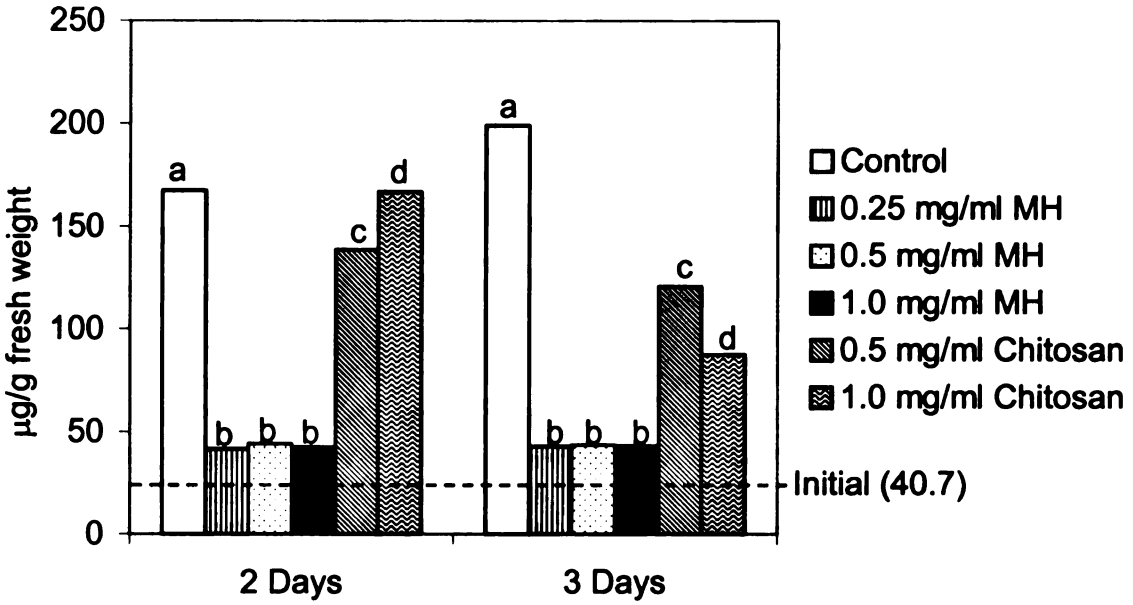


Figure 2-14: Steroid glycoalkaloid levels in tuber tissue 2 and 3 days after water (control), MH, and chitosan treatment. Initial indicates steroid glycoalkaloid levels at time of treatment (0 days). The letters indicate significant differences ($P < 0.05$) between treatments.

Systemic Effect of Chitosan and *P. infestans* Mycelium Homogenate Treatment of Tuber Tissue on *F. sambucinum* Infection

Tuber tissue treated with 1.0 mg/ml chitosan and challenged three days after treatment with *F. sambucinum* had a significant decrease in the depth of infection by *F. sambucinum* 5 days after challenge in layer 2, 0.5 cm from the treated surface, and layer 3, 1.0 cm from the treated surface (Figure 2-15). MH at 0.25, 0.5 and 1.0 mg/ml and chitosan at 0.5 mg/ml did not reduce infection systemically (Figure 2-15).

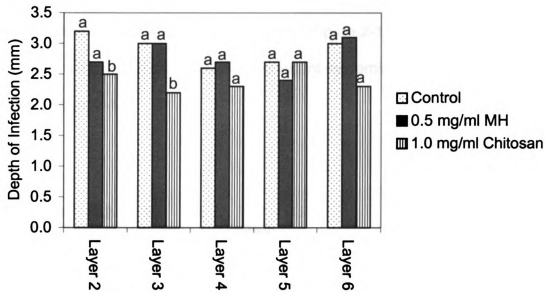


Figure 2-15: Depth of infection by *F. sambucinum* in layers of tuber tissue taken in 0.5 centimeter increments from the treated surface of tuber cylinders. Cylinders of tuber tissue were prepared and the surface was immediately treated with water (control), MH, and chitosan. Tissue layers were made and challenged with *F. sambucinum* 3 days after treatment. Depth of infection was measured 5 days after challenge. The letters represent significant differences ($P<0.05$) between treatments.

Systemic Effect of Chitosan and *P. infestans* Mycelium Homogenate Treatment of Tuber Tissue on the Activity of PR-Proteins

Chitinase activity was increased at least 2.5 cm from the treated surface, when treated with 0.25, 0.5, and 1.0 mg/ml MH (Figure 2-16) and at least 3.5 cm from the treated surface 3 days after treatment with 0.5 and 1.0 mg/ml chitosan (Figure 2-16 and 2-17). Increases in activity decreased as distance from the treated surface increased (Figure 2-16 and 2-17). Increases were higher at all depths in tissue treated with MH and were greater at higher concentrations of MH (Figure 2-16).

Glucanase activity significantly increased 1.0 cm from the treated surface 3 days after treatment with 0.25, 0.5 and 1.0 mg/ml MH (Figure 2-18). MH

treatment increased glucanase 1.5 - 2.0 cm, however, the increases were not always significantly different than the control (Figure 2-18). Chitosan treatment at 0.5 and 1.0 mg/ml did not cause a significant systemic increase in glucanase activity (Figure 2-18).

