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Integrated Management Strategies and Pathogen Detection in the Potato-*Streptomyces* Spp. Pathosystem

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INTEGRATED MANAGEMENT STRATEGIES AND PATHOGEN DETECTION IN THE POTATO-*STREPTOMYCES* SPP. PATHOSYSTEM

By

Elise C. Hollister

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ABSTRACT

INTEGRATED MANAGEMENT STRATEGIES AND PATHOGEN DETECTION IN THE POTATO-*STREPTOMYCES* SPP. PATHOSYSTEM

By

Elise C. Hollister

The cultivated potato, *Solanum tuberosum* L., is the world's most important dicotyledonous food crop. It is surpassed only by wheat, rice, and corn as the world's fourth most important human food source. This research examined the potential use of environmentally friendly disease management tools in reducing the severity of an important bacterial disease, potato common scab, as well as examined the potential use of molecular methods for detection and quantification of the pathogens that cause this disease (*Streptomyces* spp.).

Controlled environment and field experiments were conducted to determine the effectiveness of cultivar resistance and soil moisture in reducing disease incidence and severity. Cultivar resistance against common scab appeared to be the most effective means of decreasing scab incidence and severity. When cultivar resistance was combined with carefully maintaining soil moisture, near field capacity, control of the disease was slightly enhanced. Common scab reduction also occurred for scab-susceptible cultivars when soil moisture was maintained at near field capacity; however, the amount of control was not as effective as the reduced disease incidence and severity of the intermediately resistant and resistant cultivars.

The use of resistance activators for elicitation of systemic acquired resistance (SAR) in potato to common scab in field experiments produced variable results. While

many of the elicitor treatments resulted in more scab free tubers in one year, the following two years no significant differences were noted. Temperature and soil moisture may have been important variables that led to the interannual variability in these experiments. Analysis of tuber tissue for the pathogenesis related (PR) protein, chitinase, was carried out to determine if the activity of this enzyme (a marker for SAR) was increased. No increase in chitinase activity was observed, thus suggesting that resistance was not induced by the activators.

PCR and real time PCR were used as methods of pathogen detection and quantification, respectively. The *nec1* gene, which is associated with pathogenicity in *Streptomyces* spp., was used to rapidly screen isolates obtained from soil from fields cropped to potato in the major potato production counties of Michigan. The pathogen was isolated and detected in each county sampled. Real time PCR was used to quantify pathogen populations from three Michigan fields. Results indicated slight differences in pathogen population between the fields. PCR was found to be the better method for identification of the causal agent, while real time PCR was useful when quantification was needed. These findings demonstrate that cultivar resistance is the primary method for managing common scab of potato. Soil moisture application is also an important management strategy, especially when scab-susceptible cultivars are planted. SAR may provide more scab-free tubers if combined with other management methods. As a stand-alone disease management tool, it offered no control against this particular genus of pathogens. Also, detection of the pathogenic *Streptomyces* spp. was achieved more economically and accurately by PCR. Real time PCR was a reliable method for quantification, but is much less economic if it is to be used as a diagnostic tool.

"Scab remains one of the most important and least satisfactorily controlled of potato diseases."

John C. Walker (1969)

To my grandmothers, Elizabeth Ruth Fulghum and Beatrice Mardell Poole, two very special and exceptionally intelligent women who can fully appreciate the opportunities that have been given to me in my lifetime.

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LITERATURE REVIEW

Importance of the Potato

The potato (*Solanum tuberosum* L.), native of South America's Andean highlands, has been cultivated for at least 7,000 years according to studies using scanning electron microscopy and radiocarbon dating (Martins, 1976). Some archaeological evidence suggests that wild potatoes were eaten 13,000 years ago in Chile (Simpson and Ozorgaly, 1995). The potato is a member of the Solanaceous plant family, which is made up of approximately 96 genera and 2800 species, with the greatest diversity in the American tropics (Walters and Keil, 1996). Solanaceae is one of the most economically important plant families. In addition to the potato, it includes the following major crops: tomato (*Lycopersicon esculentum*), eggplant, (*Solanum melongea*), bell peppers and chili peppers (*Capsicum* species.), tomatillo (*Physalis philadelphica*), and tobacco (*Nicotiana tabacum*).

Solanum, the genus to which the cultivated potato tuber belongs, contains approximately 1,000 species. Yet, the cultivated potato belongs primarily to *Solanum tuberosum*. This cultivated species is a tetraploid, which consists of two subspecies, *S. tuberosum* subsp. *tuberosum* and *S. tuberosum* subsp. *andigena*. At least six additional cultivated species: *Solanum ajanhuiri*, *S. phureja*, *S. stenotomum*, *S. chaucha*, *S. juzepczukii*, and *S. curtilobum* and more than 230 wild species are generally recognized (Harris, 1992). Due to the extensive work of plant breeders, agronomists, and plant pathologists, today the potato is grown worldwide as it has become well adapted to cool temperate zones of the high altitudes of the Andes, at sea level in temperate zones in

North America, Europe, southern Chile and Argentina, and at appropriate altitudes in intermediary latitudes. As a result of these factors, the potato ranks first as the most important dicotyledonous food crop, and is surpassed only by wheat, rice, and corn to be the world's fourth most important human food source. Hence, understanding diseases that affect the yield and market quality of such a high-value crop is extremely critical so that proper disease management strategies can be established.

Streptomyces scabies, The Primary Causal Agent of Potato Common Scab

Common scab of potato is caused by several Gram-positive filamentous species of *Streptomyces*, but *Streptomyces scabies* is the predominant causal agent. Originally named *Oospora scabies*, the organism was described as a "bacteria-like fungus" (Thaxter, 1890). Over twenty years passed before it was determined that it was in fact a bacterium, and it was renamed *Actinomyces scabies* (Gussow, 1914). Eventually the actinomycetes were subdivided, and the pathogen was placed it into the genus *Streptomyces* (Waksman and Henrici, 1948).

Streptomyces scabies is an aerobic, soil-borne bacterium that causes a range of symptoms on the surface of potato tubers including superficial cork-like lesions, erumpent cushion-like raised scabby lesions, and pitted lesions, which extend through the tuber periderm into the cortex and vascular storage parenchyma. This bacterium is the most important plant pathogen in the genus *Streptomyces* worldwide, and common scab of potato ranks fourth in severity of potato diseases (Hooker, 1981) and affects potatoes where ever they are grown. Pathogenic streptomycetes are able to cause a variety of diseases, including pod wart of peanut, scab disease on radish, carrot, red beet and sugar

beet roots, as well as root rots on the fibrous roots of many plants, such as celery, sweet potato and eggplant.

In addition to *S. scabies*, other species within the genus *Streptomyces*, such as *S. acidiscabies* and *S. caviscabies* have more recently been identified as causal agents of potato scab. Currently, there is a growing list of more than thirty *Streptomyces* species capable of enciting scab lesions on potatoes (Table 1). It is likely that there are more species that cause scab, but they have yet to be identified.

Historically, identification of the pathogen has been difficult and inaccurate due to the variability of morphological and physiological characteristics, which were used as the basis for taxonomy. Only within the last decade have the most efficient methods of DNA-based methods been used for rapid identification of the various species and strains. Hence, taxonomic studies of the genetically diverse causative organisms have only recently been consistent since the development and use of DNA-based detection methods.

TABLE 1. A list of species within the genus *Streptomyces* that are causal agents of potato scab, and their reference and year of publication.

Potato Scab Causal Agent	Reference	Year Published
Streptomyces scabies	Thaxter	1890
Streptomyces violaceus	Rossi-Doria	1891
Streptomyces intermedius	Kruger	1904
Streptomyces clavifer	Millard and Burr	1926
Streptomyces fimbriatus	Millard and Burr	1926
Streptomyces sampsonii	Millard and Burr	1926
Streptomyces setonii	Millard and Burr	1926
Streptomyces wedmorensis	Millard and Burr	1926
Streptomyces candidus	Krasil'nikov	1941
Streptomyces globisporus	Krasil'nikov	1941
Streptomyces longisporus	Krasil'nikov	1941
Streptomyces albidoflavus	Waksman and Henrici	1948
Streptomyces rimosus	Sobin *et al*.	1953
Streptomyces aureofaciens	Corbaz	1964
Streptomyces flaveolus	Corbaz	1964
Streptomyces griseus	Pridham	1970
Streptomyces atroolivaceous	Archuleta and Easton	1981
Streptomyces cinerochromogenes	Archuleta and Easton	1981
Streptomyces corchorusii	Archuleta and Easton	1981
Streptomyces diastatochromogenes	Archuleta and Easton	1981
Streptomyces lydicus	Archuleta and Easton	1981
Streptomyces resistomycificus	Archuleta and Easton	1981
Streptomyces collinus	Dey *et al.*	1981
Streptomyces acidiscabies	Lambert and Loria	1989
Streptomyces caviscabies	Goyer et al.	1996
Streptomyces turgidiscabies	Miyajima *et al*.	1998
Streptomyces europaeiscabiei	Bouchek-Mechiche *et al*.	2000
Streptomyces stelliscabiei	Bouchek-Mechiche *et al*.	2000
Streptomyces species strain VKM Ac-2124	Shashkov *et al*.	2002
Streptomyces luridiscabiei	Park *et al*.	2003
Streptomyces niveiscabiei	Park *et al*.	2003
Streptomyces puniciscabiei	Park *et al*.	2003

The Infection Process

Streptomyces scabies produces non-fragmenting substrate mycelia that colonize the soil, and it uses extracellular hydrolytic enzymes to obtain nutrition from the organic compounds present in the soil. Like fungi with non-motile spores, this bacterium produces spores for aiding in dispersal. The pathogen is spread through soil water, by wind-blown soil, and on infected seed tubers (Agrios, 2005). The spores and mycelia of the bacteria overwinter in the soil on infected plant tissue. The spores also contribute to the survival over periods of drought, freezing temperatures, and anaerobic conditions produced by water saturation (Agrios, 2005). *Streptomyces scabies* has been introduced into the majority of soils by infected seed tubers. Agricultural machinery can also be a means of dispersal for the pathogen (Agrios, 2005). Once the bacteria have been introduced into a field, they persist and may survive indefinitely.

The smooth gray spiral spore chains arise from fragmentation of the aerial hyphae formed from the substrate mycelium. As the cross walls of the aerial hyphae constrict, the spores are pinched off at the tip and eventually break away. The spores produce one or two germ tubes that are able to penetrate directly through the immature stomata and immature lenticels yet to form a protective barrier, as well as wounds on the periderm of the potato tuber, which is most susceptible to infection during early tuber development. This period of rapid growth begins when the tuber diameter has reached twice that of the stolon and will continue for six to eight weeks, depending on cultivar (Loria *et al.*, 1997). Once the bacterium has gained entry into the tuber, it grows between or through a few layers of cells, which die, and the pathogen derives nutrients from them (Agrios, 2005).

In response to the bacteria, the living cells surrounding the lesion rapidly divide and produce many cork layers around the site of entry that isolate the bacteria and many plant cells as well (Agrios, 2005). Normally, numerous groups of cork cell layers are produced, and as they are pushed outward from the periderm and sloughed off, the bacteria are able to grow and multiply in the additional dead cells, leading to the development of large scab lesions, which are darker in color than healthy skin. Such a lesion can enlarge through the growing season and produce any or all of the three major scab symptoms: a) superficial, russet, or surface scab refers to the cork-like lesions that are darker than the healthy skin; b) cushion-like, erumpent, or raised scab refers to the corky growth that protrudes from the periderm; and c) pitted or deep scab that penetrates into the tuber and produces dark, sunken lesions. If infections occur when the tuber is immature, then one or more of the lesion type(s) will increase in severity as the tuber enlarges during the growing season. The lesion depth is dependent upon the cultivar used, on soil conditions, and on the invasion of scab lesions by secondary organisms, including insects, which are able to chew the cork layers and allow *S. scabies* to invade the tuber to a much greater depth (Agrios, 2005). The mechanisms that enable the pathogen to invade the plant and trigger the host's response of cork production are not fully understood.

The Role of Enzymes in Pathogenesis

The role of degradative enzymes during host invasion is not well documented. The role of cellulolytic and pectinolytic activity of *S. scabies* has been investigated. There is a high activity of pectic acid *trans*-eliminase, but there was no strict correlation

between enzyme activity and virulence (Knosel, 1970). However, since the pathogen first attacks the middle lamellae of the cells (Lapwood, 1973), it is possible to suggest that the activity of this enzyme functions as a virulence factor. One report of the production of indoleacetic acid from tryptophan by *S. scabies* suggested that this product may be important in pathogenesis (Hirata, 1959), but the evidence is inconclusive. Slightly more focus on esterase has demonstrated that pathogenic *S. scabies* are capable of breaking down suberin with this heat-stable enzyme (McQueen and Schottel, 1987). Since suberin, a complex polymer composed of fatty acid polyesters and phenolic compounds, acts as a physical barrier to pathogens, it has been suggested that esterase produced by *S. scabies* may play a role in pathogenicity. Further investigation of the role of enzymes during the infection process is required because informative correlations between enzymes and pathogenesis are currently unavailable.

The Role of Thaxtomins in Pathogenesis

In contrast to the role of enzymes, much attention has been paid to scab phytotoxins in pathogenesis. *S. scabies* produces a class of phytotoxins, known as thaxtomins, which have been associated with potato scab. Thaxtomins were first isolated from potato tubers and were characterized as unique 4-nitroindol-3-yl-containing 2,5 dioxopiperazines (King *et al.*, 1992). Thaxtomin A (Figure 1) is the predominant phytotoxin produced by both *S. scabies and S. acidiscabies*, but there are 10 additional related compounds that have been isolated and characterized (King and Lawrence, 1996; King *et al.*, 1992).

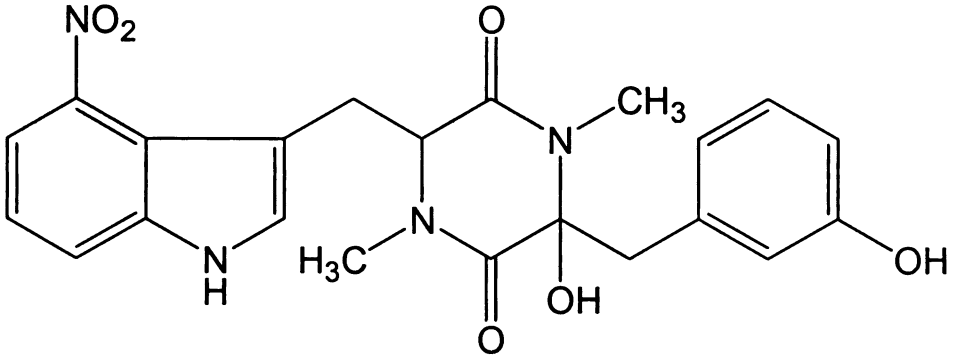

Figure 1. Structure of Thaxtomin A, a phytotoxin associated with virulence in plant pathogenic *Streptomyces* species.

Most literature has reported thaxtomin synthesis as a necessary pathogenicity factor. A gene, *nec1*, was cloned from a *S. scabies* strain and subsequently used to transform *Streptomyces lividans*, which is not pathogenic to potato. The transformation conferred the ability of *Streptomyces lividans* to necrotize and colonize potato tuber disks and produce scab like symptoms on immature tubers (Bukhalid and Loria, 1997). The *S. lividans* expressing *nec1* did not produce thaxtomin A, but did produce an unidentified, extracellular, water-soluble compound that caused necrosis on potato tuber disks. In another study, this gene was structurally conserved among all but one pathogenic *Streptomyces* spp. strains (Bukhalid et al., 1998). The pathogenic strain that failed to hybridize to *nec1* and the transformed *S. lividans* suggested that the function of the gene is not always directly involved in thaxtomin A biosynthesis, but that it is a greater than 99% predictor of pathogenicity (Bukhalid and Loria, 1997). The complete nucleotide sequences of two peptide synthetase genes (*txtA* and *txtB*) were determined from *S. acidiscabies* (Healy *et al.*, 2000). They found that the involvement of the thaxtomin A and thaxtomin B peptide synthetase catalytic domains was consistent with the formation

of *N*-methylated cyclic dipeptides such as thaxtomins. Based on high-performance liquid chromatography analysis, thaxtomin A production was abolished in *txtA* gene disruption mutants, which were avirulent on potato tubers. The authors demonstrated that the introduction of the thaxtomin synthetase cosmid into a *txtA* mutant restored pathogenicity and thaxtomin A production. Most studies conclude that thaxtomin biosynthesis is necessary for pathogenicity. However, a recent study where *S. scabies* and *S. acidiscabies* isolates were screened for thaxtomin production, one of the pathogenic isolates was unable to produce thaxtomin A *in planta* (El-Sayed, 2000). The production of other thaxtomins was not assessed. This suggests that thaxtomin production increases a pathogen's virulence and is not a required component of pathogenicity. Another recent study by Kers *et al.* (2005) supports the notion that thaxtomins aid in virulence. Although thaxtomins may not be determinants of pathogenicity, they have been well studied and characterized. The pathogenicity factors required for infection in plant pathogenic *Streptomyces* species are yet to be identified.

Common Scab Management Strategies

Cultivar Resistance

One of the most important management strategies in control of common scab is the use of resistant cultivars; hence, the extensive effort of breeders and agronomists is necessary for improved development of cultivars that display favorable agronomic traits and resistance to common scab. Potato cultivars are well documented to widely vary in their susceptibility to scab infections caused by *Streptomyces* species (Blodgett and Stevenson, 1946; McKee, 1958; McKee, 1963; and Douches *et al.*, 1996). If the lesions

cover more than five percent of the tuber surface, the potatoes fail to make U.S. No. 1 grade (USDA-Agricultural Marketing Service, 1991). Methods for evaluation of scab infections have been developed to compare levels of scab resistance. These rating systems have been devised because the percentage of surface area covered and the type of lesion can vary from tuber to tuber within a plot.

One rating system rated each tuber and classified the percentage of tuber surface infected with scab into five categories (Emilsson and Gustafson, 1953). They designated lesion categories: superficial, ordinary, deep, and elevated, as well as computed the total percentage of tuber surface covered with scab, but chose the most prevalent type of lesion in the plot to rate the type of lesion. Another similar rating system was devised for each tuber, but then created an overall scab index combining the percentage surface area covered and the type of lesion (Bjor and Roer, 1980). However, a superior system for rating resistance to scab with two separate indices was created: surface area covered (AI) and the type of lesion (LI) (Goth *et al.*, 1993). It was found by cluster analysis on both AI and LI were superior to the overall index of Bjor and Roer in comparisons for discriminating among potato cultivars (Goth *et al.*, 1993). Numerous laboratory (King *et al.*, 1989; Lawrence *et al.*, 1990) and greenhouse (Bjor and Roer, 1980; Hooker, 1950; McKee, 1958; Wiersema, 1970) methods for screening scab resistance in breeding material have been proposed. However, field trials are necessary to evaluate the true value of resistance to scab because numerous variables, such as the variation in aggressiveness of scab isolates (Elesawy and Szabo, 1979; Keinath and Loria, 1989), soil temperature (Stevenson *et al.*, 2001), soil moisture levels (Jellis, 1974; Lapwood *et al.*, 1971), and soil pH (Davis *et al.*, 1974), are known to heavily influence the severity of

scab. These variables additionally make the quantification and categorization of scab resistance in breeding material extremely difficult.

An important method in breeding selection is one that is measured by the size of response to selection in a breeding population. This method works for quantitative traits, whose response to selection is dependent upon the degree of genotypic and phenotypic variance in the population, heritability, and intensity of selection (Tai *et al.*, 1996). Diploid interspecific hybrids that are selected for their resistance to scab and their ability to produce 2n male gametes were a good source of resistance (Tai *et al.*, 1996). The examination of the transmission of resistance from diploid parents to their tetraploid progenies through 4x-2x matings indicated that the selection for scab resistance in potato is based on individual seedlings or best families.

The principal cultivated potato (*Solanum tuberosum* subsp. *tuberosum*) is a tetraploid that displays tetrasomic inheritance (Harris, 1992). As a consequence, genetic analysis has proved difficult, particularly for quantitative traits. The first well-documented report of the genetic nature of scab resistance was proposed with the assumption that scab resistance was due to a single, dominant gene (*Sc*); (Krantz and Eide, 1941). They divided the segregating progeny of a tetraploid cross into five genetic groups ranging from nulliplex to quadruplex for *Sc*. Lauer and Eide (1963) reported that a single gene could explain scab resistance, but that for effective resistance, the gene must be a duplex. Cipar and Lawrence (1972) concluded that the scab resistance in fact involved more than one locus and that inheritance was relatively simple at the diploid level. Alam (1972) hypothesized that two independent loci were required for scab resistance where one locus, one or more dominant alleles confer resistance (Sc_1) and the

second locus, homozygous recessive alleles confer resistance (sc_2). Murphy *et al.* (1995) studied the transmission of scab from diploid parent to the tetraploid level and was able to support Alam's hypothesis.

Estimates of broad-sense heritability (Nyquist, 1991) are important because both additive and non-additive genetic variances are usable. The magnitudes of the additive and nonadditive components of these genetic variances are not known. The high broad-sense heritability estimate suggested that a genotype identified as scab resistant would remain scab resistant in many different growing environments (Haynes *et al.*, 1997). This group documented significant genotype x environment interaction for both AI and LI. The genotypic material whose resistance to disease was close to immune remained fairly stable in different environments. However, for both of the indices, the genotype x environment variance was only one-half to one-third of the variance due to the environment.

Tuber Periderm Development. The mechanisms of scab resistance have been closely associated with tuber periderm development. Lutman (1919) concluded that the thickness of the tuber skin determined its resistance to scab. Darling (1937) noted that the lenticel was the primary avenue of infection and that resistant cultivars had lenticels that were smaller and consisted of small, closely associated, suberized cells whereas those of the susceptible cultivars were large and composed of loosely arranged, spherical cells. Fellows (1927) proposed that the tuber must be actively growing if infection is to occur. This was based on scab infections occurring only in immature stomata and lenticels that are formed in new and rapidly growing protective tissue (Fellows, 1927). Most attention has been given to younger tubers since Sanford (1926) had shown that the

infection occurred during the early stages of tuberization. Histological studies showed that the outer-most cell layers of the periderm were remaining in a living, nucleated condition in a resistant cultivar, while those of the susceptible lines were not (Cooper *et al.*, 1954). They proposed resistance to be closely associated with the nature of the development of the periderm during tuberization.

Chlorogenic Acid Content. The role of chlorogenic acid in cultivar resistance to scab has been studied. Johnson and Schaal (1952) demonstrated a close correlation between the amount of chlorogenic acid in the tuber's peripheral layers and scab resistance. Susceptible tubers possessed about half the quantity of the acid of the resistant cultivars. They suggested that chlorogenic acid lowers the pH of the cells, thereby acting as an unsuitable medium for the bacteria or that the acid itself or its quinone may be directly or indirectly involved in the formation of the suberized periderm. Emilsson and Gustafsson (1953), however, were unable to support the Johnson and Schaal (1952) study. They found no correlation with scab resistance and chlorogenic acid. Further investigations on the role of chlorogenic acid and phenol oxidase in cultivar resistance to scab produced no correlations (Holm and Adams, 1960).

Thaxtomin Sensitivity. The relative susceptibilities of cultivars to *S. scabies* and their corresponding sensitivities to thaxtomin A have shown to be positively correlated (Acuna *et al.*, 2001). This suggests that different degrees of modification of the phytotoxin to the tuber tissues to yield less bioactive metabolites may be an important component of cultivar resistance. Such assumptions are consistent with the fact that many xenobiotics, such as herbicides, are metabolized after being introduced into plant tissue. The metabolism by oxidation, hydrolysis, or conjugation (usually to

carbohydrates, glutathione, or amino acids), often leads to detoxification of the original active compounds (Kaufman, 1976). Some of these transformations can be accompanied by microbial agents. King and Lawrence (2000) described microbially induced conjugation of thaxtomin A and thaxtomin B to make *O*-glucosides 3 and 4, respectively. They noted a relative loss of bioactivity when these conjugates were applied to the surfaces of aseptically cultured potato minitubers.

Chemical Control

Decreased scab incidence was reported when soil was treated with the fungicide pentachloronitrobenzene, (Hooker, 1954). Pentachloronitrobenzene (PCNB) was found to vary in effectiveness depending on the method and rate of application. The reduction of scab severity using this fungicide was effective at exceedingly high rates according to Davis, *et al.* (1974). Analogues of daminozide (*N*-dimethylaminomaleamic and *N*-(dimethylamino)-methylsuccinamic acids) were found to decrease scab incidence when applied as a foliar spray or applied directly to soil (McIntosh and Bateman, 1979). Another study tested 30 compounds related to an effective scab control foliar spray, 3,5-dicholorphenoxyacetic acid. None of the compounds were more effective than 3,5-D, and those that were as effective also demonstrated negative effects on tuber yield, number, and shape (McIntosh *et al.*, 1985). Substituted benzoic and picolinic acids reduced scab in greenhouse experiments; however, the foliage in some of the treatments was damaged (McIntosh and Bateman, 1988), suggesting that systemic acquired resistance may have been involved in the scab reduction.

Sulfur, when combined with 90% available soil moisture, was effective in reducing scab incidence and severity (Davis *et al.*, 1974b). Other studies confirmed these

results (Davis *et al.*, 1974a and Davis, *et al.*, 1976). However, sulfur applied without added soil moisture was ineffective in reducing scab infection in all studies and the possible effects of sulfur on yield were not published in any of these studies. Sulfur was effective in reducing *Streptomyces* soil rot of sweet potato, a disease caused by a closely related pathogen; however, yields were decreased (Ristaino and Averre, 1992).

Captafol (McIntosh, 1977) and ethionine (McIntosh and Burrell, 1980) were effective in decreasing scab severity, with little reduction in yield. However, none of these are currently available in commercial formulations to control scab disease. Attempts to control *Streptomyces* soil rot, a disease of sweet potato caused by a related pathogen, *Streptomyces ipomoea*, with sulfur and soil fumigation with Telone C-17 reduced disease. Fumigation proved effective, but the sulfur treatments significantly reduced yield (Ristaino and Averre, 1992). More recently, efforts to control common scab with sodium hypochlorite and thiophanate-methyl seed treatments provided no significant reductions in scab infections (Errampalli and Johnston, 2000). Currently, there are no conventional pesticides registered to control common scab; therefore, the focus and establishment of other control methods is essential.

Soil Moisture

One study that initiated the use of irrigation to control scab was conducted by Sanford (1923). Soil moisture treatments applied in high amounts were shown to significantly reduce the severity of scab infection by *S. scabies*. Consequently, many studies using irrigation as a means to control scab followed Sanford. Irrigation applied during the early stages of tuberization was shown to speed the swelling of the tubers and significantly decreased incidence of scab (Lapwood *et al.*, 1970). At high water

potentials at or near field capacity, the actinomycete populations on the lenticels were low, and the bacterial populations were high, which suggested that irrigation might decrease the population of *S. scabies* in tuber lenticels by increasing populations of scab-antagonistic bacteria.

The amounts of irrigation that effectively control scab, the appropriate timing of irrigation, and the reduction in scab infection are variable between reports. Some have reported that the incidence of potato scab increased with soil moisture (Starr *et al.*, 1943) or that precipitation, which led to high soil moisture content, did not reduce scab infection on tubers (Goto, 1985). However, this may be due to the soil type, since dry soils have been shown to increase incidence and severity of disease or could be due to evaporation of precipitation. This also may be due to the water repellent nature of sand, which is exacerbated by high temperatures. Although relatively few studies associate increased scab infection with increased soil moisture, the majority of reported irrigation experiments to control scab demonstrate that *S. scabies* is inhibited in soils with high moisture levels. Irrigation for four weeks after tuber initiation at a 15 mm soil moisture deficit was found to decrease scab most effectively in one study (Lapwood, 1973). According to another experiment, five weeks of irrigation following tuber initiation was found to substantially reduce scab, while two weeks of irrigation after initiation was not sufficient to provide management of scab (Lewis, 1970). Although there is variation in relation to timing of irrigation, which is most likely due to environmental and cultivar effects, irrigation that provides adequate soil moisture at or near field capacity during tuberization is one of the most important crop management methods of controlling this disease without significantly reducing yield. However, a study by Davis *et al.* (1974b)

underscores the importance of combining irrigation with other methods to control common scab. They irrigated soils to greater than 90 percent available soil moisture during the early stages of tuber development and this was not sufficient alone to produce tubers of adequate quality for market standards. Studies that combine soil moisture management and cultivar resistance are needed. Yet, maintenance of sufficient soil moisture should not be overlooked and is particularly important because there are no resistant cultivars completely immune to the disease and there are no commercially available effective chemicals that have been developed to control this disease.