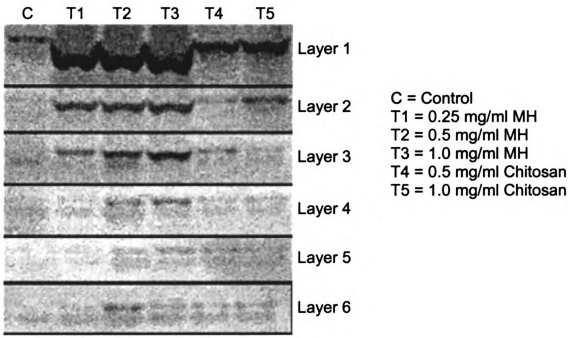
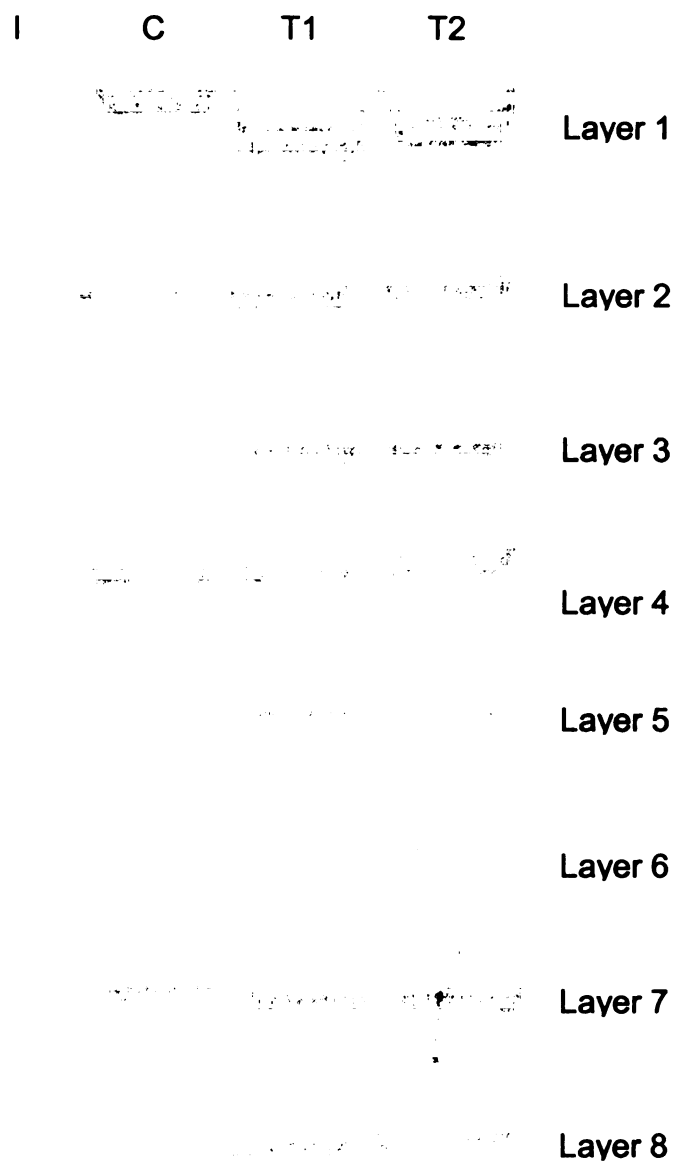


Figure 2-16: Chitinase activity in layers of tuber tissue taken in 0.5 centimeter increments from the treated surface of tuber cylinders. Chitinase activity was assessed 3 days after treatment of the surface of the tuber tissue cylinders with water (control), MH, or chitosan.



I = Initial C = Control T1 = 0.5 mg/ml Chitosan T2 = 1.0 mg/ml Chitosan

Figure 2-17: Chitinase activity in layers of tuber tissue taken in 0.5 centimeter increments from the treated surface of tuber cylinders. Chitinase activity was assessed 3 days after treatment of the surface of the tuber tissue cylinders with water (control) or chitosan.

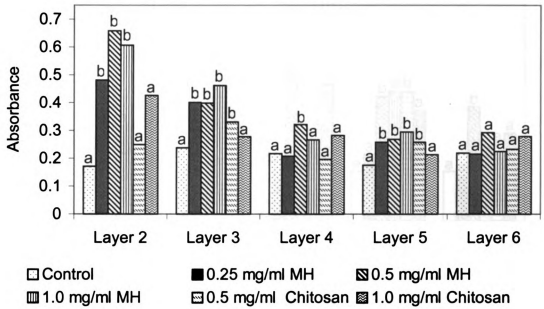


Figure 2-18: Glucanase activity in layers of tuber tissue taken in 0.5 centimeter increments from the treated surface of tuber cylinders. Glucanase activity was assessed 3 days after treatment of the surface of the tuber tissue cylinders with water (control), MH, or chitosan. The letters represent significant differences ($P < 0.05$) between treatments.

Systemic Effect of Chitosan and *P. infestans* Mycelium Homogenate Treatment of Tuber Tissue on the Activity of Oxidative Enzymes

After 3 days peroxidase activity increased 2.5 cm from the treated surface, when treated with 0.25, 0.5 and 1.0 mg/ml MH and 0.5 and 1.0 mg/ml chitosan (Figure 2-19). However, increases were not consistent and were not always significantly different (Figure 2-19).

Polyphenoloxidase activity increased 0.5 cm below the tissue surface 3 days after treatment with 0.5 and 1.0 mg/ml chitosan (Figure 2-20).

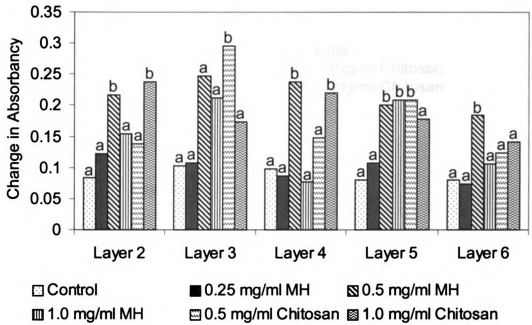


Figure 2-19: Peroxidase activity in layers of tuber tissue taken in 0.5 centimeter increments from the treated surface of tuber cylinders. Peroxidase activity was assessed 3 days after treatment of the surface of the tuber tissue cylinders with water (control), MH, or chitosan. The letters represent significant differences ($P < 0.05$) between treatments.

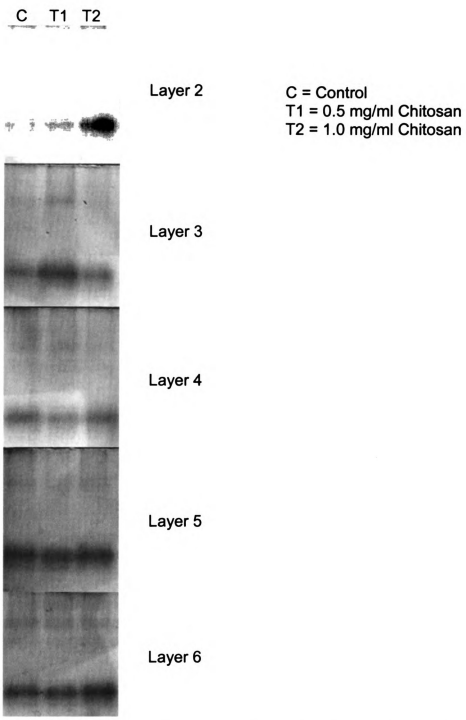


Figure 2-20: Polyphenoloxidase activity in layers of tuber tissue taken in 0.5 centimeter increments from the treated surface of tuber cylinders. Polyphenoloxidase activity was assessed 3 days after treatment of the surface of the tuber tissue cylinders with water (control) or chitosan.

Systemic Effect of Chitosan and *P. infestans* Mycelium Homogenate Treatment of Tuber Tissue on Steroid Glycoalkaloid Levels

Steroid glycoalkaloids (SGA) did not significantly decrease below the treated surface of the tuber tissue 3 days after treatment with 0.5 and 1.0 mg/ml chitosan (Figure 2-21).

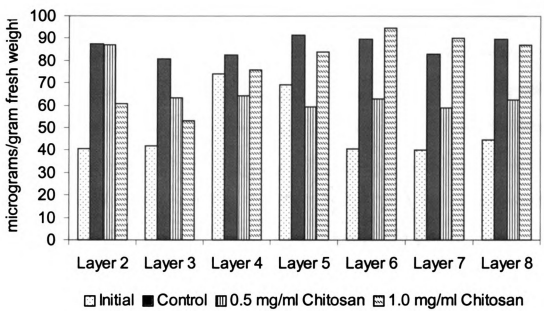


Figure 2-21: Glycoalkaloid levels in layers of tuber tissue taken in 0.5 centimeter increments from the treated surface of tuber cylinders. Glycoalkaloid levels were assessed 3 days after treatment of the surface of the tuber tissue cylinders with water (control) or chitosan.

DISCUSSION

Chitosan has been reported to inhibit growth of a number of fungal pathogens including various *Fusarium* species (Allen and Hadwiger, 1979; Stössel and Leuba, 1984). This research has shown that chitosan also inhibited growth of *F.*

sambucinum in vitro at concentrations of 0.5 and 1.0 mg/ml (Figure 2-2). It is unlikely that the antifungal activity of chitosan accounts for the increased resistance to *F. sambucinum* observed in potato tuber tissue treated with chitosan. Increases in resistance were not observed until 2 days after chitosan treatment (Table 1-10), suggesting resistance is not caused by the direct affect of chitosan, but more likely an induced plant response. Additionally, *F. sambucinum* spore germination on tuber tissue was not affected by chitosan treatment supporting a role of plant defense mechanisms in *F. sambucinum* resistance. However, these results do not eliminate any role for the antifungal activity of chitosan in the increased resistance observed after chitosan treatment.

Chitosan and the *P. infestans* mycelium homogenate (MH) treatment locally activate a number of puntative defense mechanisms including the pathogenesis-related proteins chitinase and β-1,3-glucanase and the oxidative enzymes peroxidase and polyphenoloxidase (Figure 2-3, 2-4, 2-7, 2-8, 2-9, 2-12, and 2-13). Additionally, steroid glycoalkaloid levels were suppressed in treated tissue (Figure 2-14).

Chitinase and β-1,3-glucanase are induced in potato upon pathogen attack suggesting they may have a role in defense against pathogens (Kombrink *et al*, 1988; Godoy *et al*, 1996). The cell walls of the hyphae of *Fusarium* species are typically composed of β-1,3-glucan and chitin (Gooday, 1994). β-1,3-glucanase and chitinase may therefore directly inhibit growth and infection by *F. sambucinum* by breaking down the cell walls of *F. sambucinum* hyphae. It has also been suggested that β-1,3-glucanase and chitinase may release elicitors

from fungal cell walls that can induce plant defense reactions (Mauch and Staehelin, 1989; Bowles, 1990; Boller, 1995). Chitinase activity increased within two hours (Figure 2-6) of treatment. However, resistance was not observed until 2 days after treatment. This suggests that if chitinase has a role in defense against *F. sambucinum* it may not be a direct role. Additionally, higher levels of chitinase activity occurred when the tuber tissue was treated with MH verses chitosan (Figure 2-5). However, MH conferred less resistance to *F. sambucinum* than chitosan. The size of chitosan oligomers has been shown to affect the ability of chitosan to induce defense responses (Kendra and Hadwiger, 1984; Doares *et al*, 1995). Potentially, the size of chitin fragments produced by chitinase may also affect induction of plant defenses. MH, which induces a higher level of chitinase, may produce smaller chitin fragments that might be less effective at inducing plant defense mechanisms, thus explaining why chitosan treatments are more effective at controlling *F. sambucinum* than MH.