Biological Control

Scab decline due to a biological factor was first demonstrated by the application of 1 percent suppressive soil plus 1 percent alfalfa meal into disease conducive soils (Menzies, 1959). Several *Streptomyces* species isolated produced bacteriocins against virulent isolates. They also reduced the number of scab lesions on potato tubers grown in a field experiment (Lorang, 1988). Strains of *Streptomyces* antagonistic to the pathogen were isolated from potatoes grown in a soil that became suppressive to scab after twenty-three years of potato monoculture (Lorang et al., 1989). The decreased severity of potato scab caused by the antagonistic *Streptomyces* strains did not affect yield in the tuber plots (Liu *et al.*, 1995). All of the suppressive isolates have been found in soils that have been cropped to potatoes for over 20 successive seasons and had subsequently become naturally suppressive to the pathogen (Menzies, 1959). All of the strains are non-pathogenic and are members of the genus *Streptomyces*. It has been demonstrated *in vitro* that these isolates produce antibiotics that are inhibitory toward pathogenic *S. scabies* strains (Liu *et al.*, 1996; Lorang *et al.*, 1995). Organisms in this genus produce a

variety of antibiotics with a diversity of structures, including polyketides, β-lactams and peptides in addition to many other secondary metabolites that have anti-fungal, anti-tumor, and immunosuppressive activities (Chater and Hopwood, 1993).

The antibiotics produced by the suppressive isolates, which have been found in the naturally suppressive soils, may play a primary role in inhibiting the potato scab pathogen. The mechanism of antibiosis as a biological control measure has been well understood in other systems (Kerr, 1980; Silo-Suh, *et al.*, 1994). Understanding the nature and diversity of the streptomycete antibiotics is necessary for characterizing the mechanism of biological control of potato scab. Also, the availability of these antibiotics for application to disease-conducive soils may be an effective way to potentially augment the activity of these biocontrol organisms. It seems that the suppressive strain would either need to outcompete pathogenic *S. scabies* for the available resources or be able to inhibit the pathogen directly (Liu *et al.*, 1996). A recent attempt to disinfest seed potatoes of *S. scabies* with a *Streptomyces* phage *in vivo* significantly disinfested seeds and hence, reduced contamination of soil from seed-tuber-borne inoculum and reduced infection of daughter tubers (McKenna *et al.*, 2001). Since no chemicals are available for scab control, effective scab-antagonistic biocontol agents should be further explored.

Induced or Acquired Resistance

A phenomenon in many plant species is demonstrated when a plant is able to respond to pathogen infection due to the induction of a broad-spectrum resistance to subsequent infection by the same or other pathogens. This phenomenon is known as systemic acquired resistance (SAR), or induced resistance. The broad immunity of SAR arises as a result from infection with a necrotizing pathogen, a naturally occurring

compound in nature, or a synthetic chemical compound. SAR development is triggered by a virulent or avirulent pathogen, a natural elicitor, or a synthetic chemical elicitor. Following the induction of resistance is usually an accumulation of salicylic acid and pathogenesis-related (PR) proteins. Van Loon *et al.* (1994) proposed a PR-protein nomenclature, which comprises 11 families of proteins. PR-protein examples are chitinases (PR-3 family), B-1,3 glucanases (PR-2 family), and osmotin (PR-5 family), which all show antimicrobial activity *in vitro*.

Early work on the induced resistance phenomenon was reviewed by Chester (1933), and he termed it physiological acquired immunity. Pioneering studies by Ross (1966) experimentally proved that a localized, necrotic infection could lead to resistance against further infection by the same or by many different pathogens. The Food Quality Protection Act, signed into legislation in 1996, has led to an increased interest in development of biopesticides, some of which elicit the SAR pathway of resistance. Since the EPA annually reviews the conventional pesticides, each year many are unregistered for use, due to persistence in environment, carcinogenicity, and toxicity leading to negative impacts on human health, environmental quality, and endangered species. Therefore, much interest has been focused on development of natural inducers of plant resistance, which, unlike conventional agricultural pesticides, are environmentally friendly (Ozeretskovskaya, 1995 and Hammerschmidt and Dann, 1997).

Chitosan, a polymer of β-1,4-D-glucosamine, is non-toxic, biodegradable, and biocompatible. Chitosan has been used as an elicitor for plant protection from pathogens (Hadwiger *et al.*, 1984). Treatment with chitosan has enhanced resistance of tomato to *Fusarium oxyporum* f. sp. *radicis-lycopersici* (Benhamou and Thériault, 1992), has been

an effective elicitor of resistance in potato to late blight (Vasyukova *et al.*, 2000), effectively reduced leaf rust of peanut against *Puccinia arachidis* (Sathiyabama and Balasubramanian, 1998), and also activated protective mechanisms against the root-knot nematode, *Meloidogyne incognita* (Vasyukova *et al.*, 2001). Beausejour *et al.* (2003) found that combining chitosan with *Streptomyces melanosporofaciens*, a biocontrol agent, led to a greater reduction of common scab incidence and severity than by applying them as separate treatments. Their findings support the general notion that induced resistance should not be used as a stand-alone tool in the management of plant diseases.

Harpin, a protein isolated from *Erwinia amylovora*, the bacterium that causes fire blight of pome fruits, was identified as an elicitor of plant defenses by Wei *et al.* (1992). The protein is responsible for eliciting the development of the hypersensitive response (HR), which is a rapid, localized cell death. The HR is associated with many broad-spectrum host defensive responses, including generation of reactive oxygen species and lignification of the host cell wall. In addition to the hypersensitive response, the resistance activator harpin has been correlated with induction of mitogen-activated protein kinase activity (Desikan *et al.*, 1999), which is an important component of signaling during plant pathogen interactions (Ligterink *et al.*, 1997). SAR induction by harpin is mediated through salicylic acid and the *NIM1* gene (Dong *et al.*, 1999). One study found that transgenic potato plants expressing harpin protein had reduced the lesion growth rate of *Phytophthora infestans*, and concluded that the hypersensitive response in potato was a promising approach to enhance disease resistance (Li and Fan, 1999). Like chitosan, harpin is able to protect against a broad range of phytopathogens. Also, it is a

low risk biopesticide that is much safer than conventional pesticides and is biodegradable.

A recent study reported that benzothiadiazole (BTH) effectively induced resistance in greenhouse-grown potato plants against *Alternaria solani* and *Erysiphe cichoracearum* (Bokshi *et al.*, 2003). BTH was also effective in reducing the severity of early blight in field-grown plants and reduced the severity of dry rot of field-grown tubers (Bokshi *et al.*, 2003). Increased β-1,3-glucanase activity in all plant organs, except roots, was detected for up to 45 days after treatment. The potential use of these naturally derived elicitors has not been thoroughly investigated in relation to induction of resistance to potato common scab. Further investigations that combine resistance activators with other management methods are required.

As previously mentioned, there are no chemicals registered for scab control and current management strategies are limited, not always practical and not always fully effective. Current disease management recommendations to growers for control of potato common scab include the following: plant disease-free seed, use resistant cultivars (most potatoes currently demanded by the market are susceptible) and apply careful irrigation during early tuber development. The primary goal of this thesis is to investigate the usefulness of management strategies and molecular diagnostic methods for potato scab. The four main objectives of this study are to: 1) assess the effects of combining cultivar resistance with soil moisture application on potato common scab severity (Chapter II); 2) determine the effects of four compounds on induction of resistance in potato to common scab (Chapter III); 3) evaluate two PCR-based methods for detection and quantification

of pathogenic *Streptomyces* species (Chapter I); and 4) determine the effects of antimicrobial compounds on the growth of *Streptomyces scabies in vitro* (Chapter IV).

Chapter I

EVALUATION OF PCR-BASED DETECTION OF PATHOGENIC *STREPTOMYCES* SPECIES IN SOIL POPULATIONS OF MICHIGAN'S MAJOR POTATO PRODUCTION COUNTIES AND ASSESSMENT OF REAL TIME PCR AS A METHOD FOR PATHOGEN QUANTIFICATION.

INTRODUCTION

Fresh market standards for potatoes permit very few blemishes on the surface of U.S. Grade A potatoes (U.S.D.A., 1991). The specifications for potato common scab infection are less than 5% surface area of the tuber can be covered by scab lesions for U.S. Grade A. By identifying fields with the lowest levels of inoculum, production of higher quality tubers is more likely. Fields with high levels of inoculum should be avoided and when used, require a judgment between risk and value loss (Wale, 2004).

Current methods of pathogenicity testing for *Streptomyces* species on tuber and tap root crops are based on laborious thaxtomin or melanin production assays (Leiner *et al.*, 1996; Lawrence *et al.*, 1990; Kinkel *et al.*, 1998; Goth and Webb, 1986; Keinath and Loria, 1990; and Wanner, 2004), which are partial indicators of pathogenicity, or are based on inconvenient, time-consuming potato tuber pathogenicity tests (Schaad, 2001).

Soil-borne plant pathogens, particularly persistent ones in which long rotations have little to no effect, create a severe risk to potato growers if environmental conditions are favorable for disease development and susceptible cultivars are planted. Determining disease risk based on populations of soilborne pathogens, such as *Streptomyces scabies*, may be a valuable tool that can reduce the epidemics associated with soilborne diseases.

The *nec1* gene, closely correlated with pathogenicity in *S. scabies,* was suggested for use in detection of causal agents of scab (Bukhalid *et al.*, 1998). Recent studies using *nec1* as a screening tool in PCR assays have successfully identified *nec1* as a pathogenicity indicator by Bukhalid *et al.*, 1998; Bukhalid *et al.*, 2002; Park *et al.*, 2003; and Wanner, 2004 . The most current recommendation for *S. scabies* identification is PCR using *nec1* (Schaad *et al.*, 2001). Recent development of conventional and quantitative real time PCR assays in other pathosystems in identification of soil-borne plant pathogens in soil and plant material suggests that this approach may be useful in quantifying pathogenic *Streptomyces* spp. directly in soil (Lees *et al.*, 2002; Cullen *et al.*, 2002; Cullen *et al.*, 2001;and Bell *et al.*, 1999). Rapid detection of this pathogen is critical so that disease risk assessment can be established. Methods using *nec1* (presumed to be a likely predictor of plant pathogenicity; Bukhalid *et al.*, 1998) in PCR detection of isolates obtained from soil and in DNA extracted directly from soil using real time PCR were evaluated. The objectives of this study were to 1) evaluate PCR detection of pathogenic *Streptomyces* species obtained from soil populations of Michigan's major potato production counties; and 2) assess the usefulness of real time quantitative PCR as a method for pathogen quantification in soil sampled from a controlled environment experiment and from Michigan potato production soils.

MATERIALS AND METHODS

PCR DETECTION OF PATHOGENIC *STREPTOMYCES* SPP. IN MICHIGAN

Soil Sampling

Soil samples were obtained from Michigan potato fields in major potato production counties for isolation of *Streptomyces* spp. to be used for PCR detection experiments. In 2000, soil samples were collected from commercial potato production fields or from Michigan State University potato research fields within the following counties: Bay, Delta, Dickinson, Kalkaska, Luce, Monroe, Montcalm, Otsego, Presque Isle, Saint Joseph, and Sanilac (Figure 1-1). In 2001, soil samples were collected from commercial potato production fields or from Michigan State University potato research fields within the following counties: Clinton, Delta, Dickinson, Huron, Luce, Mecosta, Montcalm, Otsego, Presque Isle, Saginaw, Saint Joseph, and Tuscola (Figure 1-1). In 2002, soil samples were collected from commercial potato production fields or from Michigan State University potato research fields within the following counties: Dickinson, Kalkaska, Luce, Monroe, Montcalm, Otsego, Presque Isle, and Saint Joseph (Figure 1-1). In 2003, soil samples were collected from commercial potato production fields or from Michigan State University potato research fields within the following counties: Luce, Marquette, Monroe, Montcalm, Otsego, and Presque Isle (Figure 1-1). Soil was taken from one location per field (from at least 10 m from edge of field inside the plant canopy). Debris was removed from the upper layer of soil and soil was collected at a depth of 15 cm with a hand shovel until approximately 700 g of soil was obtained from each field.

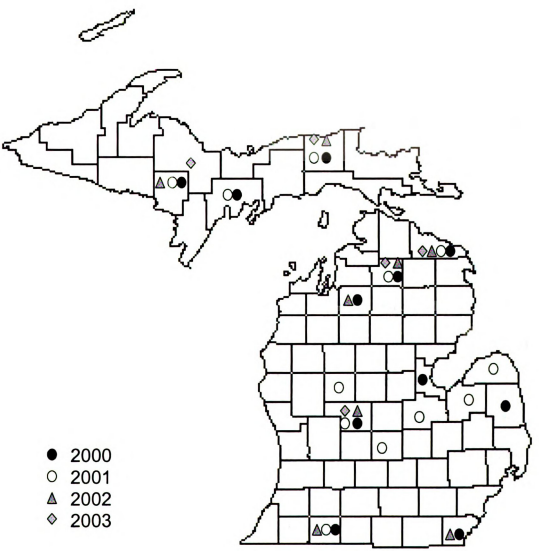

Figure 1-1. Map of Michigan counties from which soil was collected from potato fields for isolation of *Streptomyces* species in 2000, 2001, 2002, and 2003.

Isolation of *Streptomyces* spp. From Soil

Plant debris and stones were manually removed from the soil samples. The soil samples were air dried for 10 days at room temperature to reduce non-filamentous bacteria. Soil (10 g) was placed in sterile 500 ml Erlenmeyer flasks and 90 ml of 0.1% weight/volume sterile water agar was added. The Erlenmeyer flasks were placed on an orbital shaker at 200 rpm for 2 hours. Serial 10-fold dilutions were made in 0.85% sodium chloride. The first dilution was made in 9 ml of the saline solution. Subsequent dilutions were prepared in 900 ml volumes. Aliquots of 100 µl were applied to water agar (WA; 14 g of agar per liter of sterilized distilled water adjusted to a pH of 7.2 with 1 M NaOH) and STR, a semi-selective *Streptomyces* medium developed by Conn *et al.* (1998) with a glass spreader. STR medium is (per liter of distilled water): soluble potato starch, 5.0 g; yeast extract, 4.0 g; bacto peptone, 0.6 g; protease peptone number 3, 0.6 g; NaCl, 10 g; K_2HPO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; agar, 17 g; and 1 ml of a micro-element stock solution ($FeSO_4$, 10 mg; $ZnSO_4$, 1 mg; and MnCl, 1 mg dissolved in distilled water). The pH of STR is 7.2. After autoclaving, the following antibiotics were added: 200 ppm cycloheximide (dissolved in 70% ethanol), 5.6 ppm nystatin (dissolved in 95% ethanol), 0.6 ppm rifampicin (dissolved in methanol), and 15 ppm nalidixic acid (dissolved in 0.1 N sodium hydroxide). Cultures were incubated at 30°C for at least 10 days. Colonies were selected for further analysis (DNA extraction and PCR) based on having morphological features characteristic of *Streptomyces scabies*. These features included substrate and aerial mycelium formation and sporulation on STR and WA.

Genomic DNA Extraction

In 2000 and 2001, DNA was extracted from isolates with typical *Streptomyces scabies* colony morphology by the following method. Cultures of each single colony isolate were prepared in 100 ml of oatmeal broth in sterile 250 ml Erlenmeyer flasks. To prepare oatmeal broth, rolled oats (20 g) in distilled water (1 l) were boiled for 10 minutes and then strained through 4 layers of cheesecloth, yielding 800 ml of filtrate. Micronutrient stock solution (1ml) ($FeSO_4 \cdot 7H_2O$, 0.1 g; $MnCl_2 \cdot 4H_2O$, 0.1 g; $ZnSO_4 \cdot 7H_2O$, 0.1 g; distilled H_2O, 100 ml) was added to the filtrate, and the volume was brought to 1 l. The pH was adjusted to 7.2 with 1M sodium hydroxide before sterilization by autoclaving. Cultures were incubated on an orbital shaker at 185 rpm for at least 72 hours at room temperature. Cells were collected from liquid cultures and centrifuged for 1 minute at 6,000 rpm. Cells were subsequently washed with 2 ml of 0.1X SSC (1X SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate). Cell lysis buffer (1 ml) was added (0.1 M sodium phosphate (pH 7.0) plus 20 % weight/volume sucrose) and 2.5 mg/ml of lysozyme (from chicken egg white) per sample and incubated for at least three hours at 37°C. DNA extraction buffer (400 µl) was added to each sample (200 mM Tris pH 7.0, 25 mM EDTA, 0.5% weight/volume sodium dodecyl sulfate). The supernatant was collected (1 ml) by centrifugation at room temperature for 2 minutes at 13,000 rpm and was extracted twice with a 500 µl of a phenol: chloroform: iso-amyl alcohol solution (25:24:1) and each concluded with centrifugation at room temperature for 5 minutes at 13,000 rpm. A one volume of iso-propanol was added and mixed well by inversion, the samples were centrifuged at room temperature for 5 minutes at 13,000 rpm and the DNA was vacuum-dried for 20 minutes. Vacuum-dried samples

were resuspended in 50 µl of sterile nuclease free water and stored at –20°C. In 2002 and 2003, DNA was extracted from isolates with typical *S. scabies* colony morphology by the following method. Single colony isolates were streaked onto 5 Petri dishes (100 mm x 15 mm) of potato dextrose agar (PDA). PDA was purchased and prepared as described by Sigma Chemical Company at 39.0g/l of distilled water, Saint Louis, Missouri, USA, and the pH was adjusted to 7.2 with 1 M NaOH. Cultures were incubated at room temperature or 30°C for approximately 10 days and spores were collected with a sterile spatula and placed into a sterile 1.5 ml microcentrifuge tube. A total of 300 mg of spores was obtained per isolate. The spores were suspended in 500 ml of AutoGen CTAB (purchased from AutoGen, Holliston, Massachusetts, USA), vortexed, and incubated for at least 1.5 hours at 65°C. The lysed spore suspension was transferred to sterile AutoGen tubes and submitted to the Genomics Technology Support Facility at Michigan State University, East Lansing, Michigan, USA, for genomic DNA extraction using the AutoGenprep 850alpha® robot using the machine's plant/fungal protocol and reagents. Gel electrophoresis was used to verify the DNA extraction process before proceeding with PCR. In all years DNA samples (10 µl) were stained in a Ficoll® -based loading dye (15% Ficoll® type 400 (purchased from Amersham Biosciences, Buckinghamshire, England), 0.25% bromophenol blue, and 0.25% xylene cyanol FF) and loaded into 1% agarose gels made of 1X TAE (1X TAE is 40 mM Tris, 40 mM acetic acid, and 1 mM EDTA) and electrophoresed. Agarose gels were then stained for at least 10 minutes in an ethidium bromide stock solution (10 mM Tris-HCl, 1 mM EDTA, 1 mg/ml ethidium bromide) and viewed under UV light.

PCR Detection of *Streptomyces* spp. and *necl*

The sets of primers used to screen the isolates for the genus *Streptomyces* were developed by Bukahlid *et al.* (1998) and Edwards *et al.* (1989). The sequences of these primers are If: 5'-GTTGTCTTCGG CGAGGGCGTGCAGG-3' and Ir: 5'-AGCG GAAGGATTTGCGACCACCAACG-3' and pA: 5'-AGAGTTTGAT CCTGGCTCAG-3' and pH: 5'-AAGGAGGTGATCCAGCCGCA-3' (Table 1-1). In 2000 and 2001, two subsamples per field were screened until 20 bacterial isolates were positively identified as *Streptomyces* spp. In 2002 and 2003, one sample per field was screened until 20 isolates were identified as *Streptomyces* spp. These isolates were then subjected to further screening for pathogenicity using a set of primers for amplification of *necl*, a gene highly correlated with plant pathogenicity in *Streptomyces* spp and highly conserved among plant pathogenic *Streptomyces* species. These primers were developed by Bukahlid, Chung, and Loria (1998). The sequences of these primers are Nf (5'-ATGAGCGCGAACGGAAG CCCCGGA-3') and Nr (5'-GCAGGTCGTCACG AAGGATCG-3') (Table 1-1). PCR amplification was carried out with RED*Taq*® DNA polymerase (5.0 U; Sigma Chemical Company, Saint Louis, Missouri, USA) using all RED*Taq*® reagents (10X PCR buffer: 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM MgCl2 and 0.01% gelatin; 10 mM dNTP mix: 10 mM each of dATP, dCTP, dGTP, and dTTP) with sterile water double processed for tissue culture (Sigma Chemical Company, Saint Louis, Missouri, USA). The thermal cycler parameters used for all PCR reaction was: DNA denaturing at 94°C for 1 min., annealing at 50°C for 2 min., and elongation at 72°C for 2 min. for 25 cycles. DNA from the *S. scabies* strain DP and sterile water was included in all PCR experiments as positive and negative controls, respectively. The

amplified DNA was electrophoresed on 1% agarose gels made of 1X TAE or 1X TBE (1X TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA). Agarose gels were then stained for at least 10 minutes in an ethidium bromide stock solution (10 mM Tris-HCl, 1 mM EDTA, 1 mg/ml ethidium bromide) and viewed under UV light.

TABLE 1-1. Primers used in PCR and real time PCR targeted against *nec1*, the genus *Streptomyces* and the pBluescript plasmid.

Primers	Nucleotide sequence (5'-3')	Initial [ ] in PCR (nM)	Reference
nec1			
Nf	ATGAGCGCGAACGGAAGCCCCGGA	250	Bukhalid *et al.* 1998
Nr	GCAGGTCGTCACGAAGGATCG	250	Bukhalid *et al.* 1998
Streptomyces			
If	GTTGTCTTCGGCGAGGGCGTGCAGG	250	Bukhalid *et al.* 1998
Ir	AGCGGAAGGATTTGCGACCACAACG	250	Bukhalid *et al.* 1998
Streptomyces			
pA	AGAGTTTGATCCTGGCTCAG	150	Edwards *et al*. 1989
pH	AAGGAGGTGATCCAGCCGCA	150	Edwards *et al*. 1990
real time *nec1*			
q*nec1* f	GCCGCTCGGAGAGTACGA	300	This study[a]
q*nec1* r	AAGCGCTGGGCATTGTTG	300	This study[a]
real time pBlue			
pBluef	TGGGCTGTGTGCACGAAC	300	This study[a]
pBluer	TCAAGACGATAGTTACCGGATAAGG	300	This study[a]

[a] Designed by using Primer Express (version 1.0) software (PE Applied Biosystems)

QUANTIFICATION OF PATHOGENIC *STREPTOMYCES* SPP. SOIL POPULATIONS

I. Assessment of Pathogen Populations in a Controlled Environment Experiment

Soil Sampling

Soil was sampled from the second trial of the controlled environment continuous soil moisture experiment conducted in Chapter 2. Soil was taken from one location per treatment at a depth of 15 cm with a hand shovel until approximately 300 g of soil was obtained from each treatment.

Soil DNA Extraction

Plant debris and stones were manually removed from the soil samples. DNA was extracted from soil by a combination of bead beating and chemical lysis methods developed by McVeigh *et al.* (1996) and Krsek and Wellington (1999). TNPE (50 mM Tris-HCl, 10 mM EDTA, pH 8.0, 100 mM NaCl, 1% (w/w) polyvinylpolypyrolidone) and 0.5 g glass beads (diameter 0.1 mm) were added to 1 g of soil and bead beaten on a paint shaker at full speed for 10 minutes. Samples were cooled on ice for 1 hour. Lysozyme (5 mg/ml) was added and samples were incubated at 37°C for 1 hour and then incubated with sodium dodecyl sulfate at final concentration 1% at 65°C for 30 minutes. Samples were centrifuged at 10 minutes at 3,600 x *g* at room temperature to recover supernatant that contained the DNA. The supernatant was extracted with a 1:1 volume of Tris-equilibrated phenol, followed by two extractions with a 1:1 volume of phenol/chloroform/iso-amyl alcohol (25:24:1). A 1/10 volume of 5 M NaCl plus 1 volume of 30% polyethylene glycol were added. Samples were placed on ice for at least 15 minutes and subsequently centrifuged for 20 minutes at 10,000 x g at room temperature. Supernatant was discarded, and samples were vacuum dried and resuspended in 50 µl of sterile nuclease free water and stored at -20°C. Gel electrophoresis was used to verify the DNA extraction process before proceeding with PCR. DNA (10 µl) was stained in a Ficoll®-based loading dye (15% Ficoll® type 400 (purchased from Amersham Biosciences, Buckinghamshire, England), 0.25% bromophenol blue, and 0.25% xylene cyanol FF) and loaded into 1% agarose gels made of 1X TAE (1X TAE is 40 mM Tris, 40 mM acetic acid, and 1 mM EDTA) and electrophoresed. Agarose gels were then stained for at least 10 minutes in an ethidium

bromide stock solution (10 mM Tris-HCl, 1 mM EDTA, 1 mg/ml ethidium bromide) and viewed under UV light.

Classical PCR

Classical PCR was performed as described above in *PCR Detection of Streptomyces spp. and nec1*, except 2 µl of bovine serum albumin (100 ng/µl) was added to correct for possible interference from contaminating substances (e.g., humic substances, fulvic acid, and phenolic compounds) in the DNA samples extracted from the soil) to verify the use of the primers before proceeding with real time PCR.

Real Time PCR

PCR primers to quantify pathogenic *Streptomyces* spp. populations were derived from the *nec1* gene (Table 1-1). The primers used were developed using the Perkin Elmer Applied Biosystems (Foster City, California, USA) primer design software Primer Express® version 1.0 under the parameters required for use with the ABI Prizm® 7700 sequence detection system. The primer sequences for the forward and reverse primers based on *nec1* were N1f 5'- GCCGCTCGGAGAGTACGA –3' and N1r 5' – AAGCGCTGGGCATTGTTG –3' (Table 1-1). Primer and template concentrations for real time PCR using the SYBR® green PCR master mix (purchased from Perkin Elmer Applied Biosystems, Foster City, California, USA) were optimized empirically. Initial primer concentrations of the real time PCR reactions were 300 µM. All PCR reactions were carried out in MicroAmp® Optical 96-well reaction plates and optical caps (purchased from Perkin Elmer Applied Biosystems, Foster City, California, USA) using 1X SYBR® green PCR master mix and sterile nuclease free PCR-grade water (purchased

from Sigma Chemical Company, Saint Louis, Missouri, USA). pBluescript® (purchased from Stratagene, La Jolla, California, USA) was used as a genetic normalizer and to correct for possible interference from PCR-inhibiting substances (e.g., humic substances, fulvic acid, and phenolic compounds) in the DNA samples extracted from the soil in all of the real time PCR reactions as a linearized plasmid (10 µl pBluescript® was digested with 1.5 µl *EcoR V*, purchased from Invitrogen Corporation, Carlsbad, California, USA). Sterile nuclease free water and DNA from *Streptomyces scabies* were used as negative and positive controls, respectively. The real time PCR parameters used via the Perkin Elmer Applied Biosystems Prizm® 7700 sequence detection system were 50°C for 2 minutes for one cycle; 95°C for 10 minutes for one cycle; and 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. All real time PCR reactions were run in repetitions of 4 reactions per DNA sample and all sets of reactions were repeated. Cycle threshold (Ct) values were established based on when real time PCR reactions were in transition between linear and log phases.

II. Assessment of Pathogen Populations in Three Michigan Potato Fields

Soil sampling

Soil was sampled from three Michigan potato production fields in Montcalm county. The three fields varied in the amount of common scab infection that was observed on tubers (cultivar Snowden) at the end of the growing season and were classified by the grower as extremely low infection rate, moderately low infection rate, and severe infection rate (field not harvested due to severe scab infection). Soil was collected in 20 locations per field in a zigzag pattern at a depth of 15 cm with a hand shovel until approximately 700 g of soil was obtained from each sample per field location.

Soil DNA Extraction

DNA was extracted from soil using a BIO 101 FastPrep® bead mill homogenizer (purchased from BIO 101, Carlsbad, California, USA) and the corresponding FastDNA® SPIN kits for soil (purchased from BIO 101, Carlsbad, California, USA). DNA was extracted from 2.5 g of soil per soil sample using the above materials as described by BIO 101 with the FastPrep® bead mill homogenizer at speed 5.5 for 40 seconds, followed by an incubation on ice for 10 minutes, repeated 5 times, with a final incubation on ice for 1 hour. Gel electrophoresis was used to verify the DNA extraction process before proceeding with PCR. DNA (10 µl) was stained in a Ficoll®-based loading dye (15% Ficoll® type 400 (purchased from Amersham Biosciences, Buckinghamshire, England), 0.25% bromophenol blue, and 0.25% xylene cyanol FF) and loaded into 1% agarose gels made of 1X TAE (1X TAE is 40 mM Tris, 40 mM acetic acid, and 1 mM EDTA) and electrophoresed. Agarose gels were then stained for at least 10 minutes in an ethidium bromide stock solution (10 mM Tris-HCl, 1 mM EDTA, 1 mg/ml ethidium bromide) and viewed under UV light.

Classical PCR

Classical PCR was performed to determine which samples detected *necl* before proceeding with real time PCR. Classical PCR was performed as described above in ***PCR Detection of Streptomyces spp. and necl.***

Real Time PCR

PCR primers to quantify pathogenic *Streptomyces* spp. populations were derived from the *necl* gene (Table 1-1). These were the same primers used in the controlled

environment experiment in this chapter (Table 1-1). Primer and template concentrations for real time PCR using the SYBR® green PCR master mix (purchased from Perkin Elmer Applied Biosystems, Foster City, California, USA) were optimized empirically. Initial primer concentrations of the real time PCR reactions were 300 μM. All PCR reactions were carried out in MicroAmp® Optical 96-well reaction plates and optical caps (purchased from Perkin Elmer Applied Biosystems, Foster City, California, USA) using 1X SYBR® green PCR master mix and sterile nuclease free PCR-grade water (purchased from Sigma Chemical Company, Saint Louis, Missouri, USA). Sterile nuclease free water and DNA from *Streptomyces scabies* were used as negative and positive controls, respectively. The real time PCR parameters used via the Applied Biosystems Prizm® 7700 sequence detection system were 50°C for 2 minutes for one cycle; 95°C for 10 minutes for one cycle; and 95°C for 15 seconds and 60°C for 1 minute for 40 cycles. All real time PCR reactions were run in repetitions of 4 reactions per DNA sample and all sets of reactions were repeated. Cycle threshold (Ct) values were established based on when real time PCR reactions were in transition between linear and log phases.