Peroxidase has previously been reported to increase in potato tuber tissue in response to *F. sambucinum* infection suggesting it may have a role in potato tuber defense against *F. sambucinum* (Zeng, 1993). A specific role of peroxidase in plant defense against pathogens has not been demonstrated (Chittoor *et al*, 1999). However, peroxidase activity has been correlated with a number of puntative plant defense mechanisms including lignification and suberization of cell walls, cross-linking of hydroxyproline-rich glycoproteins in cell walls, and production of toxic phenolic free radicals (Chittoor *et al*, 1999). In potato tuber tissue, peroxidase has been associated with suberization (Espelie *et*

al, 1986; Espelie and Kolattukudy, 1985). The formation of suberin and wound periderm has been reported to be an important defense mechanism of potato tuber tissue against *F. sambucinum* (O'Brien and Leach, 1983). Treatment with arachidonic acid, a component of the MH has previously been reported to stimulate peroxidase activity and increase accumulation of lignin (Bostock and Schaeffer, 1986). Induction of resistance caused by MH and chitosan may in part be caused by increased suberization and wound healing caused by increased peroxidase activity. It is also possible that increases in peroxidase activity caused by MH and chitosan treatments may produce toxic levels of phenolic free radicals inhibiting *F. sambucinum* growth. Peroxidase activity increased within 12 hours of chitosan treatment and continued to increase for at least 7 days (Figure 2-10 and 2-11). The timing and duration of increased peroxidase activity would allow for accumulation of phenolic free radicals as well as changes in the structure of the cell wall before induction of resistance was observed two days after treatment (Hammerschmidt, 1984).

Similar to the PR-protein response, peroxidase activity also increased more in MH treated tissue than in tissue treated with chitosan (Figure 2-8 and 2-9). However, the chitosan treated tissue was more resistant to *F. sambucinum* infection. This indicates that other defense responses are at least partially responsible for the increase in resistance. However, higher levels of peroxidase may not always lead to increased resistance because the substrate of peroxidase may be the limiting factor in peroxidase induced defense responses and not the peroxidase enzyme. On the other hand, chitosan appears to

enhance the wound response while MH may induce isozymes similar to those induced during the HR (Figure 2-8 and 2-9). Therefore, peroxidase isozymes induced by MH may have a greater role in plant defense against *F. sambucinum* than those induced by chitosan.

The oxidative enzyme polyphenoloxidase increases upon infection by pathogens and therefore has been implicated in host defense, however, the role of polyphenoloxidase in defense in unclear (Thygesen *et al*, 1994; Mayer and Harel, 1979). Polyphenoloxidase catalyzes the conversions of phenols into quinones (Vaughn *et al*, 1988). The quinones can react with proteins and are often more toxic to pathogens then the unoxidized phenols used to produce them (Hammerschmidt and Schultz, 1996). Increased polyphenoloxidase activity may increase quinones levels and contribute to resistance against *F. sambucinum* by inhibiting growth.

Steroid glycoalkaloids (SGA) accumulate in response to wounding of potato tuber tissue and are generally considered a defense mechanism against herbivores (Kuc, 1984; Valknonen *et al*, 1996). SGA may also be important in defense against pathogens (Valkonen *et* al, 1996; Kuc, 1984). The SGAs α-solanine and α-chaconine were reported to inhibit *F. sambucinum* spore germination and growth (Zeng, 1993). SGAs were reported to be suppressed upon infection by *F. sambucinum* (Zeng, 1993) and in response to *P. infestans* and the fatty acid components of *P. infestans* mycelium arachidonic acid and eicosapentaenoic acid (Ishizaka and Tomiyama, 1972; Tjamos and Kuc, 1982). This research showed SGA was suppressed in response to chitosan treatment

and by an even larger degree by MH treatment (Figure 2-14). It is unlikely that SGA contributes to the resistance response since levels in the MH and chitosan treated tissue were substantially lower than the control tissue, which was not resistant to *F. sambucinum*.

Steroid glycoalkaloid and sesquiterpenoid biosynthesis share a common biosynthetic pathway until farnesyl pyrophosphate at which point they diverge (Figure 2-22). Suppression of steroid glycoalkaloids is often associated with increases in the sesquiterpenoid phytoalexins (Kuc, 1984). However, sesquiterpenoid phytoalexins are probably not involved in resistance of potato tuber tissue to *F. sambucinum* because *F. sambucinum* has the ability to detoxify and tolerate high levels of the sesquiterpenoid phytoalexins rishitin and lubimin (Desjardins and Gardner, 1989; Desjardins *et al*, 1989).

Chitosan and MH treatments triggered systemic changes in addition to the localized changes. Chitosan treated tuber tissue showed increased resistance 0.5 cm below the treated surface (Figure 2-15) and exhibited an increase in chitinase throughout the entire 3.5 cm depth of the tuber tissue cylinder (Figure 2-17) and polyphenoloxidase 0.5 cm from the treated surface (Figure 2-20). MH treated tissue also exhibited an increase in chitinase throughout the entire 2.5 cm depth of the tuber tissue cylinder (Figure 2-16) as well as an increase in β-1,3-glucanase 1.0 cm from the treated surface (Figure 2-18), however, there was no increase in resistance below the treated surface (Figure 2-15). The ability of chitosan to induce resistance below the surface may account for the higher resistance levels obtained by chitosan treatment verses MH treatment. Hyphae

that penetrate the surface tissue of chitosan treated tissue may encounter mechanisms that would prevent further penetration.