Data Analysis

An analysis of variance (ANOVA) was run to test the effects of county and year for 1) the ratios of *Streptomyces* species obtained and 2) the percentages of *nec1* detected for the evaluation of PCR-based detection of pathogenic *Streptomyces* species in soil populations of Michigan's major potato production counties.

An ANOVA was run to test the effects of soil moisture application on quantity of presumed pathogenic *Streptomyces scabies* populations as determined in the assessment of real time PCR as a method for pathogen quantification in the controlled environment experiment. A t-test with comparison of least significant difference was used to determine the differences in quantities between samples.

An ANOVA was run to determine whether there were differences in amount of scab observed at harvest between three Michigan potato fields in the assessment of real time PCR as a method for pathogen quantification.

For all statistical analyses, an outcome was deemed statistically significant if the probability for a Type I Error was 5% or less (alpha = 0.05). The GLM (general linear model) for ANOVA and LSD (least significant difference) of the means for t-tests were conducted using SAS® version 8 software (SAS Institute, Inc.; Cary, North Carolina).

RESULTS

EVALUATION OF PCR-BASED DETECTION OF PATHOGENIC STREPTOMYCES SPECIES IN SOIL POPULATIONS OF MICHIGAN'S MAJOR POTATO PRODUCTION COUNTIES

In 2000, *Streptomyces* species were isolated from all of the eleven counties sampled (Figure 1-2). Because the isolates of the *Streptomyces scabies* morphology obtained from soil were screened with PCR primers specific for the genus *Streptomyces* until 40 *Streptomyces* spp. were identified per county, the ratios of *Streptomyces* spp. per total isolates screened was highly variable between counties and hence, no trends were found of the total number of actinomycetes isolated. The PCR protocols indicated that a range of 26-36% from counties 2, 3, 8, 9, and 10 were *Streptomyces* spp.; from 46-54% for counties 1, 4, 7, and 11 were *Streptomyces* spp.; and 57% and 75% were *Streptomyces* spp. from counties 5 and 6, respectively (Figure 1-2). In 2000, *Streptomyces* spp. with *necl* were detected in each county sampled. Most of the *Streptomyces* spp. screened with PCR primers for *necl* made up less than 50% of the streptomycete population. The *necl*-containing *Streptomyces* spp. from counties 3, 5, and 8 ranged from 23% to 28% of the total streptomycete isolates analyzed; from 38-48% of the streptomycetes recovered from counties 1, 7, 9, and 10 and streptomycetes isolated from four of the fields were positive for *necl* at or over 50% and was 70% for county 11 (Figure 1-3).

In 2001, *Streptomyces* spp. were again isolated at variable ratios to the total actinomycete isolates recovered. The actinomycete collections were screened until 40 isolates tested positive *Streptomyces* spp. per county, which again led to no obvious trends across the counties tested in relation to the percentages of this genus detected. The actinomycete isolates ranged from 28-34% *Streptomyces* spp. for counties 1, 3, 5, and 12;

from 42-50% streptomycetes for most of the other counties; and greater than 50% streptomycetes for counties 6 and 8 (Figure 1-4). In 2001, *nec1* was detected in isolates collected from all fields. The *nec1* gene was most commonly found in isolates representing less than 50% of the streptomycetes recovered for most of the counties screened (Figure 1-5). Isolates from nearly half of the counties sampled tested positive for *nec1* at frequencies between 30-40% for counties 1, 3, 5, 7 and 10; between 48-55% for counties 4, 6, 11, and 12; and 88% and 75% for counties 8 and 9, respectively, which was significantly higher than the presence of *nec1* in the other counties (Figure 1-5).

In 2002, variable percentages of total streptomycetes (as percentages of actinomycetes) were screened until 20 isolates tested positive for the genus *Streptomyces* per county. *Streptomyces* spp. were most commonly isolated at or less than 50% of the time from the actinomycetes recovered (Figure 1-6). The actinomycete isolates ranged from 34-50% *Streptomyces* spp. for counties 1, 2, 3, 6, 7, and 8; and 57% and 63% were identified by PCR as *Streptomyces* spp for counties 4 and 5 (Figure 1-6). In 2002, *nec1* was detected in *Streptomyces* spp. from all of the counties sampled. Most of the isolates contained *nec1* around or less than 50% of the total screened *Streptomyces* spp. (Figure 1-7).

In 2003, percentages were variable for the isolates screened until 20 *Streptomyces* spp. were obtained per county. *Streptomyces* spp. were isolated from each of the counties. Actinomycete isolates were identified by PCR as 28-38% *Streptomyces* spp. for counties 4, 5, 6, and 7; and 51%, 47%, and 50% *Streptomyces* spp. from counties 1, 2, and 3, respectively (Figure 1-8). The *nec1*gene was detected in *Streptomyces* spp. from each of the counties sampled in 2003. In 2003, *nec1* was detected in 30-50% of the

Streptomyces spp. isolates obtained from counties 3, 4, 5, and 7; and in 65%, 60%, and 70% of the *Streptomyces* spp. from counties 1, 2, and 6, respectively (Figure 1-9). Detection of *nec1* by PCR is shown below (Figures 1-10 to 1-21).

An analysis of variance (ANOVA) determined that for the ratios of *Streptomyces* species obtained that neither county nor year resulted in a significant effect on differences of ratios obtained. An ANOVA determined that there was a significant county effect in relation to *Streptomyces* species in which *nec1* was detected, but that year effect was not significant.

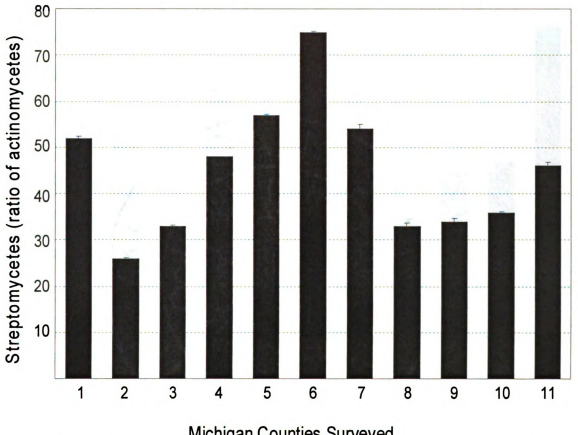

Figure 1-2. Ratios of actinomycetes obtained from 11 Michigan counties identified as *Streptomyces* species by PCR detection in 2000.

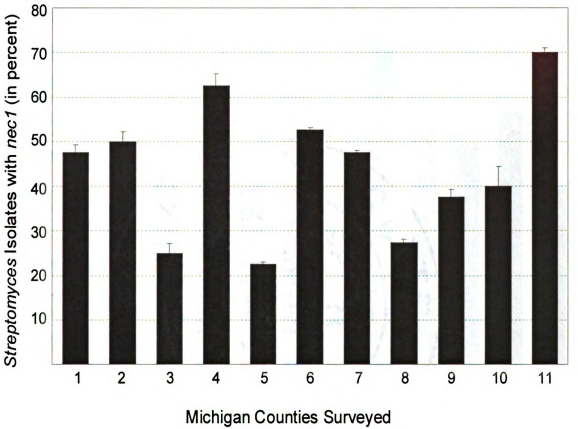

Figure 1-3. Percentages of *Streptomyces* spp. isolates obtained from 11 Michigan counties in which *nec1* was detected by PCR in 2000.

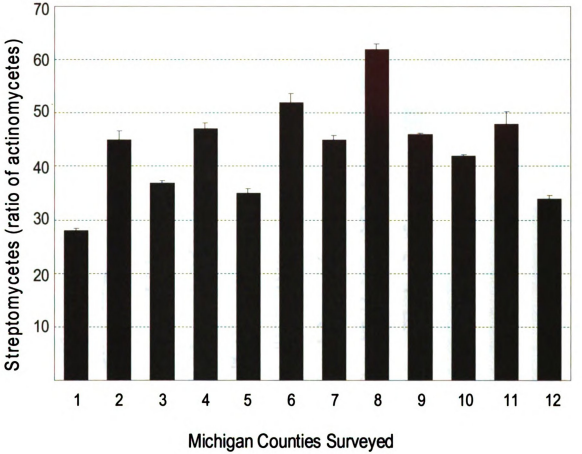

Figure 1-4. Ratios of actinomycetes obtained from 12 Michigan counties identified as *Streptomyces* species by PCR detection in 2001.

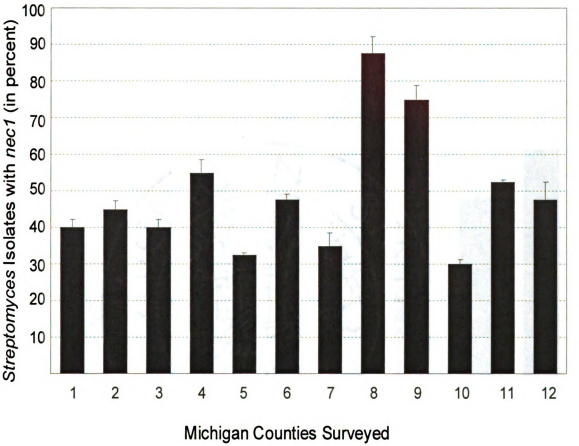

Figure 1-5. Percentages of *Streptomyces* spp. isolates obtained from 12 Michigan counties in which *nec1* was detected by PCR in 2001.

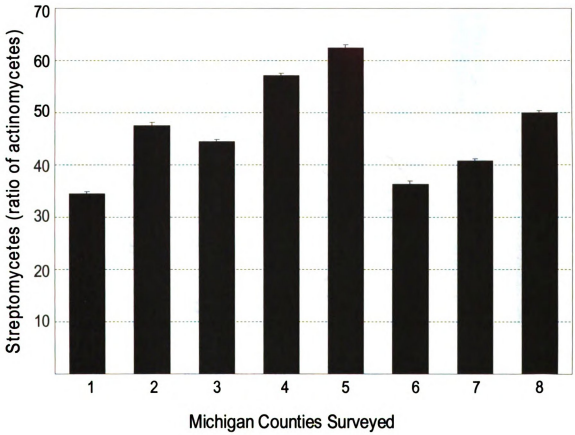

Figure 1-6. Ratios of actinomycetes obtained from 8 Michigan counties identified as *Streptomyces* species by PCR detection in 2002.

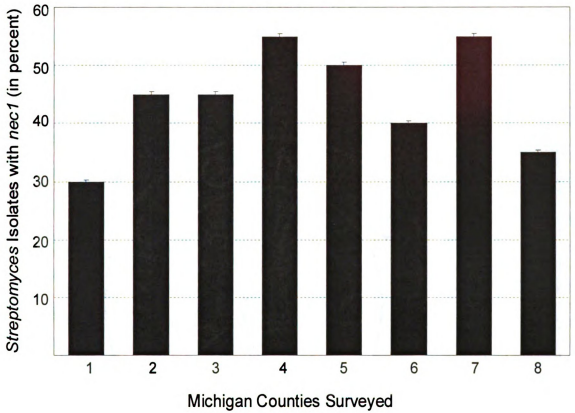

Figure 1-7. Percentages of *Streptomyces* spp. isolates obtained from 8 Michigan counties in which *nec1* was detected by PCR in 2002.

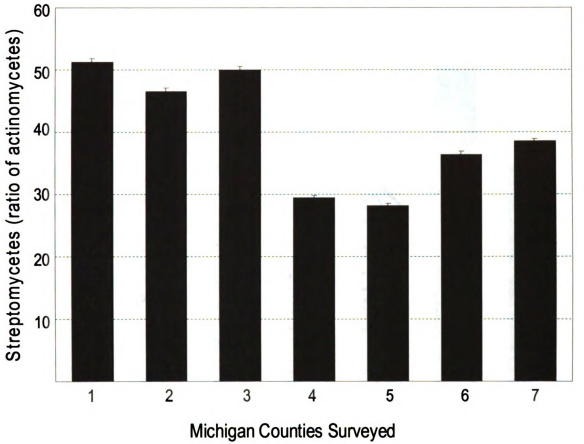

Figure 1-8. Ratios of actinomycetes obtained from 7 Michigan counties identified as *Streptomyces* species by PCR detection in 2003.

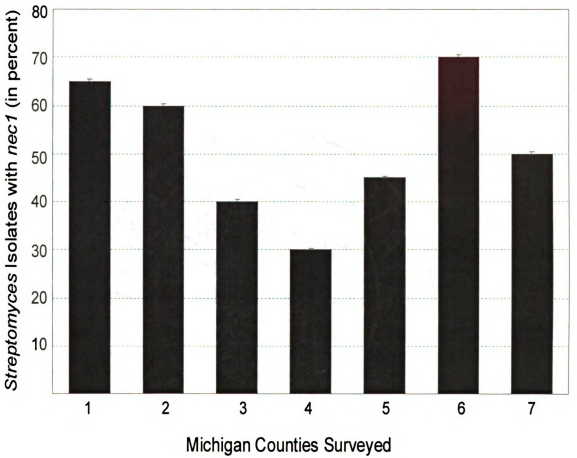

Figure 1-9. Percentages of *Streptomyces* spp. isolates obtained from 7 Michigan counties in which *nec1* was detected by PCR in 2003.

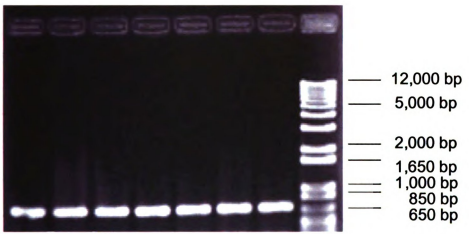

Figure 1-10. Picture of an agarose gel taken under UV light exhibiting seven of the *nec1*-containing *Streptomyces* isolates obtained from county 5 in 2001. The PCR products from *nec1* amplification are 720 bp.

ASSESSMENT OF REAL TIME PCR AS A METHOD FOR PATHOGEN QUANTIFICATION

Controlled Environment

Quantification experiments of DNA extracted directly from soil from the first trial of the continuously applied soil moisture in a controlled environment experiment conducted in Chapter 2 led to significant differences (as determined by t-test) between the amounts of pathogen DNA detected for the irrigation treatments. Real time PCR primers derived from *nec1* sequence detected differences in the mean quantity of DNA obtained from the different irrigation regimes. The two drier soil treatments contained slightly more DNA than the two more moist soil treatments. The detection of *nec1*-containing *S. scabies* averaged $1.5E^{-5}$ pg/ml DNA for the least irrigated moisture treatment (9% volumetric water content) (Figure 1-11). The corresponding mean cycle threshold value for this soil moisture treatment was 26 cycles (Figure 1-11). *Streptomyces scabies* with *nec1* were detected at an average of $1.0\ E^{-5}$ pg/ml DNA for the 14% volumetric water content irrigation treatments (Figure 1-11). This irrigation treatment also had an average cycle threshold value of 26 cycles (Figure 1-11). The mean quantity of pathogen DNA from samples obtained from the 21% volumetric water content was extremely low (near zero) and averaged $2.9\ E^{-7}$ pg/ml (Figure 1-11). The cycle threshold for 21% volumetric water content averaged 29 cycles (Figure 1-11). The samples from the most irrigated treatment (29% volumetric water content) had an average cycle threshold value of 33 cycles and contained a mean quantity of DNA near zero of $2.4\ E^{-7}$ pg/ml of pathogenic *S. scabies* DNA (Figure 1-11).

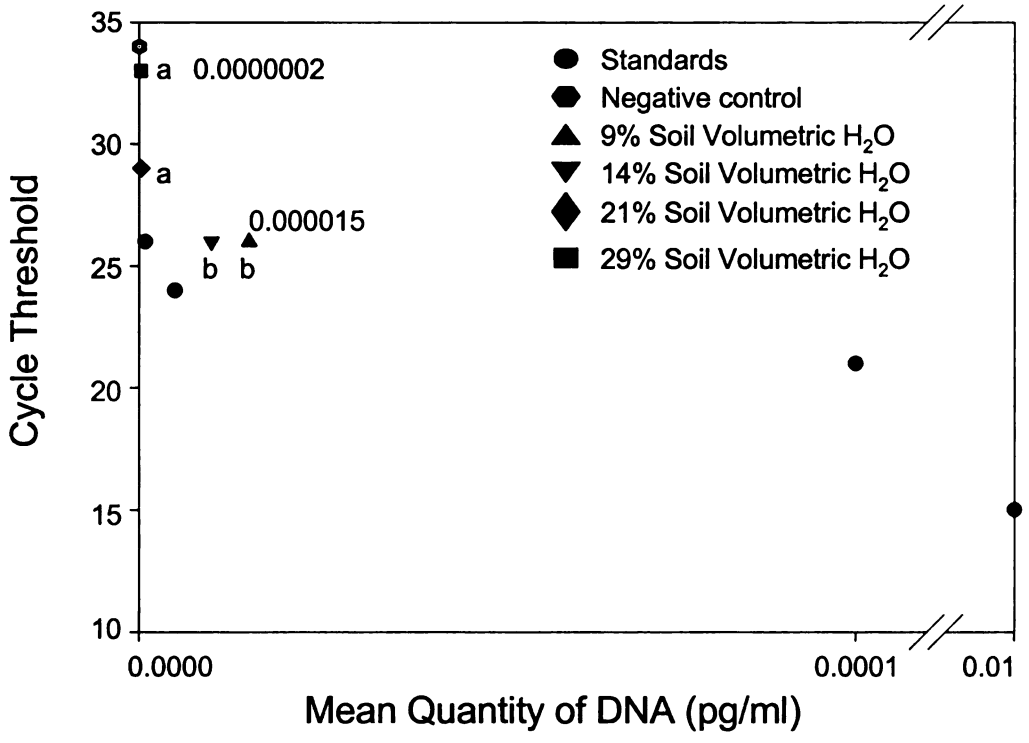

Figure 1-11. Mean quantity of DNA from *nec1*-containing *S. scabies* detected by real time PCR from soil samples collected from a controlled environment experiment.

Three Michigan Potato Fields

Pathogenic *Streptomyces* spp. were detected by classical PCR in all three of the Michigan potato fields tested. There were differences in the twenty samples per field that were screened for presence of pathogenic *Streptomyces* spp. Seven out of 20 (35%) of the samples screened from field one, which had the highest amount of scab infection on tubers, contained pathogenic *Streptomyces* spp. DNA (Figure 1-12). Field two, which had a moderate amount of scab infections at harvest, had 4 of 20 samples (20%) with pathogenic *Streptomyces* spp. DNA (Figure 1-12). Only 2 of 20 samples (10%) from the third field, which had low levels of infection at harvest, contained pathogenic *Streptomyces* spp. (Figure 1-12). The differences in *nec1* detection were not statistically significant between the three fields as determined by a one-way ANOVA. Differences in the quantity of pathogenic *Streptomyces* spp. DNA as determined by real time PCR were detected between the three fields. The relative quantities of pathogenic *Streptomyces* spp. DNA per sample as determined by cycle thresholds generated by real time PCR were somewhat consistent between the 7 samples from field one. Six of the seven samples had average Ct values that ranged from 10.4 to 14.4 cycles (Figure 1-13). One of the samples from field one had a lower average Ct value of 23.6 cycles (Figure 1-13). Two of the samples from field two had Ct values that averaged 12.6 and 12.2, respectively (Figure 1-13), which were similar values to the majority of samples from field one. The other two samples from field two had higher Ct values that averaged 20.1 and 22.2 cycles (Figure 1-13). The samples from the third field, which had the lowest amount of scab infection, had average Ct values of 17.7 and 12.6 cycles, values that were to those detected in the other two fields (Figure 1-13).

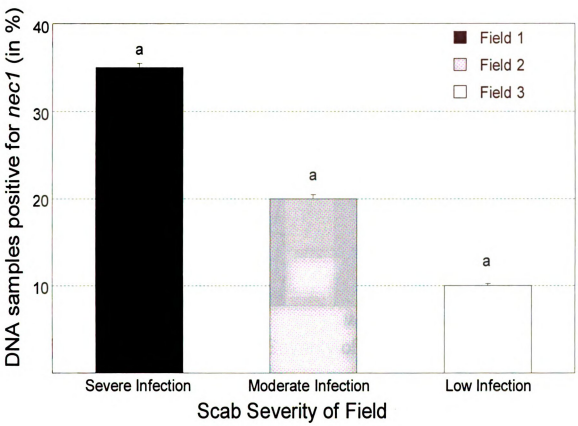

Figure 1-12. Number of samples in which *nec1*-containing *Streptomyces* spp. were detected by classical PCR in pre-screening before real time PCR analysis. Twenty samples per field were screened for *nec1* by classical PCR to determine which samples required quantification using real time PCR. The detection of *nec1* was not statistically significant between the fields as determined by a one-way ANOVA.

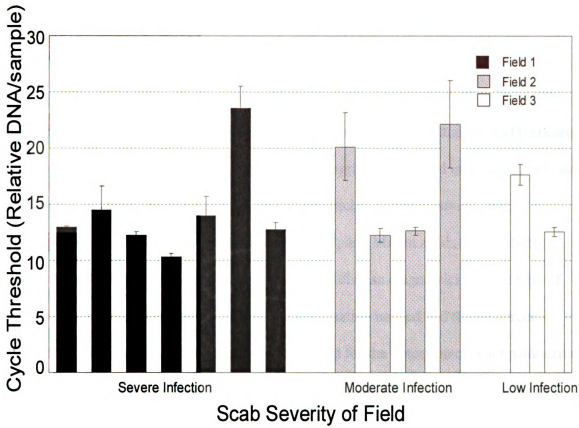

Figure 1-13. Average cycle threshold (Ct) values obtained from the three Michigan potato fields. The average quantification values (Ct values) were obtained by real time PCR using primers that were derived from *nec1* to detect the relative quantities of presumably pathogenic *Streptomyces* species DNA in each of the soil samples between the three fields.

DISCUSSION

Both of the molecular tools discussed in this chapter are helpful and are very important in current plant and animal health diagnostics. The use of PCR and real time PCR technologies in plant disease diagnostics has been extensively studied, and there are numerous reports and reviews supporting its use in agriculture (Ward *et al*, 2005; Schena *et al.*, 2004; Levin, 2004; Gachon *et al.*, 2004; Holzricher and Milanovich, 2003; McCartney *et al.*, 2003; Schaad and Frederick, 2002; and Martin *et al.*, 2000). Both technologies are helping to shape the future of plant disease diagnostics and the type of assay desired depends on requirements of the information needed and the resources available. Until recently, diagnostic procedures used for the detection of plant pathogenic *Streptomyces* species have been laborious and time consuming, often taking weeks to obtain results. The recent development of nucleic acid based detection has allowed for a rapid screening of many plant pathogens in soil and plant material, including the potato scab pathogens *Streptomyces scabies* and *S. turgidiscabies* (Lehtonen *et al.*, 2004). Furthermore, current recommendations in the manual used by many plant disease diagnosticians to identify the causal agents of common scab of potato suggest the use of *nec1* for use in PCR assays (Schaad *et al.*, 2001). *nec1* is highly correlated with plant pathogenicity in the genus *Streptomyces*.

In 2000, *Streptomyces* spp. were isolated at varying percentages of total microorganisms screened between the counties. This suggested that either other related genera with similar morphological characteristics were frequently isolated and comprised the other percentage of organisms isolated or that *Streptomyces* spp. were somewhat

more difficult to isolate from some soils versus others, particularly for the soil from counties with lower percentages of isolated streptomycetes (Figure 1-2). Other related microorganisms with similar morphological characteristics and/or commonly isolated soil actinomycetes that probably comprised the other percentages of microorganisms isolated include the following genera: *Nocardioidies*, *Streptoverticillium*, *Microbiospora, Micromonospora*, *Chainia*, *Streptosporangium* and *Saccharopolyspora*. The detection of *nec1* in 2001 was variable between the counties sampled, suggesting that although it was present in each of the counties tested, the amount of pathogens relative to *Streptomyces* spp. isolated were not consistent. Also, it is possible that for some of the counties in which high percentages of the *Streptomyces* isolates contained *nec1*, such as counties 4 and 11, that the same isolate may have possibly been predominant in that particular soil sample, could have grown on organic debris in the soil sample, may have been isolated repeatedly and does not necessarily indicate that the proportion of the scab pathogen in a sample is actually that high and prevalent in actual soil populations. This is likely since most *Streptomyces* species are non-pathogens that exist as saprophytes and because there are more than 530 species of *Streptomyces* currently described (Garrity *et al.*, 2004).

In 2001, *Streptomyces* spp. were isolated from each county, but no specific correlations were found between the percentages of this genera of bacteria and other genera with similar morphological characteristics (Figure 1-4). Similar to 2000, *Streptomyces* spp. were isolated from soil samples from most of the counties at approximately 50% of the total microorganisms isolated. Again, this suggests that other genera of soil microbes have similar morphological characteristics to *Streptomyces* were frequently isolated and that diagnostic methods for scab detection should not be based on

morphological observations of isolates alone. The percentage of these isolates varied in their levels of detection of *nec1*, but less than 50% of the population with *nec1* was the trend, which was consistent with the results of *nec1* screening of *Streptomyces* spp. in 2001 (Figure 1-3 and 1-5). Again, the higher percentages of *Streptomyces* spp. with *nec1* in certain counties suggests that multiple isolations of the same isolate may have occurred and that some isolates may have grown in the laboratory during the soil drying process and/or the isolation process.

In 2002, no consistencies were found across counties in relation to the percentage of *Streptomyces* spp. isolated in comparison to other groups of microorganisms (Figure 1-6). Again, this illustrates the fact that soil is very heterogeneous and that isolation of *Streptomyces* spp. based on morphology is not necessarily a good indicator that the organism is a streptomycete since there are related Actinobacteria with morphological characteristics that can not be easily distinguished from *Streptomyces*. The results underscore the importance of screening DNA from the organisms by PCR or an alternative nucleic acid-based detection approach (Figure 1-6). Similar to the results from the previous two years, in 2002, *nec1* detection was highly variable between the locations, farms, and soil types from the different counties. The percentages of *nec1* in the populations assessed were similar to those found in 2000 and 2001. Because some of the percentages of *Streptomyces* spp. with *nec1* were somewhat high at 50% and 55% for counties 5, and 4 and 7, respectively, it is possible that multiple isolations of the same isolate may have occurred, as was previously mentioned (Figure 1-7). Alternatively, some growth of microorganisms may have occurred in the laboratory in the soil debris

from the time between the samples were allowed to air dry and between subsequent incubation on media.

The isolation of *Streptomyces* spp. in 2003 was consistent with the other three years. Again, no trend was found in relation to the frequencies in which *Streptomyces* spp. were isolated. It is likely that *Streptomyces* spp. populations vary highly between soil samples and because only one sample was obtained from each field, results may be skewed. Similar to the previous three years, the detection of *necl* in relation to the percent of *Streptomyces* spp. containing the gene was highly variable across the counties. The percentages of *Streptomyces* spp. with detection of *necl* were relatively high for counties 1, 2, and 6, which suggests that, as noted with counties sampled in other years with high percentages of *Streptomyces* spp. containing *necl*, that growth of certain microbes may have occurred in the laboratory during sample processing periods or that multiple isolations of an individual isolate may have occurred.

Real time PCR was a useful tool in determining DNA quantities of pathogenic *Streptomyces* spp. and *Streptomyces scabies* in soil samples in which potatoes were grown in a controlled environment and actual production fields (Figures 1-11 and 1-13). Real time PCR detection based on primers derived from *necl* found similar quantities of DNA in samples across irrigation treatments. The procedure produced consistent and precise quantifications in all of the *S. scabies* inoculated samples collected from the controlled environment experiment. Furthermore, the assay was able to detect differences between the pathogenic *S. scabies* populations found in the drier versus the more moist soil irrigation treatments. The slight differences in pathogenic DNA detected indicate that addition of soil moisture may have slightly inhibited the growth of *S.*

scabies. Since the pathogen favors drier soils, it is a possibility that growth of *S. scabies* was slightly higher in the drier soils. This finding does not exclude, however, the likelihood that the nature of control of potato common scab via irrigation is solely by growth reduction of the pathogen since scab infections quickly recur when irrigation treatments are halted and because biocontrol of scab, as well as scab decline in soils cropped to potato for many years, are well-documented phenomena.

It is likely that classical PCR may be a better detection and risk assessment tool that real time PCR for scab quantification and detection. This assessment is based on the results where real time PCR was used to detect pathogenic *Streptomyces* spp. populations in three Michigan fields. Classical PCR of DNA extracted directly from soil indicated that the three fields had varying percentages of pathogenic *Streptomyces* spp. present per the 20 samples tested per field (Figure 1-12). Furthermore, there was a correlation between the amount of infection that occurred in the field at harvest and the percent at which pathogenic *Streptomyces* spp. were detected by using PCR primers for the *nec1* gene. As the severity of infection per field increased, the number of samples with *nec1* detection increased, showing a positive correlation between scab incidence and risk for a subsequent growing season for a potato crop or other susceptible host. Also, these findings have implications for cultural practices, such as rotation crops, cover crops, tillage, etc. Conventional PCR using primers for *nec1* was a good indicator of risk for the fields as well as the amount of scab infection to confirm the amount of scab infection observed at harvest. Quantitative real time PCR was able to detect differences in the amount of pathogenic *Streptomyces* spp. DNA found in each sample per field; however, no correlations were found between the amount of DNA from the pathogens per sample

and the amount of risk in the field per se. In other words, similar amounts of pathogen DNA were found between fields 1, 2, and 3, which indicates that risk may not be different. Also, because the amount of DNA varied in each sample within a county, it is difficult to know what quantity of pathogenic *Streptomyces* spp. is "too high" or "risky" and hence to be at unsuitable levels for the grower to crop a field to potato. In addition, some samples in a given field had low amounts of DNA and others high, again, making useful correlations between pathogenic *Streptomyces* spp. DNA and risk assessment very difficult, if not impossible. Furthermore, quantitative real time PCR is a time consuming and very expensive process that requires special equipment with limited availability. The use of quantitative real time PCR as a diagnostic tool is unlikely to be a favorable plant disease diagnostic method for growers because of the expense incurred to process a single sample and because classical PCR, which is a less expensive procedure than real time PCR and uses machinery that is much more available, is more likely to be an affordable tool for diagnosticians to use for identification of diseases on plants and in soil samples obtained by growers. Also, it is not the method of choice because only a small portion of a sample can be analyzed in efforts to keep the costs down. As a result, the pathogen may not be detected in a soil sample because such a small amount of the soil sample is processed. In contrast to this expense, classical PCR is much more affordable and requires machinery that is much more available, which allows for more extensive sampling within a field and allows a greater sample size and number of subsamples to be processed. Classical PCR allows more extensive profiling of plant pathogens in fields or in plant samples, while quantitative real time PCR gives precise answers to the amount of pathogen present in a sample. This is potentially very useful for determining

pathogenicity of populations of *Streptomyces* prior to cultivation of potatoes, especially because soilborne inoculum is the primary source of infection within a field and the spores are resistant to desiccation. Also, it is important because pathogenic streptomycetes are found in soils that have not previously been cropped to potato (Loria, 2001 and Loria *et al.*, 1997). Although the importance of seed-borne inoculum must not be overlooked because it is provides the introduction of pathogenic *Streptomyces* strains to new fields where they were previously not present. Furthermore, seed-borne inoculum is the primary means of dispersal of different species and strains of plant pathogenic *Streptomyces* between continents.

Chapter II

ASSESSMENT OF CULTIVAR RESISTANCE AND SOIL MOISTURE APPLICATION AS A MEANS TO MANAGE POTATO COMMON SCAB

INTRODUCTION

Sanford (1923) pioneered research on the use of irrigation to control potato common scab. Soil moisture, when applied at or near field capacity, significantly decreased the severity of scab infection. Subsequently, many studies were conducted to evaluate irrigation as a way to reduce scab (Adams *et al.*, 1987; Curwen, 1980; Davis, *et al.*, 1974; Lapwood *et al.*, 1970; Lapwood *et al.*; 1971; Lapwood and Adams, 1973; Lapwood, 1973; and Wilson *et al.*, 2001). Irrigation applied during the early stages of tuberization significantly decreased scab incidence (Lapwood *et al.*, 1970). At high water potentials, actinomycete populations on the lenticels were low and non-actinomycete bacterial populations were high, suggesting that irrigation might decrease the population of pathogenic *Streptomyces* species in tuber lenticels by increasing populations of scab-antagonists.

The amount of irrigation that effectively controls scab, the appropriate timing of irrigation, and the reduction in scab infection vary between research reports. Some have reported that the incidence of potato scab increased with soil moisture (Starr *et al.*, 1943) or that precipitation did not reduce scab infection on tubers (Goto, 1985). However, this may be due to the soil type and cultivar used, since dry soils have been shown to increase incidence and severity of disease or this could be due to rapid evaporation of precipitation. Lack of scab reduction by precipitation may be due to the water repellent

nature of sand; a phenomenon extensively studied by soil scientists (Cann, 2000; Dekker *et al.*, 2001; McKissock *et al.*, 2002; Wang *et al.*, 2000; and Yang *et al.*, 1996). The water repelling is also exacerbated by high temperatures (Dekker *et al.*, 2001 and Yang *et al.*, 1996). The Cann (2000), McKissock *et al.* (2002), and Wang *et al.* (2000) studies suggest the use of high-ponding irrigation methods and clay spreading to mediate the hydrophobic sands. Nearly all studies that used irrigation to control scab reported a decrease in scab severity in soils with high moisture contents.

Irrigation for four weeks after tuber initiation at a 15 mm soil moisture deficit was found to decrease scab most effectively in one study (Lapwood, 1973). According to Lewis (1970), five weeks of irrigation at or near field capacity following tuber initiation was found to substantially reduce scab, while two weeks of irrigation after initiation was not sufficient to manage scab. Although there is variation in relation to timing of soil moisture application, irrigation that provides adequate soil moisture at or near field capacity during tuberization is one of the most important methods of managing this disease without significantly reducing yield. However, Davis *et al.* (1974) emphasized the importance of combining irrigation with other methods to control common scab since 90% available soil moisture during the early stages of tuber development was not sufficient alone to produce tubers of adequate quality for market specifications. Although reported results are variable, maintenance of sufficient soil moisture is particularly important because there are no resistant cultivars completely immune to the disease and there are very few methods available to manage this disease. The objectives of this chapter are to 1) evaluate the combined effects of cultivar resistance and irrigation on scab; and 2) determine effects of soil- wetting and drying cycles on scab infection.

MATERIALS AND METHODS

Effects of Cultivar Resistance and Soil Moisture Application on Scab Infection

I. Controlled Environment Trials

Three cultivars that vary in scab susceptibility were used. Atlantic was selected for evaluation because it is scab-susceptible and is widely used in potato production as a chip processing cultivar (Webb *et al.*, 1978). Also selected for evaluation were Boulder, a cultivar with intermediate scab resistance (Douches *et al.*, 2003), and Liberator, a cultivar with strong resistance to scab (Douches *et al.*, 2001). The latter two cultivars were developed by the Michigan State University Potato Breeding and Genetics Program.

In a greenhouse, compartmentalized wooden boxes (30.5 cm x 60.9 cm x 91.4 cm) made of wood were constructed. The boxes were lined with plastic to ensure each soil moisture treatment was contained within the box to avoid leakage and contamination. Each box was completely filled with sterilized sand. CS 615® water content reflectometers (Campbell Scientific, Incorporated, Logan, Utah, USA) were calibrated according to the manufacturer's guidelines before use and then were placed in a vertical orientation at 15 cm soil depth. The water content reflectometers, which recorded volumetric water contents of each of the sandy soil moisture treatments, were connected to a CR 10X® data logger (Campbell Scientific, Incorporated, Logan, Utah, USA) (Figure 2-1). Soil moisture data were downloaded with a laptop computer every other week.

Figure 2-1. Compartmentalized boxes lined with plastic sheeting. Each box contains individual water content reflectometers and irrigation outlets.

Whole potatoes from each cultivar were planted into the compartmentalized boxes (3 replications per cultivar per box) and were subjected to four soil moisture treatments as a completely randomized design. The soil moisture applications were made daily with a Mini-8-G watering relay (Batrow, Incorporated, Short Beach, Connecticut, USA) at the following volumetric water contents: 9% (above permanent plant wilting point), 14% (less than field capacity), 21% (near field capacity), and 29% (near saturation). These volumetric water content values were recorded and determined with CS 615® water content reflectometers.

The sand around the base of each plant was evenly inoculated 3 days after planting with a spore suspension from *Streptomyces scabies* strain DP (scraped from cultures grown on PDA at 30°C for 14 days in the dark) at 2.3 x 10^9 spores per plant. Non-inoculated boxes were used as the negative controls.

The moisture applications to the sand began 14 days after planting (when greater than 95% of the plants had emerged) and were applied through the duration of the experiment. The sand was fertilized, based on recommendations of the soil tests, with 168 kg/ha nitrogen, 117 kg/ha phosphorus, and 308 kg/ha potassium 30 days after planting. Inflorescences were clipped from the peduncles of the plants as they appeared to promote maximum tuber development. Both independent trials of this experiment were implemented in the same boxes (to maintain separation of inoculation and control treatments) and were carried out for at least 6 weeks of irrigation treatments because early tuber development (the beginning 6-8 weeks after planting) is the most critical period of time in which potatoes are susceptible to common scab.

Disease Assessment

Tubers were harvested manually and washed carefully to remove soil and debris so that scab incidence, severity, and lesion type could be assessed. Scab infections were determined by a key developed by James (1971) (Figure 2-2). This scale determines coverage of scab on the surface of the potato as a percentage over the entire tuber surface area.

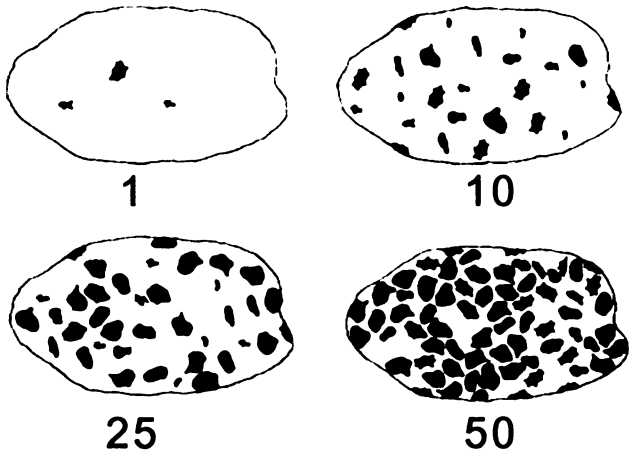

Figure 2-2. Picture of a modified disease assessment key based on the one developed by James (1971). The numbers represent the percent coverage of scab lesions on the tuber surface. This key was used for disease assessment in all experiments that required scab evaluation in this dissertation.

II. 2002 Field Trial

Potato tubers free of scab (Grade A) were manually cut into seed pieces and allowed to heal 4 days before planting. The tubers (cultivars Atlantic, Boulder, and Liberator) were planted by hand at the Michigan State University Plant Pathology Research Station, East Lansing, Michigan, USA in a Riddles-Hillsdale sandy loam on 21 May 2002. Plot dimensions were one-row by 37 m and were replicated 3 times. The plots were covered with black plastic to exclude additional rainfall. A split block design was used. Drip-tape irrigation was placed onto plots and CS 615® water content reflectometers were buried vertically under at least 15 cm of soil and were completely covered with the black plastic sheeting at planting. All rows were irrigated until emergence and were inoculated at 0.5 l per 37 m row with a suspension of *Streptomyces scabies* strain DP at 10^6 CFU/ml on 30 May 2002 and inoculation was repeated on 5 June 2002. Two inoculations were applied because the field had no history of scab and had not previously been cropped to potatoes. Non-inoculated plots were retained as negative controls. Irrigation treatments began 14 days after planting and irrigation schedules were optimized to closely correspond to the greenhouse experiment irrigation treatments:

volumetric water contents of 10% (slightly above permanent plant wilting point), 16% (less than field capacity), 21% (near field capacity), and 27% (near saturation). Soil volumetric water content was measured as described above for controlled environment experiments. Fertilizer (formulated according to results of soil tests) was drilled into plots before planting. Additional nitrogen (final N 25 kg/ha) was applied to the growing crop through the irrigation 42 days after planting. Weeds were controlled by manual weeding, hilling, and with herbicides listed in Table 2-1.

TABLE 2-1. Weed and insect management program used in the 2002 field trial.

Pesticide	Registrant	Application Rate	Application Date (days after planting)
Herbicide			
Dual 8E	Syngenta Crop Protection, Inc.[a]	2.3 l/ha	10 DAP
Basagran	BASF Wyandotte Corporation[b]	2.3 l/ha	20 and 40 DAP
Poast	BASF Wyandotte Corporation[b]	1.8 l/ha	51 DAP
Insecticide			
Admire 2F	Bayer Crop Science, L.P.[c]	1.5 l/ha	at planting
Sevin 80S	Bayer Crop Science, L.P.[c]	1.4 kg/ha	27 and 51 DAP
Thiodan 3EC	Southern Agricultural Insecticides[d]	2.7 l/ha	51 and 76 DAP
Pounce 3.2EC	Bayer Crop Science, L.P.[c]	0.6 l/ha	40 DAP

[a]Greensboro, North Carolina, USA
[b]Florham Park, New Jersey, USA
[c]Research Triangle Park, North Carolina, USA
[d]Palmetto, Florida, USA

Insects were controlled as described in Table 2-1. Bravo® (Syngenta Crop Protection, Incorporated, Greensboro, North Carolina, USA) at 1.8 l/ha was applied for management of fungal diseases weekly from 4 June to 7 September with an ATV rear-mounted R&D spray boom delivering 2.7 l/ha and using three XR11003VS nozzles per row. Vines were killed with diquat desiccant Reglone® 2EC (Syngenta Crop Protection, Greensboro, North Carolina, USA) at 46 l/ha on 26 August. Plots were mechanically

harvested on 10 September. Tubers were washed to remove soil and debris and individual treatments were evaluated for scab incidence and severity (Figure 2-2).

Effects of Drying of Soil During Early Tuber Development on Scab Infection

I. Controlled Environment Trials

Atlantic was selected for evaluation because of its susceptibility to common scab. Whole potato tubers (Grade A) were planted in a greenhouse into the compartmentalized boxes used in the previous controlled environment trials as described above in replications of 3 tubers/box. The plots were replicated twice. Soil moisture data were collected as described above. The plants were subjected to four soil moisture treatments as a completely randomized design. The soil moisture applications were made daily with a Mini-8-G watering relay (Batrow, Incorporated, Short Beach, Connecticut, USA) at the following four volumetric water contents: continuously applied 9% (above permanent plant wilting point) as an irrigation positive control, two reversing schedules of weekly alternation of 14% (less than field capacity) as a drier soil condition treatment with a weekly alternation of 21% (near field capacity) as a moist soil condition treatment, and continuously applied 29% (near saturation) as an irrigation negative control. These volumetric water content values were recorded and determined by the CS 615® water content reflectometers. The boxes that were previously infested with *Streptomyces scabies* strain DP (as described above) were implemented as the inoculation treatments. The non-inoculated boxes were used as negative controls. The soil moisture applications began 14 days after planting (when greater than 95% of the plants had emerged) and were

applied through the duration of the experiment. The soil was fertilized, based on the recommendations of the soil tests, with 168 kg/ha nitrogen, 117 kg/ha phosphorus, and 308 kg/ha potassium 30 days after planting. Inflorescences were clipped from the peduncles of the plants as they appeared to promote maximum tuber development. The second execution of this experiment was implemented in the same boxes (to maintain separation of inoculation and control treatments) and was carried out for 6 weeks of irrigation treatments. Early tuber development occurs during this time and this is when potatoes are most susceptible to common scab. Tubers were harvested manually and washed carefully to remove soil and debris so that scab incidence, severity, and lesion type could be assessed. Scab infections were determined based on the method of James (1971) (Figure 2-2).

II. 2003 Field Trial

Potato tubers free of scab were manually cut and allowed to heal 4 days before planting. Potato tubers (cultivar Atlantic) were planted by hand at the Michigan State University Plant Pathology Research Station, East Lansing, Michigan, USA in a Riddles-Hillsdale sandy loam on 27 May, 2003. Plot dimension were one-row by 37 m and were replicated 6 times and covered in black plastic to exclude additional rainfall. A split block design was used. Drip-tape irrigation was placed onto plots and CS 615® water content reflectometers were buried vertically under at least 15 cm of soil and were completely covered with the black plastic sheeting at planting. All rows were irrigated until emergence. On 28 May, 2003 and 1 July, 2003, the rows were inoculated with a suspension of *Streptomyces scabies* isolate DP (10^6 CFU/ml) at a rate of 0.5 l of inoculum per 37 m row. Two soil inoculations were applied because the field had only

previously been cropped to potatoes once and the potatoes had relatively low infection rates. Non-inoculated plots were retained as the negative control. Irrigation treatments began 14 days after planting and irrigation schedules were optimized to closely correspond to the greenhouse experiment irrigation treatments: volumetric water contents of continuously applied 10% (slightly above permanent plant wilting point) as a positive irrigation control, two alternating schedules of weekly alternation of 14% (less than field capacity) as a drier soil condition treatment with a weekly alternation of 21% (near field capacity) as a moist soil condition treatment, and continuously applied 26% (near saturation) as a negative irrigation control. Soil volumetric water content was measured as described above. Fertilizer (formulated according to results of soil tests) was drilled into plots before planting. Additional nitrogen (urea, final N 25 kg/ha) was applied to the growing crop through the irrigation 39 days after planting. Weeds were controlled by manual weeding, hilling, and with herbicides listed in Table 2-2. Insects were controlled as described in Table 2-2. For late blight control, Bravo® (Syngenta Crop Protection, Incorporated, Greensboro, North Carolina, USA) at 1.8 l/ha was applied weekly from 16 June 2003 to 18 August 2003 with an ATV rear-mounted R&D spray boom delivering 2.7 l/ha and using three XR11003VS nozzles per row. Vines were killed with diquat desiccant Reglone® 2EC (Syngenta Crop Protection, Greensboro, North Carolina, USA) at 46 l/ha on 25 August. Plots were mechanically harvested on 10 September. Potatoes were carefully washed to remove soil and debris for evaluation of the effects of the treatments on scab incidence and severity using the key by James (1971) (Figure 2-2). To evaluate the potential effects of irrigation on yield, potatoes from each harvested plot were placed onto a Tew Fruit and Vegetable processing machine with sizers (Tew

Manufacturing Corporation, Penfield, New York, USA) and weighed. US #1 potatoes were weighed separately as grade A and oversize, and all others smaller than US#1 were weighed and classified grade B.

TABLE 2-2. Weed and insect management program used in the 2003 field trial.

Pesticide	Registrant	Application Rate	Application Date (days after planting)
Herbicide			
Dual 8E	Syngenta Crop Protection, Inc.[a]	2.3 l/ha	10 DAP
Basagran	BASF Wyandotte Corporation[b]	2.3 l/ha	20 and 40 DAP
Poast	BASF Wyandotte Corporation[b]	1.8 l/ha	52 DAP
Insecticide			
Admire 2F	Bayer Crop Science, L.P.[c]	1.5 l/ha	at planting
Sevin 80S	Bayer Crop Science, L.P.[c]	1.4 kg/ha	29 and 45 DAP
Thiodan 3EC	Southern Agricultural Insecticides[d]	2.7 l/ha	52 and 72 DAP
Pounce 3.2EC	Bayer Crop Science, L.P.[c]	0.6 l/ha	40 DAP

[a]Greensboro, North Carolina, USA
[b]Florham Park, New Jersey, USA
[c]Research Triangle Park, North Carolina, USA
[d]Palmetto, Florida, USA

Data Analysis

A two-way analysis of variance (ANOVA) was run to test the effects of trial, treatment, and the interaction of trial and treatment for the evaluation of effects of cultivar resistance and soil moisture application on scab infection in the controlled environment trials. An ANOVA was run to test the treatment effects on the average scab severity observed in the field trial. A t-test with comparison of least significant difference was conducted to determine the differences in scab between treatments.

An ANOVA was run to determine whether there were differences in amount of scab observed in the controlled environment trials conducted to evaluate the effects of

drying of soil during early tuber development on scab infection. A t-test with comparison of least significant difference was conducted to determine the differences in scab between treatments for the controlled environment trials and the field trial. Yield data from the field trial were analyzed with a t-test with comparison of least significant difference to determine differences between treatments within each yield category.

For all statistical analyses, an outcome was deemed statistically significant if the probability for a Type I Error was 5% or less (alpha = 0.05). The GLM (general linear model) for ANOVA and two-way ANOVA and LSD (least significant difference) of the means for t-tests were conducted using SAS® version 8 software (SAS Institute, Inc.; Cary, North Carolina).

RESULTS

Effects of Cultivar Resistance and Soil Moisture Application on Scab Infection

Controlled Environment Trials

In trial one in the controlled environment, the cultivar Atlantic produced tubers with an average scab severity of 6-10% surface coverage when the soil moisture was maintained at 9% volumetric water content (Figure 2-3). When the volumetric water content was increased by 5%, a slight reduction in scab severity occurred; however, soil moisture application at 21% volumetric water content greatly reduced the scab severity to less than an average of 0-5% (Figure 2-3). The infections were eliminated with the most wet soil application treatment at 29% volumetric water content (Figure 2-3). The second trial in the controlled environment with Atlantic produced similar results. The scab severity increased to nearly an average of 11-25% scab coverage when soil moisture was maintained at the dry 9% volumetric water content (Figure 2-3). The amount of infections was significantly reduced with the 5% increase in soil volumetric water content (Figure 2-3). Soil moisture applied at 21% volumetric water content, like trial one, restored tubers to the marketable table stock quality with a scab severity that averaged less than 0-5% surface cover. Similar to trial one, infections did not occur with application of soil moisture at 29% volumetric water content.

The cultivar with an intermediate level of scab resistance, Boulder, had very low amounts of infection in trial one in the controlled environment (all average scab severity values were low enough for table grade tubers). When soil moisture was applied at 9% volumetric water content, the average scab severity averaged less than 0-5% (Figure 2-4).

When soil moisture was increased by 5%, the infections were nearly eliminated (Figure 2-4). No infections occurred with 21 and 29% volumetric water content (Figure 2-4) in either trial. In trial two, soil moisture maintained at 9% volumetric water content produced tubers with an average scab severity of slightly greater than 0-5% (Figure 2-4). Average scab severity decreased with the 14% soil moisture application to levels acceptable for marketable table stock (Figure 2-4). Trial one and trial two in the controlled environment with the resistant cultivar, Liberator, produced tubers with no scab infections at all soil moisture applications (Figure 2-5). All controls were free of scab infection. A two-way ANOVA determined trial, treatment, and the interaction between trial and treatment to be significant at the alpha = 0.05 level.

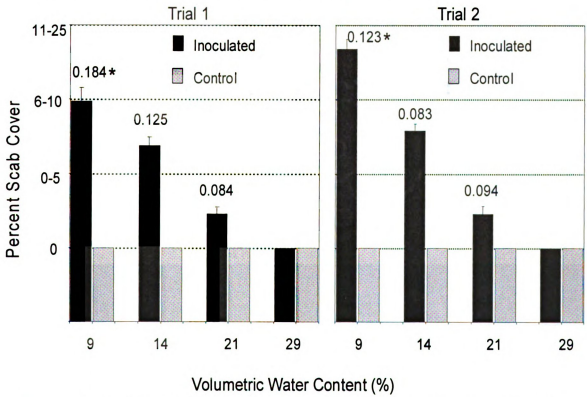

Figure 2-3. Effects of four soil moisture application regimes and a scab-susceptible cultivar, Atlantic, on average scab severity of harvested tubers in two trials conducted in a controlled environment. Asterisk denotes a statistically significant difference as determined by an ANOVA at alpha=0.05.

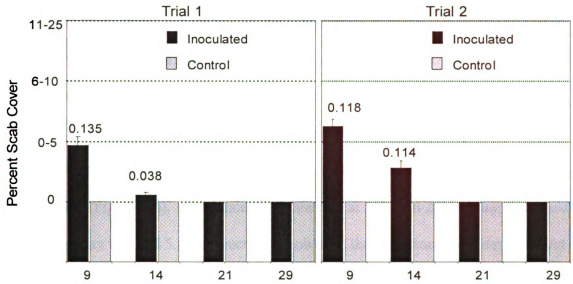

Figure 2-4. Effects of four soil moisture application regimes and an intermediately scab-resistant cultivar, Boulder, on average scab severity of harvested tubers in two independent trials conducted in a controlled environment.

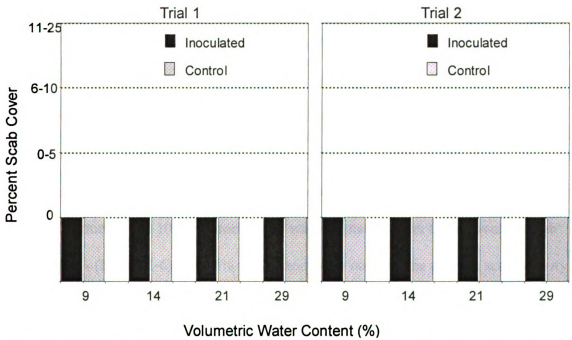

Figure 2-5. Effects of four soil moisture application regimes and a scab-resistant cultivar, Liberator, on average scab severity of harvested tubers in two independent trials conducted in a controlled environment.

2002 Field Trial

The results from the field trial showed similar trends to the results obtained from the controlled environment trials. Atlantic, the scab-susceptible cultivar, produced tubers with an average scab severity of 15% when soil moisture was maintained at 10% volumetric water content (Figure 2-6). The average scab severity was significantly reduced to approximately 9% when the soil moisture was increased to 16% volumetric water content (Figure 2-6). Maintaining soil moisture at 21% and 27% further decreased the scab severity to 6 and 5.5% respectively; however, the reductions were not sufficient for the tubers to be considered market quality for tablestock (Figure 2-6). Boulder, the intermediately scab-resistant cultivar, had very low levels of scab infection at all soil moisture treatments and tubers were of market quality for table stock at each treatment. The average scab severity was 4% when soil moisture was maintained at 10% volumetric soil water content (Figure 2-6). An increase in soil moisture application to 16% soil water content decreased the average scab severity to 2% (Figure 2-6). At 21 and 27% volumetric soil water contents, scab infections were nearly eliminated, at 1 and less than 1%, respectively (Figure 2-6). Liberator, the scab-resistant cultivar, like the intermediately scab-resistant cultivar, had very low levels of scab infection at all soil moisture treatments. At 10% volumetric soil water content, tubers had an average scab severity of 1% (Figure 2-6). At 16% volumetric water content, the average scab severity slightly decreased; however, this difference was insignificant (Figure 2-6). The greater soil moisture applications of 21 and 27% volumetric water contents produced tubers that were statistically identical to those from the two lower soil moisture applications (Figure

2-6). The non-inoculated controls for all cultivars tested were not infected with scab (Figure 2-6).

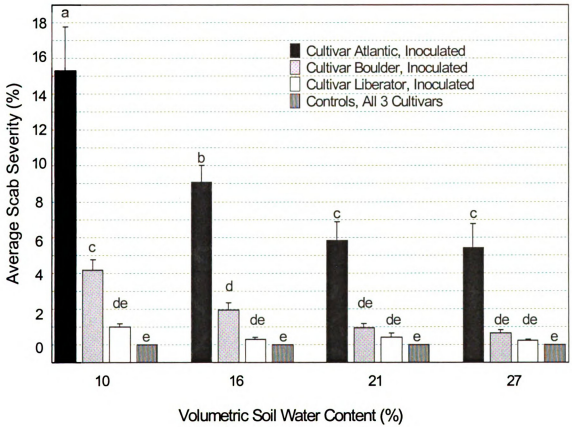

Figure 2-6. Evaluation of the effects of four soil moisture application regimes and three cultivars with varying levels of resistance to common scab on average scab severity of harvested tubers in a field trial.

Effects of Drying of Soil During Early Tuber Development on Scab Infection

Controlled Environment Trials

There was no significant difference between the two trials conducted in the controlled environment according to an ANOVA conducted at alpha = 0.05; therefore, the results from the trials were combined. The scab-susceptible cultivar, Atlantic, produced tubers with an average scab severity of 12.5% for the two trials when the soil moisture was 9% volumetric water content (Figure 2-7). When soil moisture application was applied weekly at 14% volumetric water content, alternating with a weekly 21% volumetric water content regime, the average scab severity was significantly reduced to 6.1% (Figure 2-7). When soil moisture was applied at the reverse of these two regimes (21% alternating 14% volumetric water content), the average scab severity was 8.3%, slightly higher than the previous irrigation regime (Figure 2-7). Soil moisture application at 29% volumetric water content reduced the average scab severity to less than 1% (Figure 2-7). All controls were scab-free.

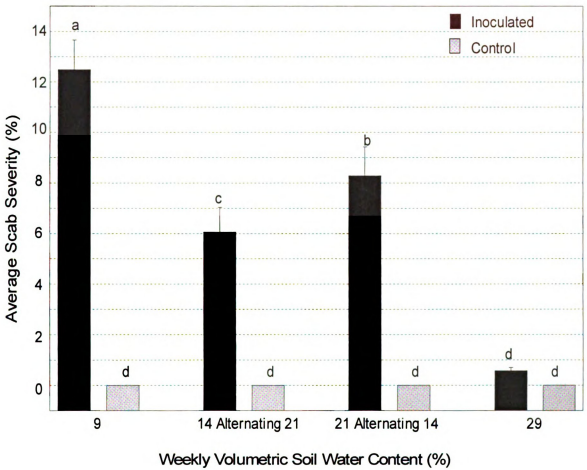

Figure 2-7. Evaluation of the effects of soil wetting and drying cycles on average scab severity of harvested tubers from cultivar Atlantic conducted in two controlled environment trials. Due to no significant trial effect according to an ANOVA at alpha=0.05, results of the two trials were combined. Two of the soil moisture regimes were applied continuously at 9% and 29% volumetric water contents. The other two regimes were applied weekly and alternated between 14% and 21% volumetric water contents on a weekly basis.