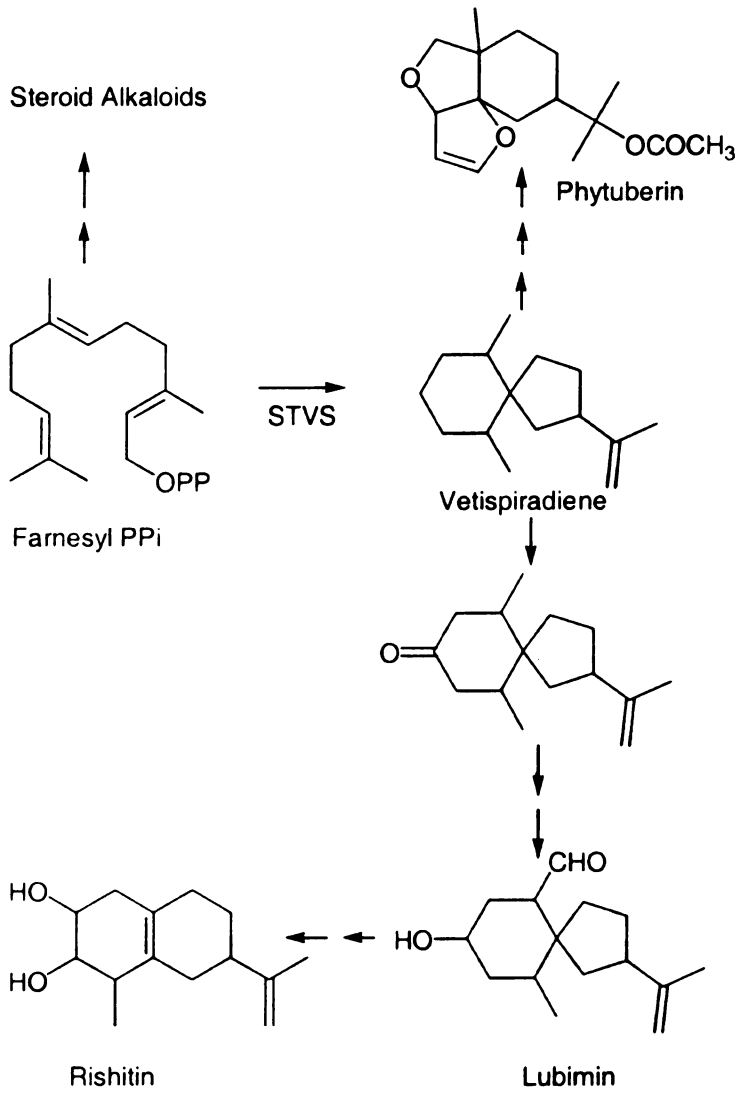


Figure 2-22: Steroid glycoalkaloid and sesquiterpenoid biosynthetic pathway.

The local and systemic responses of the tuber tissue to MH and chitosan treatments are clearly unique based on the differences in the induction of local and systemic resistance to *F. sambucinum* and induction of punatitive plant defense mechanisms. Both treatments give protection against *F. sambucinum*.

However, each treatment induces different defense responses and different levels of resistance. Treatment with MH and chitosan are promising methods of controlling *F. sambucinum*. These treatments may also prove effective against other potato tuber diseases. Both treatments induce several putative defense mechanisms both locally and systemically, which could protect the tuber against diseases that infect through the outer surface as well as those that infect the interior of the tuber. It is not likely that resistance is due to just one of these defense mechanisms, but a number of defense mechanisms working simultaneously to stop the pathogen. The multi-defense nature of this approach of controlling *F. sambucinum* will make it more difficult for the fungus to over come control of the disease as has occurred with current pesticide control methods and should allow for protection against a broad spectrum of pathogens.

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CHAPTER 3

Histochemistry and Autofluoresence Microscopy of Chitosan Treated Tuber Tissue

INTRODUCTION

Plant cell walls often respond to pathogen attack or elicitor treatment by modification of their cell walls. These changes in cell wall composition include lignification, esterification of phenols, and deposition of callose, and suberin (Hammerschmidt and Nicholson, 1999). They may be involved in host defense by increasing the mechanical strength of the cell wall, decreasing the susceptibility of the wall to cell wall degrading enzymes, preventing nutrient flow across the wall, and providing a barrier to toxins and enzymes produced by pathogens (Ride, 1978).

Phenolic compounds often accumulate in plant cells in response to pathogen attack and to resistance inducing agents and are believed to have an important role in plant defense (Nicholson and Hammerschmidt, 1992). In some cases, increased resistance has been correlated with phenol accumulation (Shiraishi *et al*, 1989; Cahill *et al*, 1989; Ryugo *et al*, 1990; Lyons *et* al, 1993; Bostock *et al*, 1999). Resistance may be attributed to the accumulation of toxic phenols, which inhibit growth of the pathogen. Additionally, polymerization of phenols into the cell wall may create a physical barrier, which can prevent pathogen infection (Nicholson and Hammerschmidt, 1992).

Histochemistry and autofluorescence microscopy are useful methods of observing phenol accumulation and deposition of phenolic cell wall material. This study investigated the effect of chitosan treatment on phenol accumulation and deposition of cell wall material in order to determine if phenols have a role in the increased resistance observed after chitosan treatment.

Callose is a polysaccaride often deposited in the cell wall in response to infection (Hammerschmidt and Nicholson, 1999). It may play a role in resistance by forming a physical barrier to pathogen ingression. Histochemical staining was also used to evaluate deposition of callose in response to chitosan treatment to elucidate the role of callose in chitosan induced resistance.

MATERIALS AND METHODS

Evaluation of Autofluorescence

Tuber discs were prepared and treated as described in chapter 1. The discs were then sliced longitudinally into 2-4 cell layer thick sections 24, 48, and 72 hours after treatment using a Hooker microtome. The sections were rinsed in water and mounted in Hoyers media (Cunningham, 1972) and observed with a light microscope under UV light. Color digital photographs were taken of the treated surface of 24 sections. The depth of fluorescence was measured from the digital photos. Three measurements were taken from each photo and averaged. The data were analyzed for significance by one-way analysis of variance, followed by Tukey's significant difference test (α=0.05), using GraphPad InStat software.

Toluidine Blue O Staining

Tuber discs were prepared and treated as described in chapter 1. The discs were sliced longitudinally into 2-4 cell layer sections 12, 24, 48, and 72 hours

after treatment using a Hooker microtome. The sections were soaked overnight in 95% ethanol in order to clear the soluble phenols. The sections were then stained with 0.1% toluidine blue O in 0.1 M sodium phosphate buffer pH 6.5 for 2 minutes. The section were then rinsed three times with water and mounted in a 50% solution of glycerol (Hammerschmidt and Kuć, 1982). Sections were observed using a light microscope and color digital photographs were taken. Three photographs were taken from each of 12 sections. The depth of toluidine blue O staining were measured for section made 48 and 72 hours after chitosan treatment and analyzed for significance by one-way analysis of variance, followed by Tukey's significant difference test (α=0.05), using GraphPad InStat software.

Lacmoid Test for Callose

Tuber discs were prepared and treated as described in chapter 1. The discs were sliced longitudinally into 2-4 cell layer thick sections 24, 48, and 72 hours after treatment using a Hooker microtome. The sections were rinsed in water and placed in a 0.1% solution of lacmoid in 50% ethanol for 24 to 48 hours (Vance and Sherwood, 1976). The sections were rinsed in water and mounted in Hoyers medium. The sections were observed using a light microscope under bright field and UV light and color digital photographs were taken.

Histochemical Staining for Lignin

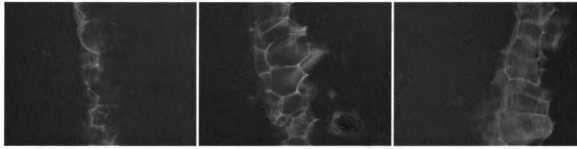
Tuber discs were prepared and treated as described in chapter 1. The discs were sliced longitudinally into 2-4 cell layer sections 24, 48, and 72 hours after treatment using a Hooker microtome. Sections were rinsed in water and soaked in 95% ethanol overnight. Sections were then stained in a saturated aqueous solution of phloroglucinol in 20% HCl for 10 minutes, rinsed in water, and washed in 70% ethanol (Hammerschmidt and Kuć, 1982). Alternatively sections were stained in a 1% aqueous solution of $KMnO_4$ and rinsed in water (Hepler *et al*, 1970). Sections were mounted in Hoyers medium and examined using a light microscope.

RESULTS

Autofluorescence Under UV-Light

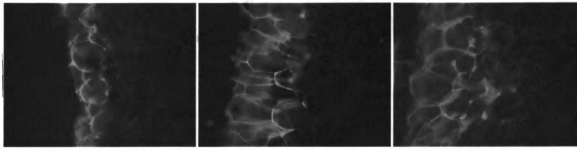
Potato tuber discs treated with 0.5 and 1.0 mg/ml chitosan or water were examined for autofluorescence 24, 48, and 72 hours after treatment. Compared to water treated tuber discs chitosan treated disks exhibited a higher intensity of autofluorescence and autofluorescent material occurred at a greater depth from the treated surface of the discs (Figure 3-1). The autofluorescence was concentrated in the cell wall area of both water and chitosan treated discs (Figure 3-1, 3-2 and 3-3). At the later time points the intensity and depth of fluorescence increased in tissue treated with both water and chitosan (Figure 3-2, 3-3, and 3-

4). By 72 hours the depth of autofluorescence in the water treated tissue begun to approach that of the chitosan treated tissue (Figure 3-4). After 48 hours the chitosan treated tissue began to exhibit a yellowish fluorescence at the tissue surface, which was not visible in the water treated tissue (Figure 3-2). This yellowish fluorescence became even more evident after 72 hours (Figure 3-3).



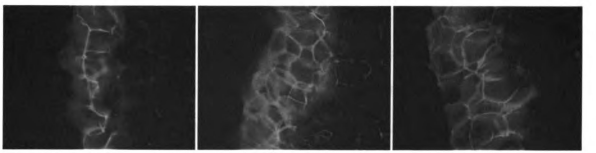
Control 0.5 mg/ml Chitosan 1.0 mg/ml Chitosan

Figure 3-1: Fluorescence micrograph of longitudinal sections of potato tuber discs 24 hours after treatment with water (control) or chitosan. Blue-green or blue fluorescence indicates phenolic deposition. 400x magnification.



Control 0.5 mg/ml Chitosan 1.0 mg/ml Chitosan

Figure 3-2: Fluorescence micrograph of longitudinal sections of potato tuber discs 48 hours after treatment with water (control) or chitosan. Blue-green, blue, or yellow fluorescence indicates phenolic deposition. 400x magnification.



Control 0.5 mg/ml Chitosan 1.0 mg/ml Chitosan

Figure 3-3: Fluorescence micrograph of longitudinal sections of potato tuber discs 72 hours after treatment with water (control) or chitosan. Blue-green, blue, or yellow fluorescence indicates phenolic deposition. 400x magnification.

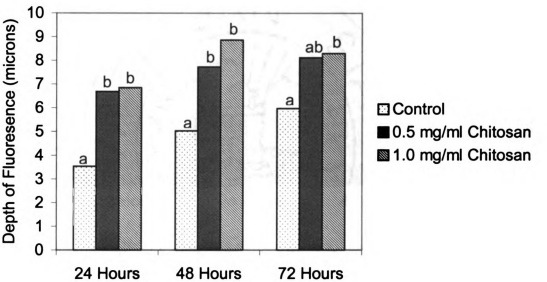


Figure 3-4: Depth of autofluorescence in longitudinal sections of potato tuber discs 24, 48, and 72 hours after treatment with water (control) or chitosan. The letters represent significant differences ($P < 0.05$) between treatments.