2003 Field Trial

The average scab severity of Atlantic tubers irrigated at 10% volumetric soil water content was much higher than the two previous trials that were conducted in the controlled environment, at 31.2% scab cover (Figure 2-8). When one of the wetting/drying the soil moisture regimes was applied at 14% weekly alternating with 21% weekly volumetric water content, the average scab severity was significantly reduced to 25.9% when compared to the driest soil (Figure 2-8). The 21% weekly volumetric soil water application regime alternating with 14%weekly volumetric soil water content produced tubers of similar infection rates (and statistically identical) to the inverse watering regime, with an average scab severity of 22.6% (Figure 2-8) surface coverage. The average scab severity was reduced to less than 3% when the soil moisture was maintained at 26% volumetric water content (Figure 2-8). The non-inoculated controls at each moisture regime had 2.5% or less average scab infections due to some possible plot overlapping from plots that were inoculated with *Streptomyces scabies* in the previous year's trial (Figure 2-8).

There was no significant difference among treatments for the total yield obtained (ranged from 10.2-12.8 kg/10 plants) (Table 2-3). Marketable yields (grade A plus oversize tubers) were similar across all treatments: 9.9-12.3 kg/10 plants (Table 2-3). The weight of tubers in the oversize class (greater than 500 g) were statistically the greatest with the alternating 21/14% (moist-dry soil) volumetric water content regime at 3.1-3.4 kg/10 plants (Table 2-3). All other oversize yields were similar to each other, except the alternating 14/21% (dry-moist soil), which produced only 1.7-2.0 kg/10 plants of oversize tubers, a statistically significant less amount (Table 2-3). Grade A yields

were significantly higher, 9.4-9.6 kg/10 plants, for plants grown under the moist soil and for the control of the alternating dry/moist soil (Table 2-3). Grade A yield was significantly lowest for plants grown under the driest of the irrigation regimes (Table 2-3). Grade B yields were similar across most of the treatments. The highest Grade B yields were obtained from plants that were inoculated and grown under the alternating 21/14% (moist-dry soil) volumetric water content regime at 1.5 kg/10 plants (Table 2-3). The lowest Grade B yields were in the non-inoculated plants under the alternating 21/14% (moist-dry soil) volumetric water content regime at 0.2 kg/10 plants (Table 2-3).

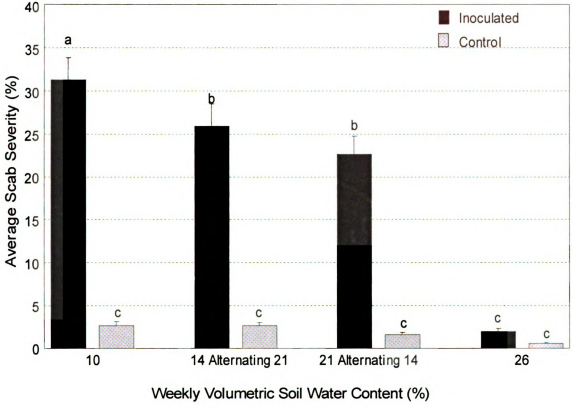

Figure 2-8. Evaluation of the effects of soil wetting and drying cycles on average scab severity of harvested tubers from cultivar Atlantic in a field trial in 2003.

TABLE 2-3. Effects of irrigation and inoculation on mean oversize, grade A, grade B, total, and marketable yields of Atlantic tubers.

	Average Yield per Category (in kg/10 plants)									
Treatment	Oversize	S.E.	Grade A	S.E.	Grade B	S.E.	Total	S.E.	Marketable	S.E.
Dry Soil										
Inoculated	2.9 abc	0.38	7.1 b	0.47	0.3 ab	0.08	10.4 a	0.82	10.0 a	0.81
Control	2.9 abc	0.48	7.0 b	0.92	0.3 ab	0.07	10.2 a	1.07	9.9 a	1.03
Alternating Moist/Dry Soil										
Inoculated	3.4 a	0.53	8.9 ab	0.85	0.5 a	0.17	12.8 a	0.80	12.3 a	0.74
Control	3.1 ab	0.54	8.8 ab	0.64	0.2 b	0.05	12.1 a	0.75	11.9 a	0.76
Alternating Dry/Moist Soil										
Inoculated	2.0 bc	0.54	8.8 ab	0.75	0.3 ab	0.11	11.0 a	0.77	10.8 a	0.82
Control	1.7 c	0.42	9.6 a	0.42	0.3 ab	0.04	11.7 a	0.25	11.4 a	0.25
Moist Soil										
Inoculated	2.3 abc	0.19	9.5 a	0.62	0.3 ab	0.07	12.1 a	0.75	11.8 a	0.70
Control	2.3 abc	0.59	9.4 a	1.17	0.3 ab	0.09	12.1 a	1.56	11.7 a	1.56

S.E. standard error of the mean

Means with the same letter are not significantly different based on t-test conducted within yield category at a=0.05.

DISCUSSION

There is an extensive literature that spans nearly 80 years on the use of irrigation for effective management of potato common scab (Sanford, 1923; Wellings and Rosser, 1969; Lapwood, 1966; Lapwood and Hering, 1968; Lapwood and Hering, 1970; Lapwood *et al.*, 1970; Lapwood *et al.*, 1971; Lapwood *et al.*, 1973; Lapwood and Adams, 1973; Lewis, 1970; Davis *et al.*, 1974; Davis *et al.*, 1976; Curwen, 1978; Curwen, 1980; Adams *et al.*, 1987; Wilson *et al.*, 2001). Irrigation has also been used to manage similar diseases, namely radish scab, carrot scab, and Streptomyces soil rot, which are caused by related pathogenic *Streptomyces* species, (Levick *et al.*, 1981; Levick *et al.*, 1985; Schoneveld, 1993; Ristaino and Averre, 1992; and Ristaino, 1993). The effects of irrigation on plant disease were thoroughly reviewed by Rotem and Palti (1969).

Sanford (1923) originally showed that increasing soil moisture consistently decreased potato common scab. The results in this research demonstrated that when soil moisture was maintained at 14%, scab infections were severe on tubers (Figure 2-3, Figure 2-6, Figure 2-7, and Figure 2-8). However, when soil moisture was increased to 22%, scab infections could be greatly reduced. When soil moisture application was further increased to 29% and 34%, infections were eliminated or very minimal and in both cases were not severe enough to reduce marketability. The results from the Sanford study (1923) are consistent with results from this study, in which irrigation regimes greater than the 9% soil moisture, incrementally decreased the average scab severity of a susceptible cultivar. Like the Sanford (1923) study, which implemented 14%, 22%, 29%, and 34% soil moisture regimes, this dissertation reports similar scab severity values at

relatively equivalent irrigation schedules of 9%, 14%, 21%, and 29% (Figure 2-3). Similar to the Sanford report (1923), this dissertation found that 29% soil moisture was sufficient to nearly eliminate scab infection on cultivars with susceptibility to common scab (Figure 2-3 and Figure 2-4). Potato growers are urged to irrigate when daily soil water approximates 65 to 70 percent available soil water (King and Stark, 1997). The amount required of irrigation applied should be at a soil water content to field capacity or less if the grower implements light, frequent irrigation (King and Stark, 1997).

Irrigation applied during the early stages of tuberization was shown to speed the swelling of the tubers and significantly decreased the incidence of scab infections (Lapwood *et al.*, 1970 and Lapwood *et al.*, 1973). The results of the Lapwood *et al.* (1970 and 1973) studies are also consistent with this study in that increased soil moisture significantly reduced scab severity of scab-susceptible cultivars (Figure 2-3 and Figure 2-4). In the Lapwood *et al.* (1970 and 1973) studies, the scab infections on the susceptible cultivars, Majestic, King Edward, and Record were reduced to less than 1-3% surface area infections under the highest soil moisture regime. The reduction was significant compared to the soils that were maintained as dry (non-irrigated) where the mean scab surface area values for these cultivars ranged 30% to 9% infection. These results were very similar to the findings in this study, in which scab infections were quite severe on the most susceptible cultivar, Atlantic, and ranged from 6-10% and 11-25% in controlled environment trials (Figure 2-3) and about 15% in the field trial (Figure 2-6).

The results of irrigation on scab infections of an intermediately susceptible cultivar, Boulder, in this study, were similar to the results with the cultivars Record and King Edward in the Lapwood *et al.* (1970 and 1973) reports where moist soils reduced

scab infection to less than 1% of the surface area. Lapwood *et al.* (1970 and 1973) found that scab infections were not significantly reduced by irrigation when applied to a scab-resistant cultivar, Pentland Crown, because scab infections were less than 1% (nearly 0%) for all irrigation regimes except the non-irrigated control, which had 1.3% scab. Their findings are similar to this study where maintaining high soil moisture volumes applied to the resistant cultivar, Liberator, did not significantly reduce infection. Scab infections were not detected on Liberator in the controlled environment trials (Figure 2-5) and were less than 2% in the field experiment, regardless of soil moisture application (Figure 2-6). The results in this study are nearly identical to Lapwood *et al.* (1970 and 1973). The inference from this study and the Lapwood *et al.* (1970 and 1973) studies is that frequent irrigation at or near field capacity during tuberization are required to manage common scab for a susceptible potato crop, but such intensive irrigation is not necessary to manage potato common scab in resistant cultivars. This has important implications for the use of soil moisture and the development of scab resistance in breeding programs, as well as leads to environmentally friendly and sustainable ecological benefits because reduced water usage leads to a reduced probability of nitrogen leaching from soils that occurs during some irrigation events. Issues such as these are critical in current agriculture. Water usage is becoming limited (Rosegrant *et al.*, 2002) and is of a growing major concern and environmental quality concerns arise from agricultural fertilizer and pesticide usage combined with irrigation and the resulting effects of leaching toxic substances into ground water (Francaviglia *et al.*, 2000; Close *et al*, 2003; Stewart and Loague, 2003; Tiktak *et al.*, 2004; Lee and Jose, 2005; Zhang *et al.*, 2005; and Vazquez *et al.*, 2005).

Irrigation for four to six weeks after tuber initiation at a 15 mm soil moisture deficit was found to decrease scab most effectively in one study (Lapwood, *et al.*, 1973). According to another report, five weeks of irrigation following tuber initiation was found to substantially reduce scab, while two weeks of irrigation after initiation was not sufficient to provide management of scab (Lewis, 1970). Although there is variation in relation to timing of irrigation, which is most likely due to environmental and cultivar effects, irrigation that provides adequate soil moisture at or near field capacity during tuberization is one of the most important crop management methods of controlling this disease without significantly reducing yield. Studies by Wellings and Rosser (1969) and Lapwood and Hering (1970) conclude that the third week after tuber initiation was the week that, if plants were non-irrigated, led to the development of the most severe scab infections compared with any other week since this was the critical time period for irrigation. This study supports the conclusions of Wellings and Rosser (1969) and Lapwood and Hering (1970): when soils were maintained with an alternating dry/wet cycle such that the third week the soil was drier, the average scab severity at the end of the field trial found tubers grown under this regime to be the most infected by scab (Figure 2-9). The effects of soil wetting and drying when studied in the field neither refute nor support the critical nature of the third week in development of scab infections.

It is apparent that maintaining sufficient soil moisture can significantly reduce scab infection on susceptible cultivars (Figure 2-3, Figure 2-4, Figure 2-6, Figure 2-7, and Figure 2-8; Lapwood *et al.*, 1970; and Lapwood *et al.*, 1973). Even if soil moisture is applied on a weekly basis, but not maintained through the duration of early tuber development, scab infections can still be severe (Figure 2-7 and Figure 2-8). The results

of this study emphasize the importance of continuous maintenance of soil moisture versus allowing soils to dry during early tuberization. The effects of soil wetting and drying on scab development in this study were very similar to results in other studies where soil moisture was not maintained continually (Lapwood and Hering, 1968; and Lapwood and Adams, 1973). Not only does the tuber quality in relation to reduced scab infection benefit from maintaining sufficient soil moisture during the early stages of tuber development, but also yield values are enhanced. In this study and others (Lapwood *et al.*, 1970; Lapwood *et al.*, 1971; and Lapwood *et al.*, 1973) yields significantly increased when soil moisture was maintained at or near field capacity compared to the drier soils with no negative effects on tuber size. In this study grade A yields were significantly greater when soil moisture was maintained at field capacity (Table 2-3).

The mechanism(s) involved in common scab management via irrigation have not been well studied and consequently are poorly understood. The effects of irrigation on scab infection are variable and highly dependent on the type of soil, cultivar planted, soil temperature, microbial populations, and other environmental factors. The stomata and immature lenticels of potato tubers are the infection courts of *Streptomyces scabies* (Fellows, 1926). The internodes of the potato tuber rapidly expand during early tuber development. Each internode is susceptible to scab for 10 to 15 days until the lenticels are fully suberized (Lapwood and Hering, 1970). Early infections on tubers of susceptible cultivars may lead to scab infections severe enough that the harvested crop is unsaleable (this study; Lapwood and Hering, 1970; W. Kirk, personal communication).

Lewis (1970) showed that when soil conditions were moist, there were high non-actinomycete bacteria to actinomycete populations and conversely dry soil conditions

favored small ratios between non-actinomycete bacteria to actinomycete populations. He hypothesized that antagonistic bacteria may be responsible for the reduction of scab infection by irrigation because lenticels had few actinomycetes at high water potentials. Also, after irrigation events Lewis (1970) commonly isolated *Pseudomonas* spp., which rapidly overgrew *Streptomyces scabies in vitro*. Adams and Lapwood (1978) also rarely found actinomycetes on tubers surfaces that were sampled from wet soils and found that in dry soils actinomycete hyphae colonized lenticels. Sanford (1926) suggested the possibility that there could be antibiotic relations between the soil microflora and that the physical or chemical properties of the soil may effect scab development. Sanford (1926) also demonstrated that certain bacteria inhibited growth of *S. scabies* by producing acidic compounds and found one that inhibited the pathogen by some other method than high acidity. It is possible that the effects of irrigation on common scab of potato are also due to decreased soil aeration since *S. scabies* is a strong aerobe. However, there is little research available to support this hypothesis.

Whether microbial antagonism that leads to competitive exclusion or antibiosis, transient anaerobic conditions, or some other suppressive factor is responsible for inhibiting scab during irrigation is yet to be determined. Nonetheless, irrigation is used by many growers to manage the disease. However, a study by Davis *et al.* (1974) highlights the importance of combining irrigation with other methods to control common scab. They irrigated soils to greater than 90 % available soil moisture during the early stages of tuber development and this alone was not sufficient to produce tubers of adequate quality for market standards. Their study emphasizes the importance of integrating other management methods and underscores the importance of the roles that

other factors play in irrigation, such as differences in managing scab obtained with irrigation vary with soil type, cultivar planted, and microbial populations. More studies that combine soil moisture management and cultivar resistance are needed. Although the importance of incorporating careful soil moisture management when a susceptible cultivar is cropped cannot be undermined, the usage of water for irrigation at high levels can be negated if a resistant cultivar is selected for cropping. This has sizeable ecological implications and will be more important as water shortages increase. Maintenance of sufficient soil moisture with susceptible cultivars should not be overlooked and is particularly important because there are no resistant cultivars with complete immunity to the disease and there are no effective chemicals that have been developed to control this disease. More research must be conducted to further understand the nature of disease control that is achieved by the use of soil moisture application. This will allow for risk assessment, as well as determination of the benefits of irrigation, and can integrate knowledge on a per field basis for cultivar selection for cropping, irrigation regimes, and other practices. If indeed the basis of scab management via irrigation is microbial antagonism, then populations of the antagonist(s) in fields should be enumerated. Also, if the organisms are not sufficiently present, then alternative disease management strategies would need to be implemented to control common scab. Furthermore, the possibility of developing a biocontrol agent could result from such research.

Chapter III

ASSESSMENT OF FOUR COMPOUNDS ON ELICITATION OF THE SYSTEMIC ACQUIRED RESISTANCE RESPONSE IN POTATO TO COMMON SCAB INFECTION.

INTRODUCTION

Systemic acquired resistance (SAR) is a broad-spectrum immunity in plants that results from treatment with a natural or synthetic chemical or from a localized infection with a necrotizing pathogen (Kessmann *et al.* 1994). Induced plants exhibit increased production of pathogenesis-related (PR) proteins, decreased lesion size and/or number, increased resistance to a broad spectrum of pathogens, and is dependent on salicylic acid signaling (Hammerschmidt, 1999). Salicylic acid-mediated induced resistance involves the rapid induction of a number of putative defense mechanisms, which may include modifications of the plant cell wall and/or production of one or more of the following: PR proteins, phytoalexins, oxidative enzymes, and active oxygen species. Induced resistance was first discovered by Ray and Beauverie in 1901. In 1933, Chester reviewed this phenomenon. Induced resistance has since been documented in numerous plant species, and has been extensively researched for more than thirty years in economically important plant families, such as Solanaceae (tobacco: Ross, 1961; Sequira and Hill, 1974; and Pritchard and Ross, 1975) and Cucurbitaceae (cucumber: Kuc *et al.*, 1975; and Hammerschmidt *et al.*, 1976).

The 1996 FQPA (Food Quality Protection Act) coupled with environmental concerns have lead to a new era of reduced pesticide application and development of alternative strategies to manage plant diseases. Unlike resistance activators, pesticides

are potentially damaging to the environment, toxic to humans and animals, provide coverage against pathogens for shorter periods of time, and usually are targeted toward a single-site metabolic mode of action (Ozeretskovskaya, 1995). Incorporating SAR into plant disease management programs will result in a safer environment, reduced risks on human and animal health, and provide long lasting, systemic protection against a wide range of pathogens while delaying the incidence of pesticide resistance.

Systemic acquired resistance in potato has been reported in response to infections caused by fungi and oomycetes (Muller and Borger, 1940; Chalova *et al.*, 1976; Chalova *et al.*, 1977; Bostock *et al.* 1986), bacteria (Dutton *et al.*, 1997), viruses (Roberts, 1979), and nematodes (Rahimi *et al.*, 1993 and Rahimi *et al.*, 1998). Muller and Borger (1940) first discovered local induced resistance in potato tubers after application of an incompatible race of *Phytophthora infestans* to compatible races of *P. infestans* and *Fusarium caeruleum*. Glucans and lipoglycoproteins isolated from mycelium of *P. infestans* elicited non-specific resistance against *P. infestans* and resulted in production of high concentrations of rishitin and lubimin, two of the potato sesquiterpenoid phytoalexins (Chalova *et al.*, 1976 and Chalova *et al.*, 1977). Chitin, chitosan, and hydrosylates of chitosan induced resistance in potato tuber tissue to *P. infestans* (Vasyukova *et al.*, 2000 and Vasyukova *et al.*, 2001). Oligogalacturonides from *Phytophthora sojae* effectively induced resistance in potato to infection by *Erwinia carotovota* ssp. *atroseptica* (Dutton *et al.*, 1997). The use of SAR against common scab has not previously been reported. The limited effectiveness reached with current potato common scab management strategies and the successful use of resistance activators in

other pathosystems warrants an investigation of this approach to manage common scab of potato.

MATERIALS AND METHODS

Experimental Design

Snowden, a scab-susceptible cultivar, was planted in a Houghton muck soil in 2001, 2002, and 2003 at Muck Soils Experiment Station in Laingsburg, Michigan, USA as a randomized complete block design with 4 replications per treatment. In 2001, harpin and chitosan were tested as seed, in-furrow, pre-emergence, and early-emergence foliar application methods. In 2002 and 2003 harpin, chitosan, formononetin, and saponins were tested as in-furrow and foliar application methods.

Resistance Activators

Harpin

Harpin was purchased as Messenger® from Eden Bioscience Corporation, Bothell, Washington, USA (Table 3-1). The active ingredient in Messenger®, 3% harpin$_{Ea}$, is a 44 kD protein isolated from *Erwinia amylovora*, the causal agent of fire blight of apple.

Chitosan

Chitosan was purchased as Elexa® 4 Plant Defense Booster from GlycoGenesys, Incorporated (formerly known as SafeScience, Inc.), Boston, Massachusetts, USA (Table 3-1). The active ingredient in Elexa® 4, 4% chitosan, is deacylated chitin, which is extracted from the shells of *Malacostraca* (crab species) after the edible portions have been removed.

Formononetin

Formononetin (potassium salt of 4'-methoxy, 7-hydroxy isoflavone) was purchased as Myconate® from VAMTech, L.L.C., Lansing, Michigan, USA (Table 3-1). The active ingredient of Myconate®, 100% formononetin, is an isoflavone extracted from the roots of *Melilotus officinalis* (clover) that were stressed by phosphorus deficiency.

Saponins

A saponin mix was purchased as Heads Up® Plant Protectant from Heads Up Plant Protectants, Incorporated, Great Falls, Virginia, USA (Table 3-1). The saponin mix is an extract of *Chenopodium quinoa* (quinoa) roots containing quinoa saponins at 49.65%.

TABLE 3-1. Trade names, active ingredients, source of active ingredients, and registrants of the naturally occurring biological chemicals evaluated for inducing resistance in potato to common scab infection in field trials.

Trade Name	Active Ingredient (AI)	AI Source	Registrant
Messenger	harpin protein	*Erwinia amylovora*	Eden Bioscience Corp.
Elexa 4	poly-D-glucosamine	*Malacostraca* shells	GlycoGenesys, Inc.
Myconate	formononetin	*Melilotus officinalis*	VAMTech, L.L.C.
Heads Up	quinoa saponins	*Chenopodium quinoa*	Heads Up Plant Protectants, Inc.

Field Experiments

2001 Field Trial

Snowden (US#1) potatoes were mechanically cut and allowed to heal 4 days before planting. Potatoes (cut seed, approximately 42-56 g; cultivar Snowden) were planted at the Muck Soils Experiment Station in Laingsburg, Michigan, USA on 11 July

into two-row by 7.6 m plots with 86.4 cm with tubers 30 cm apart replicated four times in a randomized complete block design. The two-row beds were separated by a 1.5 m unplanted row. All rows were irrigated until emergence and were inoculated at 0.1 l/7.6 m row with a spore suspension of *Streptomyces scabies* strain DP prepared at 10^6 spores/ml on 13 July. Weeds were controlled by hilling and as described in Table 3-2. Fertilizer was drilled into plots before planting, formulated according to results of soil tests and additional nitrogen was applied as described in Table 3-2. Insects and diseases were controlled as described in Table 3-2.

TABLE 3-2. Soil fertility and weed, insect and disease management programs used in the 2001 field trial.

Chemical	Registrant	Application Rate	Application Date (days after planting)
Herbicide			
Dual 8E	Syngenta Crop Protection, Inc.[a]	2.3 l/ha	10 DAP
Basagran	BASF Wyandotte Corporation[b]	2.3 l/ha	20 and 40 DAP
Poast	BASF Wyandotte Corporation[b]	1.8 l/ha	58 DAP
Insecticide			
Admire 2F	Bayer Crop Science, L.P.[c]	1.5 l/ha	at planting
Sevin 80S	Bayer Crop Science, L.P.[c]	1.4 kg/ha	31 and 55 DAP
Thiodan 3EC	Southern Agricultural Insecticides[d]	2.7 l/ha	65 and 87 DAP
Pounce 3.2EC	Bayer Crop Science, L.P.[c]	0.6 l/ha	48 DAP
Fungicide			
Bravo	Syngenta Crop Protection, Inc.[a]	1.8 l/ha	weekly 24/7 to 19/9
Fertilizer			
Urea	Al Reem, Inc.[e]	31 kg/ha	45 DAP

[a]Greensboro, North Carolina, USA
[b]Florham Park, New Jersey, USA
[c]Research Triangle Park, North Carolina, USA
[d]Palmetto, Florida, USA
[e]Detroit, Michigan, USA

Applications of harpin (Messenger®) and chitosan (Elexa® 4) were applied according to specified labeled rate as four different treatment methods: seed pieces, in-furrow, pre-emergence, and early emergence foliar applications and untreated plots were maintained as controls. Harpin seed treatments at 47 ml/45 kg and chitosan seed treatments at 40 ml/45 kg were coated in the Gustafson® metered slurry seed treater (Seedburo Equipment Company, Chicago, Illinois, USA) with the entire tuber surface being exposed (Table 3-2). In furrow applications of harpin at 0.47 kg/ha and chitosan at 2.3 l/ha were made over the seed at planting, applied with a single nozzle R&D spray boom delivering 46 l/ha at 551 kPa per treatment and using one XR11003VS nozzle per row (Table 3-2). Pre-emergence applications of harpin at 1.9 kg/ha and of chitosan at 9.1 l/ha were applied on 20 July and were repeated as early emergence foliar harpin and chitosan applications on 30 July at 1.9 kg/ha and at 9.1 l/ha, respectively (Table 3-2). Vines were killed with diquat desiccant Reglone® 2EC (Syngenta Crop Protection, Greensboro, North Carolina, USA) at 46 l/ha on 30 September. Half-plots (7.6 m row) were harvested manually on 19 October and individual treatments were evaluated based on scab incidence and severity.

2002 Field Trial

Snowden (US#1) potatoes were mechanically cut and allowed to heal 4 days before planting. Potatoes (cut seed approximately 42-56 g; cultivar Snowden) were planted at the Muck Soils Experiment Station in Laingsburg, Michigan, USA on 7 June into two-row by 7.6 m plots with 86.4 cm with tubers 30 cm apart replicated four times in a randomized complete block design. The two-row beds were separated by a 1.5 m

unplanted row. All rows were irrigated until emergence and were inoculated at 0.1 l/7.6 m row with a spore suspension of *Streptomyces scabies* strain DP prepared at 10^6 CFU/ml on 13 June. Weeds were controlled by hilling and as described in Table 3-3. Fertilizer was drilled into plots before planting, formulated according to results of soil tests and additional nitrogen was applied as described in Table 3-3. Insects and diseases were controlled as described in Table 3-3.

TABLE 3-3. Soil fertility and weed, insect and disease management programs used in the 2002 field trial.

Chemical	Registrant	Application Rate	Application Date (days after planting)
Herbicide			
Dual 8E	Syngenta Crop Protection, Inc.[a]	2.3 l/ha	10 DAP
Basagran	BASF Wyandotte Corporation[b]	2.3 l/ha	20 and 40 DAP
Poast	BASF Wyandotte Corporation[b]	1.8 l/ha	55 DAP
Insecticide			
Admire 2F	Bayer Crop Science, L.P.[c]	1.5 l/ha	at planting
Sevin 80S	Bayer Crop Science, L.P.[c]	1.4 kg/ha	30 and 55 DAP
Thiodan 3EC	Southern Agricultural Insecticides[d]	2.7 l/ha	63 and 82 DAP
Pounce 3.2EC	Bayer Crop Science, L.P.[c]	0.6 l/ha	47 DAP
Fungicide			
Bravo	Syngenta Crop Protection, Inc.[a]	1.8 l/ha	weekly 21/6 to 18/9
Fertilizer			
Urea	Al Reem, Inc.[e]	31 kg/ha	45 DAP

[a]Greensboro, North Carolina, USA
[b]Florham Park, New Jersey, USA
[c]Research Triangle Park, North Carolina, USA
[d]Palmetto, Florida, USA
[e]Detroit, Michigan, USA

Applications of harpin (Messenger®), chitosan (Elexa® 4), formononetin (Myconate®), and saponins (Heads Up®) were applied according to specified labeled rate as in-furrow and foliar treatment methods and untreated plots were maintained as

controls. In-furrow applications of harpin at 0.47 kg/ha, chitosan at 2.3 l/ha, formononetin 0.09 kg/ha, and saponins at 0.17 kg/ha were made over the seed at planting, applied with a single nozzle R&D spray boom delivering 46 l/ha at 551 kPa and using one XR11003VS nozzle per row (Table 3-2). Foliar applications of harpin at 1.9 kg/ha, chitosan at 9.1 l/ha, formononetin at 0.35 kg/ha, and saponins at 0.74 kg/ha were made over the rows, applied with a single nozzle R&D spray boom delivering 234 l/ha at 551 kPa and using one XR11003VS nozzle per row on 15 and 22 July (Table 3-2). Vines were killed with diquat desiccant Reglone® as described above on 20 September. Half-plots (7.6 m row) were machine harvested on 27 September and individual treatments were evaluated based on scab incidence and severity.

2003 Field Trial

Snowden (US#1) potatoes were mechanically cut and allowed to heal 4 days before planting. Potatoes (cut seed, approximately 42-56 g; cultivar Snowden) were planted at the Muck Soils Experiment Station in Laingsburg, Michigan, USA on 4 June into two-row by 7.6 m plots with 86.4 cm with tubers 30 cm apart replicated four times in a randomized complete block design. The two-row beds were separated by a 1.5 m unplanted row. All rows were irrigated until emergence and were inoculated at 0.1 l/7.6 m row with a spore suspension of *Streptomyces scabies* strain DP prepared at 10^6 CFU/ml on 12 June. Weeds were controlled by hilling and as described in Table 3-4. Fertilizer was drilled into plots before planting, formulated according to results of soil tests and additional nitrogen was applied as described in Table 3-4. Insects and diseases were controlled as described in Table 3-4.

TABLE 3-4. Soil fertility and weed, insect and disease management programs used in the 2003 field trial.

Chemical	Registrant	Application Rate	Application Date (days after planting)
Herbicide			
Dual 8E	Syngenta Crop Protection, Inc.[a]	2.3 l/ha	10 DAP
Basagran	BASF Wyandotte Corporation[b]	2.3 l/ha	20 and 40 DAP
Poast	BASF Wyandotte Corporation[b]	1.8 l/ha	55 DAP
Insecticide			
Admire 2F	Bayer Crop Science, L.P.[c]	1.5 l/ha	at planting
Sevin 80S	Bayer Crop Science, L.P.[c]	1.4 kg/ha	30 and 55 DAP
Thiodan 3EC	Southern Agricultural Insecticides[d]	2.7 l/ha	65 and 81 DAP
Pounce 3.2EC	Bayer Crop Science, L.P.[c]	0.6 l/ha	45 DAP
Fungicide			
Bravo	Syngenta Crop Protection, Inc.[a]	1.8 l/ha	weekly 4/6 to 7/9
Fertilizer			
Urea	Al Reem, Inc.[e]	31 kg/ha	45 DAP

[a]Greensboro, North Carolina, USA
[b]Florham Park, New Jersey, USA
[c]Research Triangle Park, North Carolina, USA
[d]Palmetto, Florida, USA
[e]Detroit, Michigan, USA

In-furrow and foliar applications of harpin (Messenger®), chitosan (Elexa® 4), formononetin (Myconate®), and saponins (Heads Up®) were applied as described above and untreated plots were maintained as controls. Vines were killed with diquat desiccant as described above on 28 August. Half-plots (7.6 m row) were machine harvested on 15 September and individual treatments were evaluated based on scab incidence and severity.

TABLE 3-5. Application methods, number of years tested, appliation rates, and active ingredient rates of the naturally occurring chemicals evaluated for inducing resistance in potato to common scab infection in field trials.

Chemical Application Method	Years Evaluated	Application Rate	Active Ingredient Rate[a] (in%)
harpin			
seed treatment	1	1.04 ml/kg	1.650
in-furrow spray	3	0.47 kg/ha	0.107
pre-emergence spray	1	1.9 kg/ha	0.400
foliar spray	3	1.9 kg/ha	0.400
chitosan			
seed treatment	1	0.89 ml/kg	0.400
in-furrow spray	3	2.3 l/ha	0.002
pre-emergence spray	1	9.1 l/ha	0.008
foliar spray	3	9.1 l/ha	0.008
formononetin			
in-furrow spray	3	0.09 kg/ha	0.012
foliar spray	3	0.35 kg/ha	0.050
saponins			
in-furrow spray	3	0.17 kg/ha	0.013
foliar spray	3	0.74 kg/ha	0.055

[a]percentage of formulated product

Evaluation of Resistance Activators

Disease Assessment

Harvested tubers were carefully washed to avoid damage to the periderm and soil and debris was removed so that common scab incidence and severity could be assessed. One hundred sixty tubers per resistance activator treatment (40 tubers per plot, replicated 4 times) were assessed for scab incidence, rate of scab infection, and scab lesion type(s) that occurred on the tuber surface. This was done by a visual determination of percent coverage of the entire tuber surface area using a key for assessment of potato common scab developed by James (1971).

Yield

Potato tubers were evaluated for yield one day after harvest to avoid underestimation of yields due to tuber rot. Potatoes from each harvested 7.6 m half-plot were placed onto a Tew Fruit and Vegetable processing machine with sizers (Tew Manufacturing Corporation, Penfield, New York, USA) and weighed. US #1 potatoes were weighed separately as grade A and oversize, and all others smaller than US#1 were weighed and classified grade B.

Plant Emergence

To determine effects of the natural compounds on plant emergence, plants in each plot were counted. The counting of plants began once emergence was apparent, usually 14 days after planting. Emergence counts continued each season until no more plant germination was evident.

Chitinase Activity

Glycol chitin preparation. A glycol chitin (1 % w/v) stock solution was prepared by a modification of the method of Molano *et al* (1979). Glycol chitosan (5 g) was dissolved in 100 ml of 10 % acetic acid and simultaneously ground in a mortar with a pestle. The solution was incubated overnight at 22°C. The solution was vacuum-filtered through Whatman® (Whatman, Incorporated, Florham Park, New Jersey, USA) number 4 filter paper and acetic anhydride (7.5 ml) was added to the filtrate and stirred until a gel formed. The gel was cut into small (1 cm^3) pieces and methanol was added until the gel pieces were completely saturated. The mixture was homogenized in a blender for 4

minutes on the highest speed and then centrifuged at 27,000 x g at 4°C for 5 minutes. The pellet was re-suspended in a 1:1 volume of methanol, re-homogenized for 4 minutes on the highest speed and centrifuged at 27,000 x g at 4°C for 5 minutes. Sterile distilled water (500 ml) containing 0.02% (w/v) sodium azide was added to the pellet and homogenized in a blender for 4 minutes on the highest speed and the solution was stored at 4°C.

Protein extraction. Tubers were removed from storage at 4°C and washed with distilled water to remove any remaining soil particles. The apical and stolon ends were removed. A 17 mm diameter core borer was used to core twice through the cut ends of the potato. The two cylinders were sliced from both the apical and stolon ends into small 5.15 mm thick disks so that the two outermost (closest to the apical and stolon ends) slices could be used for protein extraction from each cylinder (8 slices/tuber). Each slice was treated with liquid nitrogen and ground with a mortar and pestle in 3 ml of homogenization buffer (0.01 M sodium phosphate pH 6.0 containing 1 % w/w polyvinylpyrrolidone). The homogenate was filtered through a double layer of Calbiochem® miracloth (EMD Biosciences, San Diego, California, USA). The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4°C. The supernatant was stored at -20°C. Protein was extracted from 96 samples per resistance activator treatment (3 tubers per plot yielded 24 potato tuber disks, replicated 4 times).

Chitinase Activity Assay. An agarose gel1 % (w/v) was prepared with 40 ml 0.01 M sodium phosphate (pH 6.0) and was brought to a boil in a microwave. The 1 % glycol chitin stock solution (prepared as described above; 0.4 ml) was added and stirred and 30 ml of the sodium phosphate was poured into a plastic petri dish (15 cm diameter). The

agarose gel was allowed to harden for 30 minutes and 3 mm diameter wells (1.5 cm apart) were pierced with a 3 mm diameter core borer. The agarose in each well was vacuum-suctioned from the gel with a Pasteur pipette. Protein extracts were loaded at 5 µl/ protein sample in 3 replications per gel. The petri dish was incubated for 4 hours at 37°C in a water bath. The gels were stained in a solution of 50 ml of 500 mM Tris-HCl with 0.01 % Calcoflour brightener 28® (purchased from Sigma Chemical Company, St. Louis, Missouri, USA) for 10 minutes and then rinsed twice to remove the staining solution. Distilled water (sufficient to cover gel) was added to the petri dish and incubated over night at 22 °C in the dark to allow the color to develop. The following day the water was removed from the gels and the gels were photographed under UV light. The activity of chitinase was determined as a diameter expressed in cm using Adobe Photoshop® version 6.0 software (Adobe Systems Incorporated, San Jose, California, USA) under maximal contrast using crop and diameter tools. Chitinase activity was assessed by the method of Velasquez and Hammerschmidt (2004) for 288 samples per resistance activator treatment (96 potato tuber disks per treatment were analyzed in replications of 3).

Challenge Inoculations

Tuber Disk Preparation. Tubers from the different field treatments were removed from storage at 4°C and allowed to warm to 22°C overnight. The tubers were washed thoroughly with distilled water and surface sterilized by soaking for 10 minutes in a sterile beaker containing 10% sodium hypochlorite (Clorox bleach) solution prepared in sterile distilled water, followed by a 5 minute soak in sterile distilled water to remove the

solution. The apical and stolon ends of the tubers were removed with a sterile knife. A sterile 17 mm diameter core borer was used to core through the cut ends of the potato. The cylinder was sliced into six 5.15 mm thick disks, which were rinsed twice with sterile distilled water and placed into sterile 100 mm x 15 mm plastic petri dishes lined with sterile Whatman® (Whatman, Incorporated, Florham Park, New Jersey, USA) number 1 filter paper moistened with sterile distilled water.

Inoculum Preparation. Streptomyces scabies strain DP was grown on 100 mm x 15 mm diameter plastic petri dishes containing PDA (prepared as described in chapter 1) at 30°C in the dark. After an incubation period of 12 days, the spores were carefully removed from the cultures with a sterile spatula and were re-suspended as a spore suspension at 10^9 spores/ml of 0.1 % Tween® 80 (as a wetting agent). A spore lawn was created by inoculating 100 µl of the spore suspension with a sterile glass spreader onto a fresh plate of PDA and incubated for 12 days at 30°C in the dark. Small (6.17 mm) plugs containing spores were pierced through the medium with a 6.17 mm diameter core borer and were immediately used as inoculum.

Tuber Disk Challenge Inoculation. The spore inoculum (prepared as described above) was inverted and placed in the middle of the potato tuber disks (prepared as described above) on 5 of the 6 disks. The other disk was treated with a 6.17 mm diameter of PDA as a control. The disks were incubated at 30°C in the dark for 4 days and the sterile filter paper was remoistened with sterile distilled water as necessary so that the tuber disks did not dry out. The diameter of the infection was measured with a digital caliper. All samples were frozen with liquid nitrogen until needed. Chitinase activity

was evaluated for all challenged tissue (as previously described). Forty-eight samples per resistance activator treatment were evaluated.

Weather Data

Precipitation and soil temperature data collected from depths of 50.8 mm and 101.6 mm (2 in and 4 in) for 2001, 2002, and 2003 were downloaded directly from the Michigan State University Muck Research Farm Station in Laingsburg, Michigan of the Michigan Automated Weather Network database at www.agweather.geo.msu.edu/mawn/.

Data Analysis

A two-way analysis of variance (ANOVA) was run to test the effects of year, treatment, and the interaction between year and treatment for the evaluation of resistance activators and their effects on scab as determined by the disease assessment. For yield evaluation, a two-way ANOVA was run that tested the effects of year, treatment, and the interaction between year and treatment for the effects of resistance activators on yield. Yield data from the field trials were analyzed with a t-test with comparison of least significant difference to determine differences between resistance activator treatments and application methods within each yield category.

A two-way ANOVA was run that tested the effects of year, treatment, and the interaction between year and treatment to determine whether there were differences in chitinase activities detected in tuber tissue treated with the resistance activators. T-tests with comparison of least significant difference were conducted to determine the differences in chitinase activity detected between the treatments for the trials conducted

in 2001, 2002, and 2003, as well as the chitinase activity detected in challenged tuber tissue after the challenge inoculation.

For all statistical analyses, an outcome was deemed statistically significant if the probability for a Type I Error was 5% or less (alpha = 0.05). The GLM (general linear model) for two-way ANOVA and LSD (least significant difference) of the means for t-tests were conducted using SAS® version 8 software (SAS Institute, Inc.; Cary, North Carolina).

RESULTS

Evaluation of Resistance Activators

Disease Assessment

A two-way ANOVA that tested the effects of year, treatment, and year/treatment interaction on disease found year, treatment and year/treatment interaction to be significant at $\alpha = 0.05$, so each year was analyzed individually. In 2001, the majority of harvested tubers had 5% or less scab infection (Figure 3-1). Slightly more disease free tubers resulted from treatments with chitosan and harpin (Figure 3-1). However, only the pre-emergence chitosan application was significantly better than the control. More tubers of Grade A market quality (at or less than 5% scab lesions) resulted from harpin seed treatments. All other treatments were not different than the control. The incidence and severity of scab infections with greater than 5% lesions were similar regardless of treatment. Harpin and chitosan applications neither eliminated nor significantly reduced severe infections that ranged from 50-100% scab coverage (Figure 3-1).

In 2002, the majority of harvested tubers had 6-10% scab infection (Figure 3-2). Only the in-furrow application of chitosan resulted in more disease free tubers than the untreated control (Figure 3-2). Treatment with the four compounds resulted in no increase of Grade A marketable tubers when compared to the control. In fact, the foliar formononetin application resulted is significantly fewer marketable tubers than the control. All of the in-furrow applications resulted in more tubers with 11-25% scab infection than the untreated control. In furrow and foliar applications with chitosan,

saponins, harpin, and formononetin provided little to no reduction in tubers in the more severe disease categories of 26-49% and 50-100% scab cover (Figure 3-2).

In 2003, the majority of harvested tubers had 11-25% scab infection (Figure 3-3). There were no significant differences between the incidence of tubers without scab across all treatments (Figure 3-3). Tubers in the Grade A marketable category were either no different than the untreated control or were significantly less than the control. In-furrow and foliar harpin applications and foliar chitosan applications resulted in fewer tubers in the Grade A category than the untreated control (Figure 3-3). Most of the harvested tubers were classified with 11-25% scab and there were no treatment differences in this range. The incidence of severe scab infections that ranged from 26-49 and 50-100% were not reduced when treated with natural compounds (Figure 3-3).

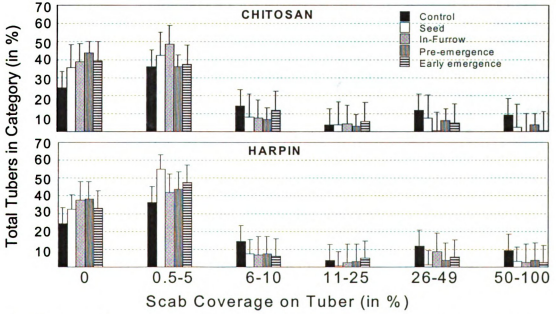

Figure 3-1. Effects of natural resistance activators and application method on scab incidence and severity of harvested tubers in 2001.

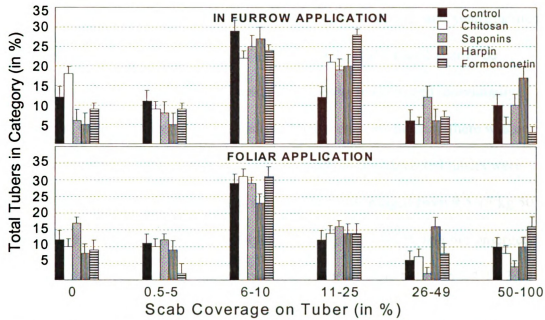

Figure 3-2. Effects of natural resistance activators, natural compounds, and application method on scab incidence and severity of harvested tubers in 2002.

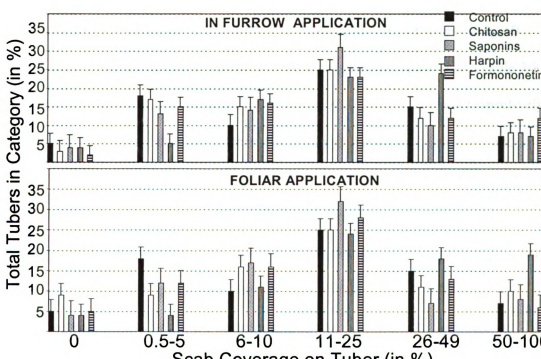

Figure 3-3. Effects of natural resistance activators, natural compounds, and application method on scab incidence and severity of harvested tubers in 2003.

Yield

A two-way ANOVA that tested the effects of year, treatment, and year/treatment interactions on marketable-, grade A-, grade B- and total-yields, found year to be significant at $\alpha = 0.05$, so each of these yield categories were evaluated individually (within years). Only oversize yield was analyzed between years because there was no significant year effect. Figure 3-4 shows plots from which the harvested tubers were obtained for yield data. In 2001, total and marketable yields ranged from 9.5-13 kg/50 plants and 7.4-9.2 kg/50 plants, respectively, for furrow, foliar, seed, and pre-emergence harpin treatments (Figure 3-5). Total and marketable yields were between 9.4-11.2 kg/50 plants and 7.3-10.3 kg/50 plants, respectively, for all chitosan treatments (Figure 3-5). Marketable and total yields for the control were 9.5 kg/50 plants and 6.8 kg/50 plants, respectively. There were no negative effects on total or marketable yields due to elicitor treatment in 2001 (Table 3-6 and Table 3-7). Oversize yields ranged from 0.0 kg/50 plants to 0.8 kg/50 plants. These differences were not significant at $\alpha = 0.05$ (Table 3-8). Grade A and B yields averaged about 8.8 kg/50 plants and 1.5 kg/50 plants, respectively across all treatments. The treatment differences were not significant (Table 3-9 and Table 3-10).

In 2002, total and marketable yields for plants treated with in-furrow applications of saponins were significantly higher (9.4 kg/50 plants and 7.1 kg/50 plants, respectively) than all other treatments (Figure 3-6). Total and marketable yields for all other treatments were similar: 6.8-8.3 kg/50 plants and 5.0-6.3 kg/50 plants, respectively. The only yields less than the control resulted from the foliar chitosan application (5.9 and 4.4 kg/50 plants for total and marketable yields, respectively) (Table 3-6 and Table 3-7).

Oversize yields ranged from 0.0-0.4 kg/50 plants and were not significantly different across all 9 treatments (Table 3-8). Grade A yields were significantly greater than the control when plants were treated with in-furrow applications of saponins (Table 3-9). All other treatments were similar, except foliar chitosan applications, which resulted in lower Grade A yields than the untreated control (Table 3-9). There were no treatment differences on yield in the Grade B category (Table 3-10).

In 2003, total yields of all treatments ranged from 8.6-12.1 kg/50 plants and marketable yields were 7.6-10.5 kg/50 plants (Figure 3-10). There were no negative effects of the natural compounds on yields in 2003 (Table 3-6 and Table 3-7). Oversize yields were not statistically significant (0.2-0.4 kg/50 plant; Table 3-8). Grade A and B yields averaged 10.1 kg/50 plants and 1.2 kg/50 plants, respectively across all nine treatments and these differences were not significant (Table 3-9 and Table 3-10).

Figure 3-4. Plots used for assessment of natural compounds at Muck Soils Experiment Station in 2003. Plots in 2001 and 2002 were similar to the ones pictured here.

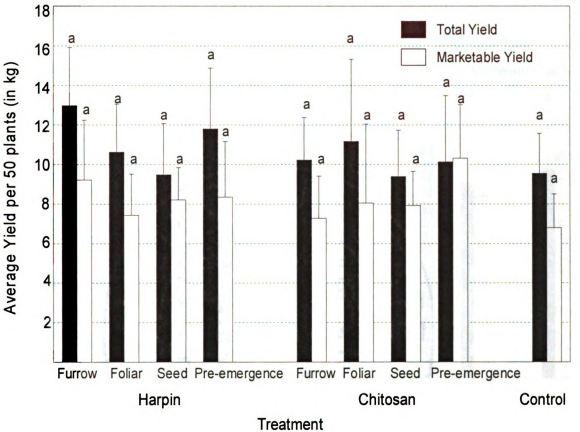

Figure 3-5. Comparison of the effects of natural resistance activators and application method on mean yields in 2001. Means with the same letter are not significantly different according to t-test at a=0.05. The t-test was conducted within yield category due to significant yield category effect as determined by a one-way ANOVA at alpha = 0.05.

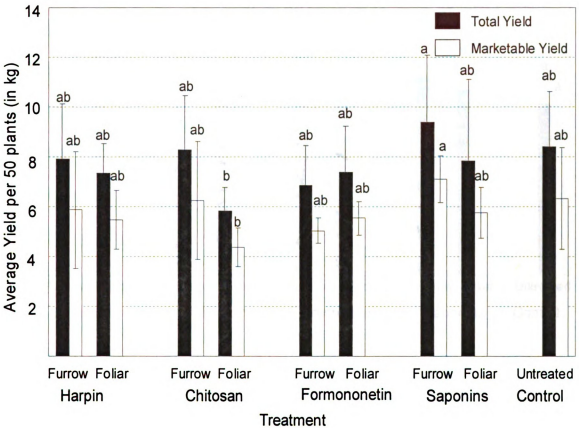

Figure 3-6. Comparison of the effects of natural compounds, resistance activators and application method on mean yields in 2002. Means with the same letter are not significantly different according to t-test at a=0.05. The t-test was conducted within yield category due to significant yield category effect as determined by a one-way ANOVA at alpha = 0.05.

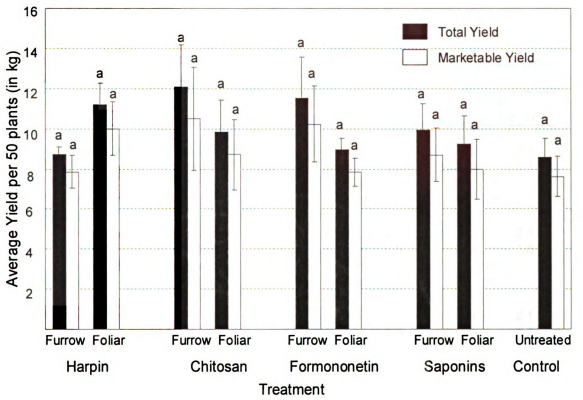

Figure 3-7. Comparison of the effects of natural compounds, resistance activators and application method on mean yields in 2003. Means with the same letter are not significantly different according to t-test at a=0.05. The t-test was conducted within yield category due to significant yield category effect as determined by a one-way ANOVA at alpha = 0.05.

TABLE 3-6. Effects of natural compounds and application method on mean total yields per 50 plants in 2001, 2002, and 2003.

Treatment	2001		2002		2003	
	Mean (kg)	Std.Error	Mean (kg)	Std. Error	Mean (kg)	Std. Error
Harpin						
In Furrow	13.0 a	2.9	7.9 ab	2.2	8.7 a	0.3
Foliar	10.6 a	2.4	7.3 ab	1.2	11.2 a	1.1
Seed	9.5 a	2.6	nt		nt	
Pre-emergence	11.8 a	3.1	nt		nt	
Chitosan						
In Furrow	10.2 a	2.2	8.3 ab	2.1	12.1 a	2.1
Foliar	11.2 a	4.2	5.8 b	0.9	9.8 a	1.6
Seed	9.4 a	2.3	nt		nt	
Pre-emergence	10.1 a	3.3	nt		nt	
Formononetin						
In Furrow	nt		6.8 ab	1.6	11.5 a	2.1
Foliar	nt		7.4 ab	1.8	9.0 a	0.5
Saponins						
In Furrow	nt		9.4 a	2.7	9.9 a	1.3
Foliar	nt		7.8 ab	3.3	9.2 a	1.4
Control	9.5 a	2.0	8.4 ab	2.2	8.6 a	0.9

nt Not Tested

Means with the same letter are not significantly different according to t-test at a=0.05.
t-test conducted within year due to significant year effect (ANOVA a = 0.05).

TABLE 3-7. Effects of natural compounds and application method on mean marketable yields per 50 plants in 2001, 2002, and 2003.

Treatment	2001		2002		2003	
	Mean (kg)	Std.Error	Mean (kg)	Std. Error	Mean (kg)	Std. Error
Harpin						
In Furrow	9.2 a	3.0	5.9 ab	2.3	7.9 a	0.8
Foliar	7.4 a	2.1	5.5 ab	1.2	10.0 a	1.3
Seed	8.2 a	1.6	nt		nt	
Pre-emergence	8.4 a	2.8	nt		nt	
Chitosan						
In Furrow	7.3 a	2.1	6.3 ab	2.4	10.5 a	2.6
Foliar	8.1 a	4.0	4.4 b	0.8	8.7 a	1.7
Seed	7.9 a	1.7	nt		nt	
Pre-emergence	10.3 a	2.7	nt		nt	
Formononetin						
In Furrow	nt		5.0 ab	0.5	10.2 a	1.9
Foliar	nt		5.5 ab	0.7	7.8 a	0.7
Saponins						
In Furrow	nt		7.1 a	0.9	8.7 a	1.3
Foliar	nt		5.7 ab	1.0	8.0 a	1.5
Control	6.8 a	1.7	6.3 ab	2.0	7.6 a	1.0

nt Not Tested

Means with the same letter are not significantly different according to t-test at a=0.05. t-test conducted within year due to significant year effect (ANOVA a = 0.05).

TABLE 3-8. Effects of natural compounds and application method on mean oversize yields per 50 plants in 2001, 2002, and 2003.

Treatment	2001		2002		2003	
	Mean (kg)	Std.Error	Mean (kg)	Std. Error	Mean (kg)	Std. Error
Harpin						
In Furrow	0.8 a	0.4	0.7 a	0.3	0.8 a	0.2
Foliar	0.6 a	0.2	0.4 a	0.1	0.7 a	0.2
Seed	0.0 a		nt		nt	
Pre-emergence	0.5 a	0.1	nt		nt	
Chitosan						
In Furrow	0.3 a	0.1	0.4 a	0.1	0.9 a	0.4
Foliar	0.2 a	0.1	0.4 a	0.1	0.3 a	0.2
Seed	0.0 a		nt		nt	
Pre-emergence	0.1 a	0.0	nt		nt	
Formononetin						
In Furrow	nt		0.2 a	0.1	0.8 a	0.3
Foliar	nt		0.4 a	0.2	0.8 a	0.4
Saponins						
In Furrow	nt		1.0 a	0.4	0.9 a	0.2
Foliar	nt		0.3 a	0.1	0.2 a	0.2
Control	0.4 a	0.2	0.3 a	0.1	0.3 a	0.2

nt Not Tested

Means with the same letter are not significantly different according to t-test at a=0.05.

TABLE 3-9. Effects of natural compounds and application method on mean grade A yields per 50 plants in 2001, 2002, and 2003.

Treatment	2001		2002		2003	
	Mean (kg)	Std.Error	Mean (kg)	Std. Error	Mean (kg)	Std. Error
Harpin						
In Furrow	10.4 a	1.6	6.4 ab	0.9	8.8 a	0.8
Foliar	8.5 a	0.8	6.2 ab	0.6	11.5 a	1.4
Seed	7.9 a	0.8	nt		nt	
Pre-emergence	9.8 a	1.3	nt		nt	
Chitosan						
In Furrow	8.6 a	0.9	7.2 ab	1.0	11.9 a	2.7
Foliar	9.5 a	1.7	4.9 b	0.4	10.3 a	1.9
Seed	8.2 a	0.7	nt		nt	
Pre-emergence	8.3 a	1.3	nt		nt	
Formononetin						
In Furrow	nt		5.9 ab	0.6	11.6 a	2.0
Foliar	nt		6.4 ab	0.9	8.7 a	0.4
Saponins						
In Furrow	nt		7.6 a	0.9	9.7 a	1.4
Foliar	nt		6.7 ab	1.3	9.5 a	1.6
Control	7.9 a	0.9	7.4 ab	1.0	9.0 a	1.1

nt Not Tested

Means with the same letter are not significantly different according to t-test at a=0.05.
t-test conducted within year due to significant year effect (ANOVA a = 0.05).

TABLE 3-10. Effects of natural compounds and application method on mean grade B yields per 50 plants in 2001, 2002, and 2003.

Treatment	2001		2002		2003	
	Mean (kg)	Std.Error	Mean (kg)	Std. Error	Mean (kg)	Std. Error
Harpin						
In Furrow	1.8 a	0.1	0.8 a	0.1	0.9 a	0.3
Foliar	1.6 a	0.3	0.7 a	0.2	1.2 a	0.1
Seed	1.6 a	0.4	nt		nt	
Pre-emergence	1.5 a	0.3	nt		nt	
Chitosan						
In Furrow	1.4 a	0.1	0.7 a	0.1	1.6 a	0.4
Foliar	1.4 a	0.3	0.5 a	0.1	1.1 a	0.1
Seed	1.2 a	0.3	nt		nt	
Pre-emergence	1.8 a	0.3	nt		nt	
Formononetin						
In Furrow	nt		0.7 a	0.2	1.3 a	0.6
Foliar	nt		0.7 a	0.1	1.1 a	0.1
Saponins						
In Furrow	nt		0.8 a	0.2	1.2 a	0.2
Foliar	nt		0.8 a	0.3	1.2 a	0.1
Control	1.3 a	0.2	0.7 a	0.1	1.0 a	0.1

nt Not Tested

Means with the same letter are not significantly different according to t-test at a=0.05.
t-test conducted within year due to significant year effect (ANOVA a = 0.05).

Plant Emergence

In 2001, the natural compounds and application methods did not effect plant emergence (Table 3-11). At 14 days after planting, more than 20% of all plants had emerged. Two weeks later, plant emergence was near 100% for all treatments (Table 3-11). In 2002, there were no significant differences between the compounds and application methods on plant emergence. At 18 days after planting, more than 50% of the plants emerged (Figure 3-12). Emergence values increased consistently over the following weeks across all treatments. The final emergence count of treatments ranged from 80-92% (Figure 3-12). In 2003, plant emergence was not negatively influenced treatment with natural compounds when compared to the control (Figure 3-13). At 19 days after planting, more than 29% of plants had emerged. By the end of the season, 99-100% of all plants emerged (Figure 3-13). This was the highest emergence rate between the 3 years.

TABLE 3-11. Effects of natural compounds on plant emergence in 2001.

Treatment	Percentage of Plants Emerged					
	7/25/01 14 DAP	7/27/01 16 DAP	7/30/01 19 DAP	8/1/01 21 DAP	8/3/01 23 DAP	8/8/01 28 DAP
Harpin						
In Furrow	25	49	87	91	93	96
Foliar	25	51	86	93	96	98
Seed	29	49	74	82	84	88
Pre-emergence	38	57	94	95	96	97
Chitosan						
In Furrow	26	55	87	94	96	98
Foliar	28	47	84	93	95	97
Seed	33	59	92	97	98	99
Pre-emergence	26	47	86	91	93	96
Control	21	34	86	96	97	98

DAP Days after planting

TABLE 3-12. Effects of natural compounds on plant emergence in 2002.