Toludine Blue Staining

Potato tuber discs treated with 1.0 mg/ml of chitosan or water were stained with toluidine blue O at 12, 24, 48, and 72 hours after treatment. Twelve hours after treatment with chitosan or water the cell walls were stained purple color,

typical of normal cell walls (Figure 3-5). After 24 hours the cell walls of the first two to three cells layers of the chitosan treated tissue stained blue-green in color; indicating deposition of phenolic compounds. The water treated tissue, however, did not stain blue-green (Figure 3-5). At 48 and 72 hours the intensity and depth of the blue-green staining increased in the chitosan treated tuber discs (Figure 3-5 and 3-6). Also, the water treated tissue, exhibited blue-green staining, but at a much lower intensity and at a lesser depth than the chitosan treated tissue (Figure 3-5 and 3-6).

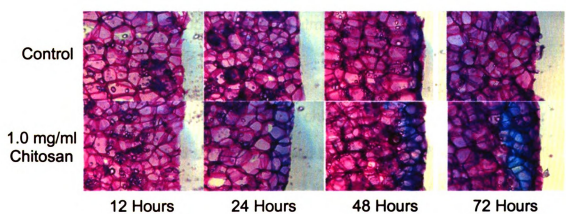


Figure 3-5: Micrograph of longitudinal sections of potato tuber discs stained with toluidine blue O 12, 24, 48, and 72 hours after treatment with water (control) or chitosan. Purple staining indicates typical cell wall material. Blue-green staining indicates phenolic deposition. 400x magnification.

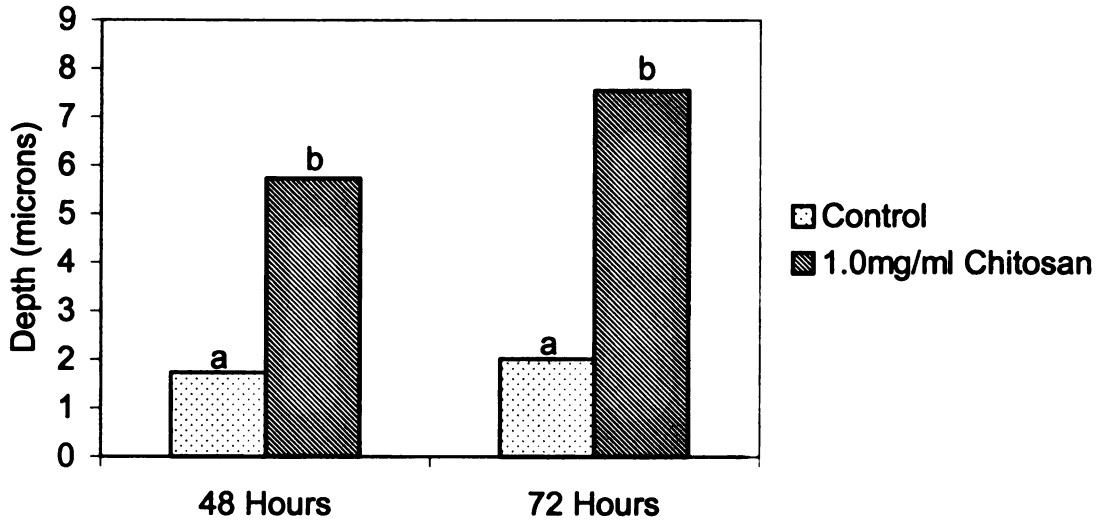


Figure 3-6: Depth of blue-green color caused by toluidine blue O staining 48 and 72 hours after treatment with water (control) or chitosan. The letters indicate significant differences ($P<0.05$) between treatments.

Lacmoid Test for Callose

Potato tuber discs treated with 1.0 mg/ml chitosan or water were stained for callose deposition. Lacmoid staining revealed that 72 hours after treatment with chitosan, a barrier of callose was deposited 2 to 3 cells layers from the treated surface (Figure 3-7). Callose deposition was not observed in water treated tuber discs (Figure 3-7). The callose deposition corresponded with the interface between tissue exhibiting autofluorescence and tissue not exhibiting autofluorescence (Figure 3-7).

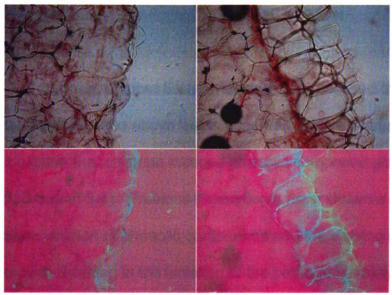


Figure 3-7: Micrographs of longitudinal sections of potato tuber discs stained with lacmoid and fluorescent micrographs of the same sections 72 hours after treatment with water (control) or chitosan. The red color of the lacmoid stain indicated the presence of callose. The blue-green, blue, and yellow fluorescence indicated deposition of phenolic compounds. 400x magnification.

Histochemical Staining for Lignin

Potato tuber tissue was stained for lignin deposition 24, 48, and 72 hours after treatment with water or chitosan using phloroglucinal HCl and 1% aqueous solution of $KMnO_4$. Neither the phloroglucinal HCl stain nor the $KMnO_4$ stain revealed lignin deposition in response to chitosan or water treatments at any of the time points.

DISCUSSION

Treatment of tuber tissue with chitosan increased the depth and intensity of autofluorescence above levels observed in tuber tissue treated with water. This indicated that chitosan induced phenolic compound accumulation (Figure 3-1, 3-2, 3-3, and 3-4). Previous experiments have shown a correlation between the accumulation of phenolic compounds and the slowing or halting of pathogen growth resulting in the isolation of the pathogens ability to further infect the plant tissue (Shiraishi *et al*, 1989; Cahill *et al*, 1989; Ryugo *et al*, 1990; Lyons *et* al, 1993; Bostock *et al*, 1999) . Therefore, increases in the concentration of phenols, as well as, increases in the area of phenol deposition observed after chitosan treatment may in part be responsible for the increase in resistance to *F. sambucinum* observed after chitosan treatment. Accumulation of phenols may completely inhibit *F. sambucinum* infection. It has also been suggested that the initial rapid accumulation of phenols slows the growth of pathogens, which allows time for the induction of other plant defenses that can completely stop invasion, by the pathogen (Mansfield, 1982 and Matern and Kneusel, 1988). Slowing the growth of *F. sambucinum* in chitosan treated tuber tissue may allow the tuber tissue to become suberized, which would prevent further infection by *F. sambucinum* (O'Brien and Leach, 1983). It has been shown to take five to seven days for tuber tissue to become suberized enough to prevent *F. sambucinum* from penetrating the tissue (Lulai and Corsini, 1998). Accumulation of phenolic

compounds may be in part responsible for slowing growth until suberization can occur.