Treatment	Percentage of Plants Emerged						
	6/25/02	7/1/02	7/8/02	7/15/02	7/22/02	8/5/02	8/7/02
	18 DAP	24 DAP	31 DAP	38 DAP	45 DAP	59 DAP	61 DAP
Harpin							
In Furrow	61	63	65	65	65	90	90
Foliar	55	57	63	66	67	88	88
Chitosan							
In Furrow	51	53	60	60	60	83	83
Foliar	53	55	58	59	60	80	80
Formononetin							
In Furrow	55	58	65	66	67	85	85
Foliar	61	64	71	71	72	90	90
Saponins							
In Furrow	59	60	67	67	67	90	90
Foliar	58	58	65	66	66	84	84
Control	59	59	65	66	68	92	92

DAP Days after planting

TABLE 3-13. Effects of natural compounds on plant emergence in 2003.

Treatment	Percentage of Plants Emerged					
	6/23/03	7/1/03	7/8/03	7/15/03	7/22/03	7/29/03
	19 DAP	27 DAP	34 DAP	41 DAP	48 DAP	55 DAP
Harpin						
In Furrow	53	84	95	98	99	100
Foliar	31	87	91	96	99	99
Chitosan						
In Furrow	29	87	89	98	100	100
Foliar	45	84	89	96	99	100
Formononetin						
In Furrow	47	86	88	99	100	100
Foliar	59	88	95	97	99	99
Saponins						
In Furrow	35	81	95	100	100	100
Foliar	57	84	95	97	98	99
Control	53	84	95	100	100	100

DAP Days after planting

Chitinase Activity

A two-way ANOVA that tested the effects of year, treatment, and year/treatment interaction on chitinase activity found year, treatment and year/treatment interaction to be significant at $\alpha = 0.05$, so each year was analyzed individually. In 2001, the average chitinase activity of tuber tissue was highest for the untreated control at 1.4 cm^2 (Table 3-14). Average chitinase activity of harpin and chitosan treatments ranged from 1.34-1.36 cm^2 and 1.28-1.39 cm^2, respectively (Table 3-14). In 2002, average chitinase activity was highest in tuber tissue from plants treated with in-furrow and foliar formononetin applications at 1.26 cm^2 (Table 3-14). Average chitinase activity of chitosan, harpin, and saponins was 1.14-1.24 cm^2, 1.24-1.25 cm^2, and 1.22-1.25 cm^2, respectively (Table 3-14). Average chitinase activity of the untreated control was lower than the other values at 1.19 cm^2 (Table 3-14). In 2003, average chitinase activity of tuber tissue was greatest for plants treated with foliar applications of harpin at 1.74 cm^2 (Table 3-14). The lowest average chitinase activity was detected in samples that were treated with in-furrow applications of saponins at 1.41 cm^2 (Table 3-14). All other activities ranged from 1.54-1.71 cm^2 (Table 3-14). Figure 3-8 shows typical results of the chitinase activity assay used to detect differences in the production of this PR-protein, which is a marker for systemic acquired resistance.

TABLE 3-14. Effects of natural compounds and application methods on mean chitinase activity of potato tuber tissue in 2001, 2002, and 2003.

Treatment	2001		2002		2003	
	Mean (cm^2)	Std.Error	Mean (cm^2)	Std. Error	Mean (cm^2)	Std. Error
Harpin						
In Furrow	1.34 cde	0.01	1.25 ab	0.02	1.65 cd	0.02
Foliar	1.34 de	0.02	1.24 ab	0.02	1.74 a	0.02
Seed	1.36 bcd	0.01	nt		nt	
Pre-emergence	1.34 cde	0.01	nt		nt	
Chitosan						
In Furrow	1.38 abc	0.01	1.24 ab	0.02	1.71 ab	0.02
Foliar	1.32 ef	0.02	1.14 d	0.03	1.67 bc	0.12
Seed	1.39 ab	0.01	nt		nt	
Pre-emergence	1.28 f	0.01	nt		nt	
Formononetin						
In Furrow	nt		1.26 a	0.04	1.62 cd	0.02
Foliar	nt		1.26 a	0.02	1.54 e	0.03
Saponins						
In Furrow	nt		1.22 b	0.02	1.41 f	0.02
Foliar	nt		1.25 ab	0.02	1.61 d	0.02
Control	1.40 a	0.01	1.19 c	0.03	1.63 cd	0.02

nt Not Tested

Means with the same letter are not significantly different according to t-test at a=0.05.

t-test conducted within year due to significant year effect (ANOVA a = 0.05).

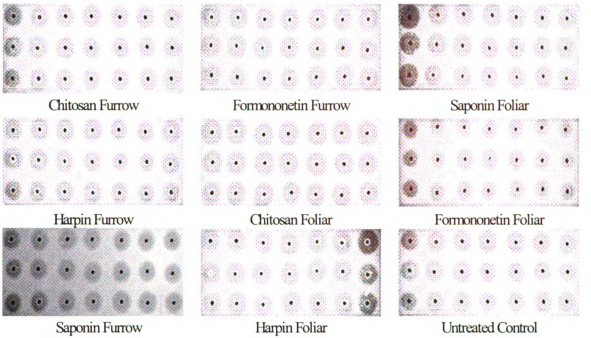

Figure 3-8. Chitinase activity assays of tuber tissue from plants treated with natural compounds in 2003.

Challenge Inoculations

Tuber slices from the untreated control challenged with *Streptomyces scabies* resulted in necrotic diameters of approximately 8 mm regardless of the depth from which the tuber slice was taken (Figure 3-9). The necrotic diameters of the other challenged tissues ranged from approximately 7-9 mm for the slices at all depths of tissue (Figure 3-9). The average chitinase activity of the challenged tuber tissue was greatest in tubers from plots that were treated with foliar applications of harpin. The average chitinase activity of this treatment was 1.68 cm^2 (Figure 3-10). Therefore, it is possible that harpin may enable the tuber to respond to pathogen attack. The average chitinase activity of challenged tissue was lower from tubers obtained from the in-furrow treatments with

harpin and formononetin than the untreated control. All other average chitinase activities were comparable to the control (Figure 3-10).

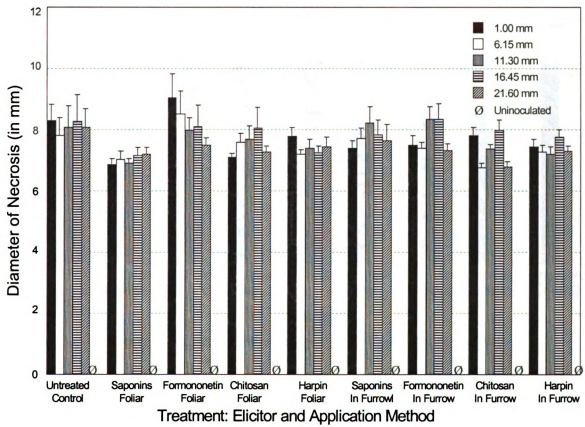

Figure 3-9. Effects of natural compounds and application method on tuber tissue slices from various depths challenged with *Streptomyces scabies*.

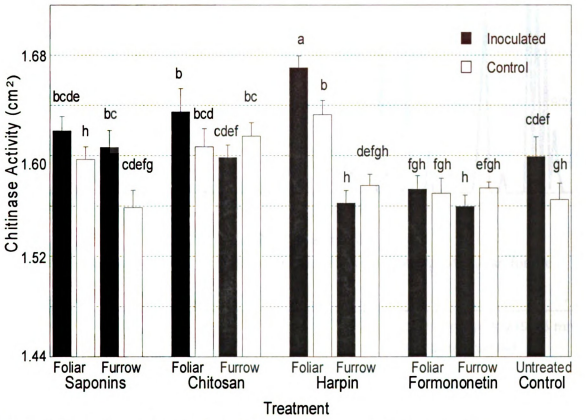

Figure 3-10. Effects of challenged tuber tissue on average chitinase activity.

Weather Data

Precipitation was greatest in 2001, with a total of 259.15 mm rainfall from 1 June to 30 September. In 2002 and 2003, the total precipitation for this time period was 219.65 mm and 154.02 mm, respectively. The soil temperatures at 50.8 mm and 101.6 mm fluctuated, but were similar for 2001, 2002, and 2003.

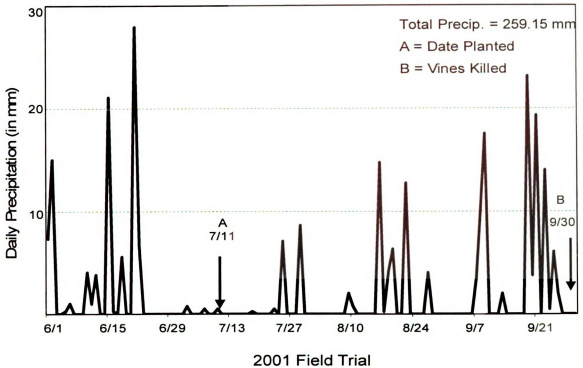

Figure 3-11. Daily precipitation data collected from Laingsburg, Michigan for the 2001 field trial.

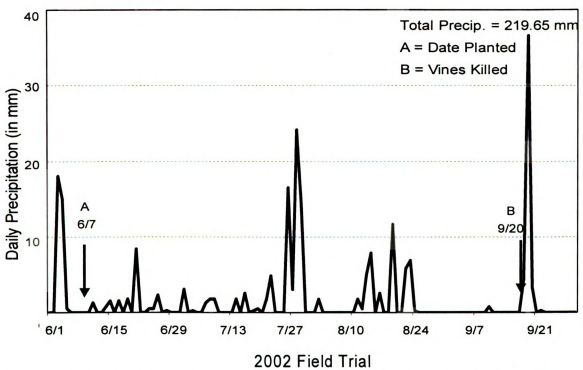

Figure 3-12. Daily precipitation data collected from Laingsburg, Michigan for the 2002 field trial.

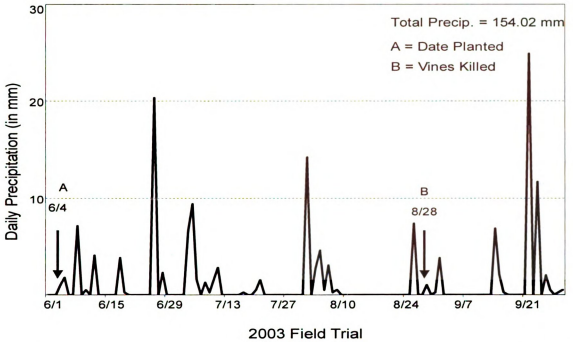

Figure 3-13. Daily precipitation data collected from Laingsburg, Michigan for the 2003 field trial.

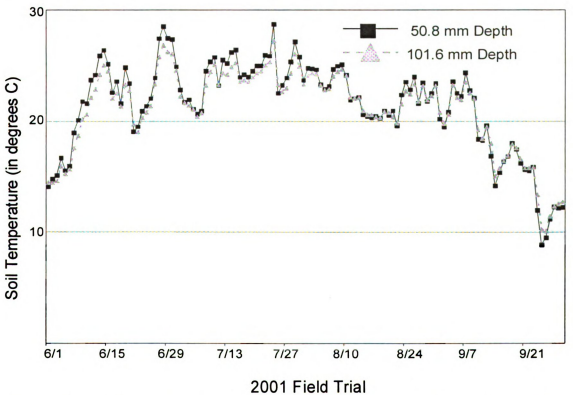

Figure 3-14. Daily average soil temperature data taken from depths of 50.8 mm and 101.6 mm collected from Laingsburg, Michigan for the 2001 field trial.

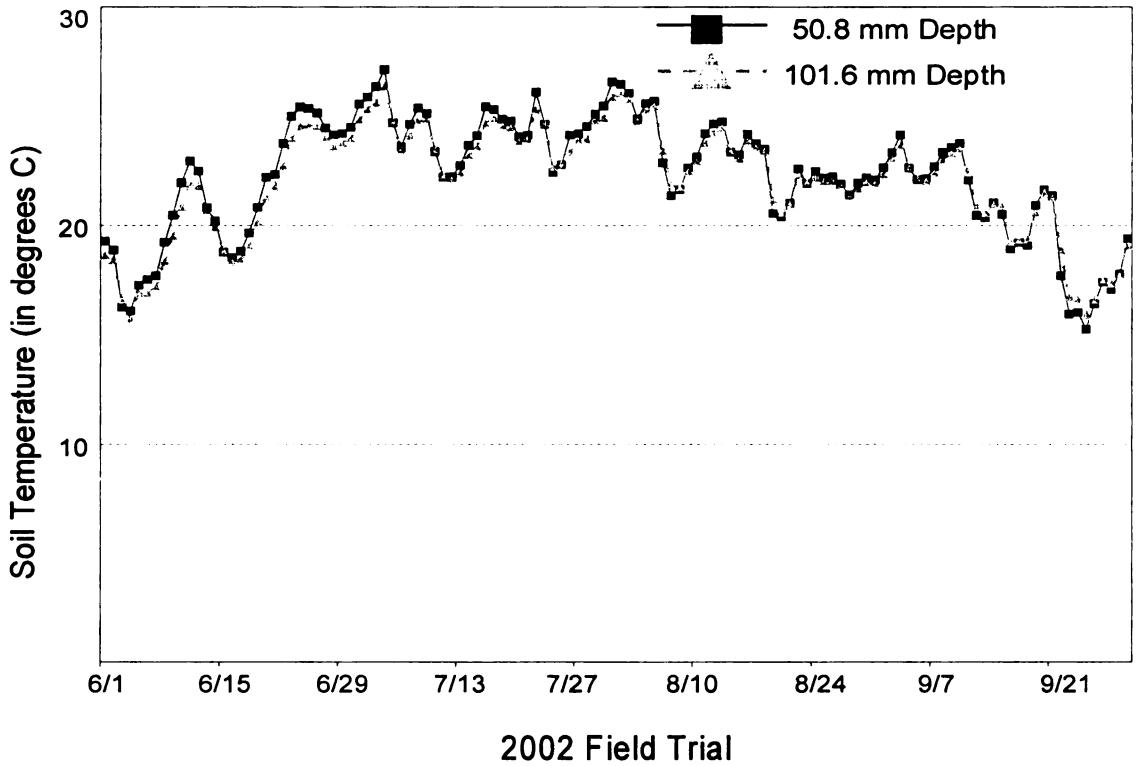

Figure 3-15. Daily average soil temperature data taken from depths of 50.8 mm and 101.6 mm collected from Laingsburg, Michigan for the 2002 field trial.

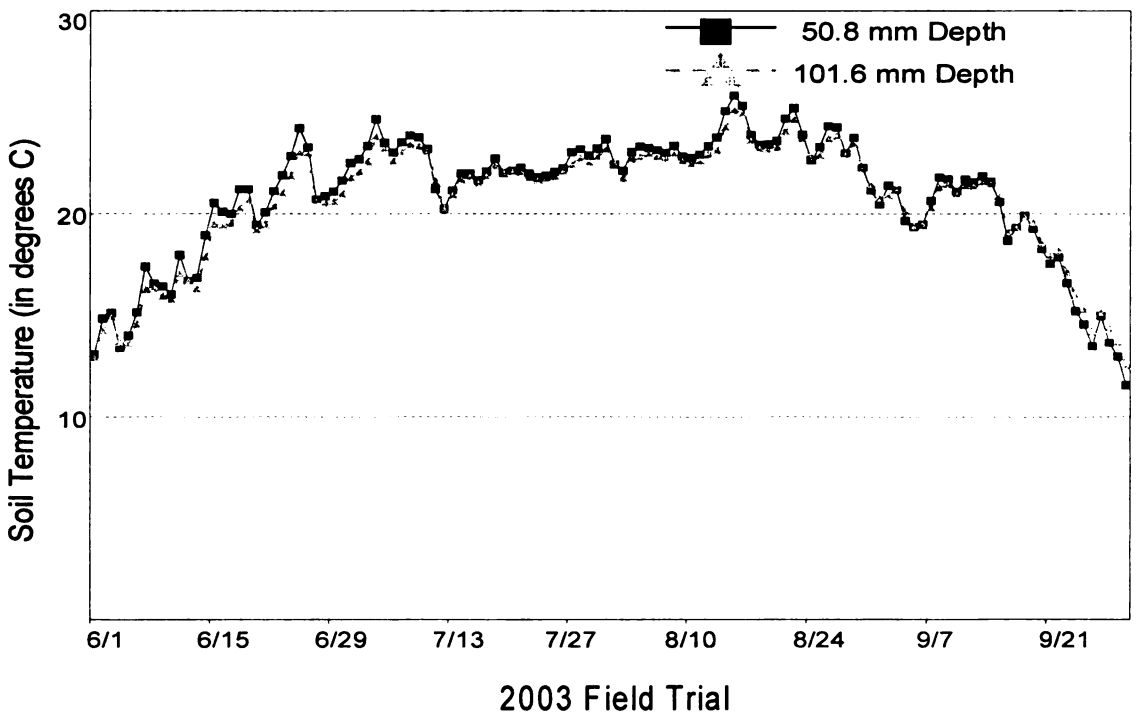

Figure 3-16. Daily average soil temperature data taken from depths of 50.8 mm and 101.6 mm collected from Laingsburg, Michigan for the 2003 field trial.

DISCUSSION

Chitosan has been used as an elicitor for plant protection from pathogens (Hadwiger *et al.*, 1984). Treatment with chitosan enhanced resistance of tomato to *Fusarium oxyporum* f. sp. *radicis-lycopersici* by causing a reduction in the number of lesions caused by the pathogen and by increasing the constitutive physical barriers in tissues (Benhamou and Thériault, 1992). Seed treatments with chitosan were effective in reducing fungal growth in plants by mechanisms characteristic of SAR, including increased production of phenolic compounds and β-1,3-glucans (Benhamou *et al.*, 1994). Chitosan elicited resistance in potato to *Phytophthora infestans* by reducing the number of necrotic cells compared to the control (Vasyukova *et al.*, 2000). Chitosan also reduced disease in peanut against leaf rust caused by *Puccinia arachidis* by reducing the number and size of lesions as well as reduced sporulation and was accompanied by increases is salicylic acid, chitinase and glucanase activity (Sathiyabama and Balasubramanian, 1998). Chitosan activated protective mechanisms in tomato against the root-knot nematode, *Meloidogyne incognita* (Vasyukova *et al.*, 2001). In this study in 2001, only the pre-emergence chitosan application produced slightly more disease free tubers than the untreated control (Figure 3-1). However, this small increase was not correlated with an increase in chitinase activity (Table 3-14), suggesting SAR was not responsible for the difference. More likely pathogen population variability and heterogeneity of soil conditions may have been more important in this difference. Although other small differences were noted, the standard error of the mean negates the other chitosan treatments as ineffective in reducing scab incidence and severity. Since the majority of

the harvested tubers in 2001 were free of disease or had 5% or less scab cover on tuber surface area, regardless of treatment (Figure 3-1), it is very likely that the environmental conditions were not highly favorable for scab development in this year. Plant emergence in 2001 was not fully uniform through early plant development at 14 and 16 days after planting (Table 3-11), but the differences had no effects on total tuber yield (Table 3-6) and were more likely due to heterogeneity within the field, seed health, or presence of seed rotting pathogens rather than effects of resistance activators. There were no negative effects of chitosan on tuber yield or tuber size in 2001 (Table 3-6, Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-5). In 2002, the in-furrow chitosan application produced slightly more disease free tubers by a few percent than the untreated control (Figure 3-2). However, because these differences were not substantially better than the control (Figure 3-2) and because only small increases in chitinase activity were detected (Table 3-14), heterogeneity of soil conditions and pathogen dispersal within the field more likely explain these differences. Foliar applications of chitosan were not effective in reducing disease severity. In 2002, the severity of scab was greater than in 2001. The majority of tubers harvested were rated with infections in the 6-10% and 11-25% scab coverage categories. The higher disease severity in 2002 suggests that perhaps disease pressure and environmental conditions were more conducive for scab infection than in the previous year, particularly precipitation (Figures 3-11, 3-12, 3-13, 3-14, 3-15, and 3-16). The low infection rates and lack of severe infections in the disease category of 50-100% scab coverage are probably better explained by weather conditions during early tuber development or other soil factors, such as pathogen distribution. Plant emergence in 2002 was somewhat uniform during early plant development, but differences were

noted 31 days after planting, but were more obvious by the time germination had ceased and plants were last counted (Table 3-12). There were no negative effects that resulted the from in-furrow application of chitosan on yield; however, there were negative effects that resulted from the chitosan foliar treatment. Plants treated with foliar chitosan applications had the lowest Grade A (Table 3-9), marketable (Table 3-7 and Figure 3-6), and total yields (Table 3-6 and Figure 3-6) than all other treatments. However, this difference is likely explained by plant emergence (Table 3-12). Plant emergence was lowest for this treatment combination as well. However, the reduced emergence for this compound and treatment method was not observed in 2001 and 2003, which suggests that poor field conditions, seed health, or presence of seed rotting pathogens rather than negative effects of the natural compound on plant germination may have been responsible for the reduced emergence and hence, yield. In 2003, neither of the chitosan applications was effective in reducing scab incidence or severity. Like 2002, the majority of tubers harvested in 2003 were classified with infections in the 6-10% and 11-25% scab coverage categories (Figure 3-3). This suggests that the similar disease severities in 2002 and 2003 may have been due to similar disease pressures and environmental conditions between these two years and less total precipitation than in 2001 (Figures 3-11, 3-12, 3-13, 3-14, 3-15, and 3-16). Plant emergence in 2003 was quite variable at 19 days after planting; however, these differences were negligible by 27 days after planting (Table 3-13). There were no negative effects of chitosan on tuber yield or size (Table 3-6, Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-7).

Harpin, a protein originally isolated from *Erwinia amylovora*, the bacterium that causes fire blight of pome fruits (Wei *et al.*,1992) is an elicitor of plant resistance. Since

its discovery, a number of other harpins have been isolated from other genera of gram negative bacteria. The harpin proteins are responsible for eliciting the development of the hypersensitive response (HR), which is a rapid, localized cell death that is associated with many broad-spectrum host defensive responses, including generation of reactive oxygen species and lignification of the host cell wall. In addition to the HR, the resistance activator harpin has been correlated with induction of mitogen-activated protein kinase activity (Desikan *et al.*, 1999), which is an important component of signaling during plant pathogen interactions (Ligterink *et al.*, 1997). SAR induction by harpin is mediated through salicylic acid and the *NIM1* gene (Dong *et al.*, 1999). One study found that transgenic potato plants expressing harpin protein resulted in reductions in the growth rate of *Phytophthora infestans*, and concluded that the hypersensitive response in potato was a promising approach to enhance disease resistance (Li and Fan, 1999). Strobel *et al.* (1996) found that a harpin protein purified from *Pseudomonas syringae* pv. *syringae* 61 was able to induce SAR in cucumber to *Colletotrichum lagenarium*, *Pseudomonas syringae* pv. *lachrymans* and tobacco necrosis virus. Tobacco leaves sprayed with harpin resulted in the HR and production of PR-proteins (Peng *et al.*, 2003). They also found that harpin, when sprayed on mutant *Arabidopsis* plants deficient in SAR regulatory genes, failed to induce SAR and plants were unable to produce the HR. This emphasizes the requirement of the HR for SAR. A recent study found that harpin, when combined with a bacteriophage, significantly reduced bacterial spot of tomato caused by *Xanthomonas campestris* pv. *vesicatoria* and increased total marketable yields (Obradovic *et al.*, 2004). Two studies found that harpin significantly reduced blue mold infection in apple caused by *Penicillium expansum* (de Capdeville *et al.*, 2002 and

de Capdeville *et al.*, 2003). Spraying plants with harpin three days before harvest in the latter study found that harpin effectively reduced the infection by the pathogen that causes fruit decay (and produces patulin, a carcinogenic mycotoxin) and could lead to fewer post-harvest fungicide applications in apple production. Like chitosan, harpin is able to protect against a broad range of phytopathogens. Also, because it is derived from a bacterium, it is a low risk, biodegradable biopesticide considered much safer than conventional pesticides. In this study in 2001, all four harpin treatments did not reduce the incidence or severity of scab infections. Slight, yet significant, differences in the production of chitinase, a commonly used marker for systemic acquired resistance, were detected in tuber tissue. However, the greatest activity of chitinase was detected in the tuber tissue from plants that were untreated; therefore, induced resistance is not correlated with the low levels of scab infection on the tubers or any increases in the amount of disease free tubers (Table 3-14). Although plant emergence in 2001 was not fully uniform through early plant development at 14 and 16 days after planting (Table 3-11), these slight differences had no effects on total tuber yield (Table 3-6) and were more likely due to heterogeneity within the field or seed health rather than effects of resistance activators. There were no negative effects of harpin on tuber yield or tuber size (Table 3-6. Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-5). In 2002, harpin foliar and in-furrow treatments did not decrease the incidence and severity of scab on tubers (Figure 3-2), but neither treatment reduced the marketability of the tubers. Plant emergence in 2002 was somewhat uniform during early plant development, but differences were noted 31 days after planting, but were more obvious by the time germination had ceased and plants were last counted (Table 3-12). There were no

negative effects of harpin on tuber size or yield in 2002 (Table 3-6, Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-6). In 2003 harpin in-furrow and foliar treatments produced more than 10% fewer tubers in the 5% or less scab infection marketable category (Figure 3-3), which suggests a possibility that harpin may have either negatively affected the host by altering its response to infection by pathogenic *Streptomyces* species or perhaps by favoring the group of pathogens in some unidentified manner. Furthermore, harpin in-furrow and foliar applications resulted in nearly 10% increases in severe scab infections in the 26-49% and 50-100% categories, respectively (Figure 3-3). This also suggests that harpin treatments may favor scab development, although it is possible that variability of pathogen populations and ideal soil conditions may have been more prevalent in plots where plants were treated with harpin. Chitinase activity of tuber tissue was highest for plants treated with foliar applications of harpin, which also produced tubers with the highest scab severity of any treatment; therefore, no correlations between chitinase activity and disease severity could be made (Table 3-14). Plant emergence in 2003 was quite variable at 19 days after planting; however, these differences were negligible by 27 days after planting (Table 3-13). There were no negative effects of harpin on tuber yield or size (Table 3-6, Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-7).

Saponins are a diverse group of anti-fungal secondary metabolites that serve as constitutive chemical defenses that ward off pathogenic microbes. Although primarily produced by dicots, the saponins produced by oats are both steroidal and triterpenoid saponins. Papadopoulou *et al.* (1999) found that oat mutants deficient in saponin production had compromised resistance to disease and concluded that saponins were

involved in disease resistance. Another study by Haralampidis *et al.* (2001) published similar findings using saponin-deficient mutants. The authors postulated that saponin production may have been lost in many modern cereals during selection and suggests that a gene involved in saponin production may be utilized in crops. Although one of the requirements of resistance activators is that they are not antimicrobial, a recent review on the mechanisms and applications of induced resistance cites examples of fungicidal compounds as effective activators of resistance (Hammerschmidt, 2004). This suggests that antimicrobial compounds may signal changes in the host response that lead to increased resistance or that the fungicide application may result in the release of elicitors by the pathogens or some other unidentified indirect mode of action. In this study, the foliar application of saponins produced slightly more disease free tubers by a few percent than the untreated control in 2002 (Figure 3-2). However, because these differences were not substantially better than the control (Figure 3-2) and because only small increases in chitinase activity were detected (Table 3-14), heterogeneity and variability of environmental conditions and pathogen dispersal within the field more likely explain these differences. Plant emergence, yield and tuber size were not negatively influenced by treatment with saponins (Table 3-12, Table 3-6. Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-6). In 2003, saponin foliar and in-furrow treatments did not reduce scab incidence or severity (Figure 3-3) and did not increase chitinase production in tubers (Table 3-14). Plant emergence in 2003 was quite variable at 19 days after planting; however, these differences were negligible by 27 days after planting (Table 3-13). There were no negative effects of the natural compounds on tuber yield or size (Table 3-6, Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-7).

Formononetin (7-hydroxy-4'-methoxyisoflavone, also known as 4'-O-methyldaidzein) is an intermediate of bioactive isoflavonoids isolated from leguminous plant species. Recently, peanut plants inoculated with *Bradyrhizobium* spp. responded by production of two phytoalexins, medicarpin and formononetin (Azpilicueta *et al.*, 2004). Formononetin was active against *Cladosporium cucumerinum* and the authors concluded that the phytoalexins were involved in defense against pathogen attack. In a recent study, formononetin crown soak and soil drench applications increased the amount of vesicular arbuscular mycorrhizae and reduced the percentage of lesions on roots on asparagus caused by *Fusarium* species (Elmer, 2002). In this study in 2002, foliar and in-furrow applications of formononetin produced tubers with the highest detected chitinase production (Table 3-14), yet with the least beneficial effects on reducing scab incidence and severity. In fact, formononetin foliar applications significantly reduced the percentage of tubers that had 5% or less scab infection when compared to the untreated control (Figure 3-2); therefore, negatively affecting tuber marketability. Plant emergence, yield, and tuber size were not affected by formononetin foliar and in-furrow applications (Table 3-12, Table 3-6, Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-6). In 2003, neither of the formononetin treatments substantially reduced scab infection rate, severity, or induced resistance (Figure 3-3). Plant emergence in 2003 was not influenced by formononetin treatments (Table 3-13). There were no negative effects of formononetin on tuber yield or size (Table 3-6, Table 3-7, Table 3-8, Table 3-9, Table 3-10, and Figure 3-7).