Phenolic compounds can also be polymerized into cell material (Nicholson and Hammerschmidt, 1992). Deposition of phenols in the cell wall may provide a mechanical barrier to pathogen penetration, prevent penetration of enzymes and toxins produced by the pathogen, and prevent nutrient flow to the pathogen (Ride, 1978). Additionally, phenolic free radicals, precursors to the polymerization of phenols into cell wall material, may be toxic to the pathogen (Nicholson and Hammerschmidt, 1992). Examination of autofluorescence in chitosan treated tuber tissue showed that the phenolic compounds were localized in the cell wall areas of the tuber tissue (Figure 3-1, 3-2, and 3-3). Toluidine-blue O staining also revealed the deposition of non-soluble phenols in the cell walls (Figure 3-5) thus confirming the autofluorescence results. These cytological studies provide evidence that chitosan may cause polymerization of phenols into the cell wall; indicates that cell wall changes may be in part responsible for the increase in resistance of tuber tissue observed after chitosan treatment.

Phloroglucinol HCl and $KMnO_4$ staining revealed that the phenolic material deposited in the cell wall was not lignin. However, it has been suggested that esterification of phenols to cell wall material may be involved in resistance against pathogens (Nicholson and Hammerschmidt, 1992).

After 48 hours, tissue treated with chitosan begun to exhibit a yellowish fluorescence in addition to the blue-green fluorescence that had been observed previously (Figure 3-2 and 3-3). The yellowish fluorescence may also indicate

cell wall deposition of phenols. The localization and timing of the yellowish fluorescence corresponded with the localization and timing of toluidine-blue O staining, thus supporting the idea that the fluorescence is caused by phenols deposited in the cell wall (Figure 3-2, 3-3, and 3-5). Alternatively, the yellowish fluorescence may indicate a change in the type of phenols accumulating in the cells. It has been suggested that chlorogenic acid, which in itself is not very toxic, may be used to synthesize other phenols that can prevent pathogen infection (Friend, 1981). Such changes may affect the fluorescent properties of the accumulating phenolics.

Lacmoid staining revealed that chitosan treated tissue developed a barrier of callose two to three cell layers from the surface of the treated tissue after 72 hours (Figure 3-7). Callose has also been implicated as a barrier to pathogen invasion (Hammerschmidt and Nicholson, 1999). Therefore, the callose barrier may prevent infection by *F. sambucinum* beyond the first two or three of cell layers.

The cytological data collectively suggests that *F. sambucinum* infection may be slowed or halted within the first few cell layers. *F. sambucinum* growth may be initially inhibited by exposure to toxic phenols and/or cell wall changes caused by the deposition of phenols that can prevent *F. sambucinum* penetration. The callose barrier appeared to correspond with the interface between those cells accumulating phenols and those in which phenolic accumulation was not evident. The phenolic accumulation may slow hyphal penetration long enough for the

callose barrier to form. The callose barrier may then prevent further penetration until suberization is complete.

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CONCLUSIONS AND FUTURE RESEARCH

Chitosan and *P. infestans* mycelium homogenate induced several putative defense mechanisms in potato tuber tissue including: oxidative enzymes, PR proteins, and cell wall alterations (Table C-1). Associated with the onset of these defenses was induced resistance to *F. sambucinum* infection. Induced resistance is therefore, a promising alternative to fungicides for the control of Fusarium dry rot. The resistance observed after treatment with chitosan and *P. infestans* mycelium homogenate is probably a result of multiple plant defense mechanisms working simultaneously to stop the ingression of *F. sambucinum*; because this resistance controls *F. sambucinum* using several defense mechanisms it may be a more stable form of resistance than fungicides which only target one aspect of fungal development.

Table C-1: Review of the effect of chitosan and MH on plant defense mechanisms.

	PR-Proteins		Oxidative Enzymes		Phytoalexins
	Chitinase	β-1,3-Glucanase	Peroxidase	Polyphenol oxidase	Steroid Glycoalkaloids
Chitosan	Local (2 hours) Systemic (2.5 cm)	Local	Local (12 hours)	Local Systemic (0.5 cm)	Local Decrease
MH	Local Systemic (2.5 cm)	Local Systemic (1.0 cm)	Local	Not Tested	Local Decrease

Further research questions include:

1. Determining if treatment with chitosan or *P. infestans* mycelium homogenate would effectively induce resistance in whole tubers and seed pieces reducing storage losses and seed decay.
2. Evaluate the ability of chitosan and *P. infestans* mycelium homogenate to reduce infection in tubers and foliage by other potato pathogens and evaluate their effect on herbivory.
3. In light of differences in the appearance of tuber tissue after chitosan and *P. infestans* mycelium homogenate and differences in the induction of putative defense mechanisms, it would be interesting to assess whether chitosan and *P. infestans* mycelium homogenate induce the same or different signally pathways.
4. Analyze phenolic and callose deposition after treatment with *P. infestans* mycelium homogenate.
5. Assess whether potato chitinase or β-1,3-glucanase inhibit *F. sambucinum* growth or if they produce elicitors that may induce other defense mechanisms.