Saponins, formononetin, chitosan, and harpin did not reduce infection when challenged with *Streptomyces scabies* (Figure 3-9). The challenged tuber tissue was

necrotized at similar severities for all four natural compounds and application methods. None of the treatments substantially reduced necrosis at any depth of tuber tissue that was tested (Figure 3-9). When the chitinase activity of the challenged tissue was assessed, none of the treatments resulted in an increased activity that correlated with a decrease in scab incidence or severity of the harvested tubers (Figure 3-3) or the challenged tuber tissue (Figure 3-9). The only treatment with significant increases in chitinase activity after challenge by *S. scabies* was the harpin foliar treatment (Figure 3-10), which also resulted in tubers with the highest scab incidence and severity of any of the nine treatments in all 3 years tested. In this study, none of the treatments with natural compounds were effective in reducing scab incidence or severity, inducing resistance, or decreasing infections after pathogen challenge. This indicates that other natural compounds should be assessed, perhaps in combination with other scab disease management strategies. It is likely that integrating strategies, such as combining resistance activators with biocontrol agents, soil moisture management, cultivar resistance, composts, or compounding more than one resistance activator may be effective in reducing the severity of scab infections. The combined effects of harpin and a bacteriophage were effective in reducing bacterial spot tomato (Obradovic *et al.*, 2004). The combined effects of *Streptomyces melanosporofaciens* and chitosan, a resistance activator and growth stimulator of streptomycetes, (Clermont and Beaulieu, 2003) were effective in reducing potato common scab. As these studies, and others, have demonstrated, integrating strategies is critical to achieve effectiveness when using resistance activators.

Chapter IV

EVALUATION OF FOUR ANTIMICROBIAL COMPOUNDS ON GROWTH INHIBITION OF THREE *STREPTOMYCES SCABIES* STRAINS *IN VITRO.*

INTRODUCTION

Chemical control of common scab of potatoes has been extensively studied, yet there are no chemicals currently registered for scab control. The fungicide pentachloronitrobenzene (PCNB) has been used to control common scab in many studies (Curwen, 1980; Nugent, 1956; Curwen, 1978; Davis *et al.*, 1974; Davis *et al.*, 1976; Fink, 1956: Menzies, 1957; Potter *et al.*, 1959: Hooker, 1954; and Houghland and Cash, 1954). However, treatment with PCNB led to decreases in yield and phytotoxicity In addition, PCNB was ineffective at low soil water potentials and required excessively high rates for efficacy.

Analogues of daminozide (*N*-dimethylaminomaleamic and *N*- (dimethylamino)-methylsuccinamic acids) were found to decrease scab incidence (McIntosh and Bateman, 1979). Another study tested 30 compounds in an attempt to identify an effective scab control foliar spray. Of those tested, 3,5-dicholorphenoxyacetic acid (3,5-D), was the most effective. None of the other compounds tested were more effective than 3,5-D. Some compounds that were as effective also demonstrated phytotoxicity (McIntosh *et al.*, 1985). Substituted benzoic and picolinic acids reduced scab in greenhouse experiments, but they were also phytotoxic (McIntosh and Bateman, 1988). Forty quinones, polyhydroxybenzenes and related derivatives were also tested against common scab (McIntosh, 1976). Unsubstituted and phenyl-, chloro-, 2,5- and 2,6-dichloro-compounds

were effective as quintozene and 4-*tert*-butyl catechol was more effective, but yields were sometimes reduced.

Captafol (McIntosh, 1977 and McIntosh, 1973) and ethionine (McIntosh and Burrell, 1980) were effective in decreasing scab severity, with little to no reduction in yield, but these are unavailable to control scab disease. Sulfur and soil fumigation with Telone C-17 reduced *Streptomyces* soil rot, a disease of sweet potato caused by a related pathogen, *S. ipomoea*. Fumigation proved effective, but the sulfur treatments significantly reduced yield (Ristaino and Averre, 1992). More recently, efforts to control common scab with sodium hypochlorite and thiophanate-methyl seed treatments provided no significant reductions in scab infections (Errampalli and Johnston, 2000).

Few studies have evaluated antimicrobial chemicals in relation to *in vitro* toxicities of *Streptomyces scabies*. Daminozide, even at high concentrations in potato dextrose agar, was only weakly toxic to three *S. scabies* isolates. Quintozene (PCNB) was much more toxic to *S. scabies* (McIntosh and Bateman, 1979). Six dichlorobenzoic acids were tested against *S. scabies*. The 2,5-isomer was the most effective in controlling scab in plants, yet was the least toxic in culture tests (McIntosh *et al.*, 1988), indicating that the control attained was not due to antibiotic activity of the compound and was possibly an effect on the plant. McIntosh *et al.* (1981) and Burrell (1982) showed that 3,5-D was no more toxic *in vitro* than other disubstituted acids that were only slightly active against scab development in field tests. Both studies suggested the mode of action of the compound is alteration of the host, suggesting systemic acquired resistance could have been involved in the reduction of infection. Due to the apparent ability of *S. scabies* to grow and survive at high concentrations of broad ranges of microbicides, the goal of

this study is to examine the effects of four antimicrobial compounds on the growth of three *S. scabies* strains *in vitro*.

MATERIALS AND METHODS

Rationale for the Design of Experiment

Four antimicrobial compounds were evaluated for their ability to inhibit growth of *Streptomyces scabies*. Vancomycin, a glycopeptide antibiotic produced by *Streptomyces orientalis* that interferes with cell wall synthesis (Nieto and Perkins, 1971), and gentamicin, an aminoglycoside antibiotic produced by *Micromonospora purpurea* that inhibits protein synthesis by binding to L6 protein of the 50 S ribosomal subunit (Lorian, 1986, Korzybski *et al.*, 1978), were selected for evaluation. Both antibiotics target gram-positive bacteria and they each have different modes of action. Pentachloronitrobenzene (PCNB), which inhibits protein synthesis by reacting with $-NH_2$ groups and –SH groups of proteins (Agrios, 1997), and copper, which inactivates some enzymes and denatures non-specific proteins (Cu^{2+} reacts with the sulfhydryl groups of cysteine) (Agrios, 1997), were selected for evaluation because both antimicrobial compounds are well-documented agricultural pesticides. These four compounds were tested at five concentrations against three *Streptomyces scabies* strains: 88-21, DP, and Onaway (Table 4-1).

Antimicrobial Compound Screening

PDA was purchased from Sigma Chemical Company (St. Louis, Missouri, USA) and prepared as described by Sigma (39.0 g PDA mixture/l distilled water). Vancomycin hydrochloride, gentamicin solution, PCNB, and copper (II) sulfate, pentahydrate (all purchased from Sigma Chemical Company) were prepared at 0.1, 1, 10, 100, and 1000

ppm stock solutions. After sterilization when the PDA had cooled to 50°C, vancomycin, gentamicin, PCNB, and copper sulfate were added at the five concentrations and stirred with a magnetic stirrer to ensure equal distribution of the compound throughout the medium. Each solution was applied in 25 ml aliquots into 100 mm x 15 mm polysterene petri dishes. The medium was allowed to cool and surface was dried before use. Spores were harvested from 12 day old cultures of *Streptomyces scabies* strains 88-21, DP, and Onaway (grown at 30°C on PDA on 100 mm x 15 mm petri dishes in the dark) with a sterile spatula. Spores were suspended in 0.1 % Tween® 80 (Sigma Chemical Company, St. Louis, Missouri, USA) as a wetting agent due to their hydrophobicity. The concentration was adjusted to 10^{10} spores/ml with a hemacytometer. Two hundred microliters of the spore suspension were inoculated onto the medium containing the antimicrobial compounds with a glass spreader. The agar surface of the petri dishes was allowed to dry, sealed with Parafilm® M (purchased from Sigma Chemical Company, St. Louis, Missouri, USA), and stored in the dark at 22°C. Uninoculated media and unamended media were used as average reflective intensity (see below) and negative controls, respectively. Seven days after inoculation, growth of the isolates was assessed. Twenty replications per each compound per concentration were tested against each *S. scabies* strain in two independent trials.

TABLE 4-1. Concentrations, modes of action, and antimicrobial spectra of four antimicrobial compounds tested *in vitro* against three *Streptomyces scabies* strains.

Antimicrobial Compound and Concentrations (in ppm)	*Streptomyces scabies* Strains	Mode of Action	Antimicrobial Spectrum
Pentachloronitrobenzene			Fungi;
0.1			Gram-positive
1		protein	bacteria;
10	88-21, DP, Onaway	synthesis	Gram-negative
100		inhibitor	bacteria;
1000			mycoplasmas
Vancomycin hydrochloride			
0.1			
1		cell wall	Gram-positive
10	88-21, DP, Onaway	synthesis	bacteria
100		inhibitor	
1000			
Gentamicin sulfate			
0.1			Gram-positive
1		protein	bacteria;
10	88-21, DP, Onaway	synthesis	Gram-negative
100		inhibitor	bacteria;
1000			mycoplasmas
Copper (II) sulfate			
0.1		enzyme	
1		inactivation;	Fungi;
10	88-21, DP, Onaway	protein	Gram-negative
100		denaturation	bacteria;
1000			

Image Analysis of Growth Inhibition

Seven days after inoculation, petri dishes were scanned using a HP Scanjet® 3670 Scanner (Hewlett-Packard Development Company, L.P., Palo Alto, California, USA) using the highest resolution and color photograph settings so that the images could be used for image analysis. Two parameters, average reflective intensity (ARI) values and area of growth, were used to evaluate the effects of the compounds on the growth

inhibition of the *Streptomyces scabies* strains. ARI values, which are based on how light or dark a pixel is and the intensity of light that is reflected from each pixel, were determined using the Sigma Scan Pro5® image analysis software program (SPSS UK, Surrey, UK). All petri dish images were calibrated based on size and analyzed with the same light intensity and manual threshold settings for the ARI measurement to ensure all images were identically measured . Growth area measurements were made using the APS Assess® image analysis software (American Phytopathological Society Press, St. Paul, Minnesota, USA). All petri dish images were calibrated based on size and analyzed under identical settings of intensity and leaf as area of interest setting (medium and bacteria were analyzed as leaves and lesions, respectively) to ensure consistency amongst measurements.

Data Analysis

Average growth area and average reflective intensity data from the trials of the four compounds were analyzed with a t-test with comparison of least significant difference to determine the differences between growth inhibition compared across the concentrations tested. For all statistical analyses, an outcome was deemed statistically different at the confidence interval of 5% or less (alpha = 0.05). The LSD (least significant difference) of the means for t-tests were conducted using SAS® version 8 software (SAS Institute, Inc.; Cary, North Carolina).

RESULTS

Copper (II) sulfate

Copper sulfate at 0.1 ppm only slightly reduced the growth of the *Streptomyces scabies* strain DP from 7,652 mm^2 to 7,397 mm^2 (Table 4-2). Although this difference in growth was significant from the untreated control, 0.1 ppm clearly had no apparent inhibitory effects on DP. An increase in copper sulfate to 1.0 ppm further inhibited growth of DP to 7,115 mm^2. However, a ten-fold increase to 10 ppm did not substantially decrease growth any further than 0.1 ppm since statistical analysis found 1.0 and 10 ppm to result in similar areas of growth (Table 4-2). At 100 ppm, the average growth area of DP was further inhibited to 6,659mm^2. Only at 1,000 ppm was the growth of DP completely inhibited (Table 4-2). When average reflective intensity (ARI) data were used to analyze the growth of DP, no significant differences were found between the untreated control (0 ppm) and 0.1 ppm copper sulfate, which were 156 units and 153 units, respectively (Table 4-3). Detectable reductions in growth were found with ARI values of DP when grown on copper sulfate at 1.0 ppm (Table 4-3). ARI measurements, like the growth measurements, found 10 ppm and 100 ppm to have similar effects of growth inhibition of DP (Table 4-2 and Table 4-3). ARI values for DP grown on medium amended with 1,000 ppm copper sulfate were drastically lower than all of the other ARI values determined for the other concentrations of this compound (Table 4-3).

The *S. scabies* strain 88-21 responded similarly to DP when grown on medium amended with 0.1 ppm copper sulfate. Although growth was statistically different from the untreated control (reduced from 7,730 mm^2 on unamended medium to 7,395 mm^2 on 0.1 ppm copper sulfate), the difference in growth was not substantial (Table 4-2). Copper

sulfate at 1.0 and 10 ppm significantly reduced the growth of strain 88-21 to levels that were statistically identical at 6,993 mm^2 and 6,803 mm^2, respectively (Table 4-2). Like DP, 88-21 was even further inhibited to 6,486 mm^2 as the concentration of copper sulfate was increased to 100 ppm (Table 4-2). The growth of strain 88-21 was completely inhibited at 1,000 ppm copper sulfate (Table 4-2). When ARI values are used to compare the effects of copper on growth of 88-21, no significant differences were found between 0 and 0.1 ppm (Table 4-3). Differences in growth were detected when copper sulfate was used at 1.0 ppm, as the ARI values for the untreated control and 0.1 ppm copper sulfate were 154 units, and was reduced to 145 units with the ten-fold increase to 1.0 ppm (Table 4-3). Copper sulfate at concentrations of 10 and 100 ppm decreased ARI values both significantly and incrementally (Table 4-3). The no growth ARI values for strain 88-21 were significantly reduced at 1,000 ppm copper sulfate when compared to 100 ppm (Table 4-3).

Similar to the results of the other two *S. scabies* strains, the growth of Onaway was significantly reduced from 7,661 mm^2 to 7,023 mm^2 when grown on medium amended with 0.1 ppm copper sulfate (Table 4-2). Copper sulfate at 1.0 ppm significantly reduced the growth of the Onaway strain even further to 6,819 mm^2 (Table 4-2). Unlike the other strains, however, the average growth area of Onaway at 10 ppm and 100 ppm of copper sulfate produced statistically similar results at 6,523 mm^2 and 3,633 mm^2, respectively. For the other two strains, 100 ppm copper sulfate resulted in a statistically significant reduction in growth when compared with 10 ppm (Table 4-2). Consistent with the results of the other two strains, medium amended with 1,000 ppm copper sulfate completely inhibited the growth of Onaway (Table 4-2). When ARI data

were used to compare the effects of copper sulfate on growth inhibition of Onaway, quite different results were obtained when compared to the strains DP and 88-21. ARI values were not significantly different from the untreated control and 0.1, 1.0, and 10 ppm copper sulfate at 168, 169, 167, and 161 units, respectively (Table 4-3). Differences in growth were detectable with ARI values obtained for 100 ppm copper sulfate (Table 4-3). Consistent with the other strains, ARI values for Onaway were significantly reduced at 1,000 ppm copper (Table 4-3).

TABLE 4-2. Comparison of effects of five concentrations of copper (II) sulfate on the average area of growth of three *Streptomyces scabies* strains.

S. scabies strain	0.1 ppm A (mm^2)±SE	1.0 ppm A (mm^2)±SE	10 ppm A (mm^2)±SE	100 ppm A (mm^2)±SE	1000 ppm A (mm^2)±SE	0 ppm A (mm^2)±SE
DP	7397 54.2 b	7115 40.5 c	7080 40.3 c	6659 96.0 d	0 e	7652 31.1 a
88-21	7395 66.8 b	6993 73.0 c	6803 64.8 c	6486 147.5 d	0 e	7730 29.6 a
Onaway	7023 81.1 b	6819 76.3 b	6523 43.4 c	6366 130.7 c	0 d	7661 39.2 a

A area
SE standard error of the mean
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test at a = 0.05.

TABLE 4-3. Comparison of effects of five concentrations of copper (II) sulfate on the growth of three *Streptomyces scabies* strains as determined by average reflective intensity.

S. scabies strain	0.1 ppm ARI ± SE	1.0 ppm ARI ± SE	10 ppm ARI ± SE	100 ppm ARI ± SE	1000 ppm ARI ± SE	0 ppm ARI ± SE
DP	153 2.0 a	151 1.9 ab	140 1.9 b	141 2.6 b	42 (NG) 0.4 c	156 1.7 a
88-21	154 3.0 a	145 2.8 ab	137 2.5 bc	128 3.1 c	43 (NG) 0.4 d	154 3.3 a
Onaway	169 1.8 a	167 1.4 a	161 1.9 a	129 5.4 b	43 (NG) 0.3 c	168 2.0 a

ARI average reflective intensity
SE standard error of the mean
NG No growth detected
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test at a = 0.05.

Gentamicin

Without antibiotics, strain DP grew at an average area of 7,652 mm^2 (Table 4-4). Addition of 0.1 ppm gentamicin to the medium significantly reduced the growth to 6,999 mm^2 (Table 4-4). A ten-fold increase of gentamicin to 1.0 ppm did not further decrease the growth of DP, and the amount of growth at this concentration was very similar to the growth that resulted from 0.1 ppm gentamicin (Table 4-4). Gentamicin at 10, 100, and 1,000 ppm completely inhibited the growth of DP (Table 4-4). The ARI of DP without gentamicin was 156 units (Table 4-5). This value was significantly reduced to 135 and 136 units for 0.1 and 1.0 ppm gentamicin, respectively (Table 4-5). The ARI values did not detect differences in growth between 0.1 and 1.0 ppm concentrations. ARI values

were similar across all three gentamicin concentrations that resulted in no growth (Table 4-5).

The growth of *S. scabies* strain 88-21 was significantly reduced from 7,730 mm^2 on the unamended medium to 1,034 mm^2 when 0.1 ppm gentamicin was added (Table 4-4). Increasing the antibiotic concentration to 1.0 ppm further decreased the average growth area of 88-21 to 6,227 mm^2 (Table 4-4). Like DP, 88-21 was unable to grow on gentamicin at concentrations of 10, 100, and 1,000 ppm (Table 4-4). Strain 88-21 has an ARI value of 154 units when grown without antimicrobial compounds (Table 4-5). This value was significantly reduced to 148 and 140 units, respectively when 0.1 and 1.0 ppm gentamicin was added to the growth medium (Table 4-5). As with DP, differences in growth between 0.1 and 1.0 ppm gentamicin were not detected with ARI values (Table 4-5). ARI values for 10, 100, and 1,000 ppm gentamicin, concentrations that resulted in no growth, were very similar Table (4-5).

The effects of gentamicin on Onaway were similar to the 88-21 strain. Gentamicin at 0.1 ppm concentration significantly reduced growth when compared to the untreated control from 7,661 mm^2 to 6,514 mm^2 (Table 4-4). When the concentration of gentamicin was increased to 1.0 ppm, the average growth area of Onaway was further significantly inhibited to 6,018 mm^2 when compared to the growth that resulted from treatment with 0.1 ppm gentamicin (Table 4-4). Consistent with the responses to the other two strains, Onaway was unable to grow in the presence of 10, 100, and 1,000 ppm gentamicin (Table 4-4). The growth of Onaway was reduced significantly from 168 units without gentamicin to 145 units with the addition of 0.1 ppm gentamicin. Medium amended with 1.0 ppm gentamicin did not further decrease growth according to the ARI

values (Table 4-5). The ARI values were consistent at 10, 100, and 1,000 ppm, which were categorized as no growth detected (Table 4-5).

TABLE 4-4. Comparison of effects of five concentrations of gentamicin on the average area of growth of three *Streptomyces scabies* strains.

S. scabies strain	0.1 ppm A (mm2) ± SE		1.0 ppm A (mm2) ± SE		10 ppm A (mm2)±SE		100 ppm A (mm2)±SE		1000 ppm A (mm2)±SE		0 ppm A (mm2) ± SE	
DP	6999	50.6 b	6930	67.8 b	0	c	0	c	0	c	7652	31.1 a
88-21	7034	71.3 b	6227	172.9 c	0	d	0	d	0	d	7730	29.6 a
Onaway	6514	221.4 b	6018	197.5 c	0	d	0	d	0	d	7661	39.2 a

A area of growth
SE standard error of the mean
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test at a = 0.05.

TABLE 4-5. Comparison of effects of five concentrations of gentamicin on the growth of three *Streptomyces scabies* strains as determined by average reflective intensity.

S. scabies strain	0.1 ppm ARI ± SE		1.0 ppm ARI ± SE		10 ppm ARI ± SE		100 ppm ARI ± SE		1000 ppm ARI ± SE		0 ppm ARI ± SE	
DP	135	3.1 b	136	4.0 b	48(NG)	0.1 c	47 (NG)	0.2 c	43 (NG)	0.1 c	156	1.7 a
88-21	138	5.2 b	140	3.1 b	43 (NG)	0.1 c	44 (NG)	0.2 c	43 (NG)	0.1 c	154	3.3 a
Onaway	145	4.0 b	144	3.9 b	45 (NG)	0.1 c	43 (NG)	0.4 c	43 (NG)	0.1 c	168	2.0 a

ARI average reflective intensity
SE standard error of the mean
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test at a = 0.05.

Pentachloronitrobenzene

Pentachloronitrobenzene (PCNB) at 0.1 ppm significantly reduced the growth of strain DP to 7,369 mm^2 when compared to the untreated control at 7,652 mm^2 (Table 4-6). When the concentration of PCNB was increased to 1.0 ppm, growth was again significantly reduced to 7,121 mm^2; however, an increase to 10 ppm reduced the growth to 6,905 mm^2, which was not statistically different from growth that resulted from 1.0 ppm PCNB. Growth of DP was significantly reduced at 100 ppm PCNB when compared to the 1.0 and 10 ppm concentrations. Only the 1,000 ppm PCNB treatment completely inhibited the growth of DP (Table 4-6). When ARI data were evaluated, no differences in growth of strain DP were found between 0 and 0.1 ppm PCNB, which were 156 and 151 units, respectively (Table 4-7). ARI values for 1.0, 10, and 100 ppm were 122, 122, and 123 units, respectively (Table 4-7). These values were statistically identical and were significantly smaller than the ARI values determined for 0 and 0.1 ppm PCNB (Table 4-7). The ARI value for no growth (1,000 ppm), 43 units, was much smaller compared to the other ARI values for which growth did occur (Table 4-7).

The growth of strain 88-21 was significantly reduced from 7,730 mm^2 to 7,057 mm^2 when 0.1 ppm PCNB was added to the medium (Table 4-6). The average growth area was slightly reduced to 6,955 mm^2 with PCNB at a 1.0 ppm concentration; however, this difference was not statistically significant (Table 4-6). The area of growth of 88-21 was substantially further reduced to 6,430 mm^2 with PCNB at 10 ppm and was again reduced to 5,851 mm^2 when the concentration of PCNB was increased to 100 ppm (Table 4-6). PCNB completely inhibited the growth of 88-21 at the 1,000 ppm concentration (Table 4-6). The ARI value for growth of strain 88-21 grown on medium amended with

0.1 ppm was reduced from 154 units to 144 units (Table 4-7). The ARI value for the 1.0 ppm PCNB treatment was further reduced to 141 units, which was statistically different from the 0.1 ppm value (Table 4-7). The 10 and 100 ppm concentrations of PCNB further reduced ARI values of 96 and 83 units, respectively, which were both statistically significant (Table 4-7). When the concentration of PCNB was increased to 1,000 ppm, the ARI value was severely diminished to 45 units (Table 4-7).

The average growth area of Onaway was significantly reduced from 7,661 mm^2 to 7,063 mm^2 with the addition of 0.1 ppm PCNB to the growth medium (Table 4-6). When the concentration of PCNB was further increased to 1.0 ppm, the average growth area of Onaway was not significantly reduced (Table 4-6). At concentrations of 10 and 100 ppm PCNB; however, growth was significantly reduced to 5,004 mm^2 and further reduced to 4,055 mm^2, respectively (Table 4-6). Like the other two strains, treatment of Onaway with 1,000 ppm PCNB resulted in no growth (Table 4-5). The ARI values obtained for Onaway grown in the presence of 0.1 ppm PCNB were significantly reduced from 168 to 159 units when compared to the untreated control (Table 4-7). An increase in concentration to 1.0 ppm significantly reduced the ARI value to 99 units (Table 4-7). PCNB and 10 and 100 ppm concentrations each significantly reduced ARI values to 72 and 52 units, respectively (Table 4-7). The ARI values determined for 1,000 ppm PCNB were consistent with the ARI values of other treatments that resulted in no growth (Table 4-7).

TABLE 4-6. Comparison of effects of five concentrations of pentachloronitrobenzene on the average area of growth of three *Streptomyces scabies* strains.

S. scabies strain	0.1 ppm A (mm2) ± SE	1.0 ppm A (mm2) ± SE	10 ppm A (mm2) ± SE	100 ppm A (mm2) ± SE	1000 ppm A (mm2)±SE	0 ppm A (mm2) ± SE
DP	7369 56.0 b	7121 58.7 c	6905 50.5 c	6172 164.3 d	0 e	7652 31.1 a
88-21	7057 74.9 b	6955 86.9 b	6430 40.1 c	5851 198.3 d	0 e	7730 29.6 a
Onaway	7063 47.9 b	6877 42.0 b	5004 316.1 c	4055 312.9 d	0 e	7661 39.2 a

A area of growth
SE standard error of the mean
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test at a = 0.05.

TABLE 4-7. Comparison of effects of five concentrations of pentachloronitrobenzene on the growth of three *Streptomyces scabies* strains as determined by average reflective intensity.

S. scabies strain	0.1 ppm ARI ± SE	1.0 ppm ARI ± SE	10 ppm ARI ± SE	100 ppm ARI ± SE	1000 ppm ARI ± SE	0 ppm ARI ± SE
DP	151 3.0 a	122 2.3 b	122 2.6 b	123 4.5 b	43 (NG) 0.2 c	156 1.7 a
88-21	144 2.7 ab	141 2.5 b	96 1.0 c	83 1.4 d	45 (NG) 0.3 e	154 3.3 a
Onaway	159 2.5 b	99 2.2 c	72 1.4 d	52 0.4 e	42 (NG) 0.2 f	168 2.0 a

ARI average reflective intensity
SE standard error of the mean
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test a= 0.05.

Vancomycin

The average area of growth of DP was inhibited to 7,265 mm^2 when grown on a medium amended with 0.1 ppm vancomycin (Table 4-8). When the concentration of vancomycin was increased ten-fold, growth was completely inhibited (Table 4-8). DP was unable to grow at 1.0, 10, 100, and 1,000 ppm concentrations of vancomycin (Table 4-8). The ARI values obtained for growth of DP on medium amended with 0.1 ppm vancomycin averaged 113 units, which was significantly reduced when compared to the untreated control (Table 4-9). The ARI values obtained from 1.0, 10, 100, and 1,000 ppm concentrations, all which resulted in no growth, were similar (Table 4-9).

The average growth area of 88-21 was significantly reduced from 7,730 mm^2 to 7,477 mm^2 when grown in the presence of 0.1 ppm vancomycin (Table 4-8). Like DP, the 88-21 strain was completely inhibited when inoculated on medium amended with 0.1, 10, 100 and 1,000 ppm concentrations of vancomycin (Table 4-8). The averaged ARI value for 88-21 grown on medium containing 0.1 ppm vancomycin was not significantly reduced when compared to the untreated control; however, it was significantly reduced for the other concentrations (Table 4-9). The ARI values for 1.0, 10, 100, and 1,000 ppm vancomycin were consistent with each other (Table 4-9).

The average growth area of Onaway was significantly reduced from 7,661 mm^2 to 7,302 mm^2 when grown on a medium containing 0.1 ppm vancomycin (Table 4-8). As was demonstrated with the other two strains, no growth was detected for Onaway at 0.1, 10, 100, and 1,000 ppm vancomycin (Table 4-8). The ARI value for the growth of Onaway at 0.1 ppm vancomycin was 107 units (Table 4-9). This value was significantly lower than the untreated control, which averaged 168 units (Table 4-9). The ARI values

for 0.1, 10, 100, and 1,000 ppm vancomycin were significantly lower than the 0.1 ppm ARI value (Table 4-9).

TABLE 4-8. Comparison of effects of five concentrations of vancomycin on the average area of growth of three *Streptomyces scabies* strains.

S. scabies strain	0.1 ppm A (mm2) ± SE		1.0 ppm A (mm2) ± SE		10 ppm A (mm2)±SE		100 ppm A (mm2)±SE		1000 ppm A (mm2)±SE		0 ppm A (mm2) ± SE	
DP	7265	61.9 b	24	11.4 c	0	c	0	c	0	c	7652	31.1 a
88-21	7477	74.8 b	40	12.8 c	0	c	0	c	0	c	7730	29.6 a
Onaway	7302	65.6 b	31	11.9 c	0	c	0	c	0	c	7661	39.2 a

A area of growth
SE standard error of the mean
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test at a = 0.05.

TABLE 4.9. Comparison of effects of five concentrations of vancomycin on the growth of three *Stretpomyces scabies* strains as determined by average reflective intensity.

S. scabies strain	0.1 ppm ARI ± SE	1.0 ppm ARI ± SE	10 ppm ARI ± SE	100 ppm ARI ± SE	1000 ppm ARI ± SE	0 ppm ARI ± SE
DP	113 6.5 b	45 (NG) 0.6 c	45 (NG) 0.1 c	44 (NG) 0.1 c	48 (NG) 0.1 c	156 1.7 a
88-21	161 3.7 a	42 (NG) 0.2 b	42 (NG) 0.1 b	47 (NG) 0.1 b	45 (NG) 0.1 b	154 3.3 a
Onaway	107 6.7 b	42 (NG) 0.4 c	44 (NG) 0.1 c	48 (NG) 0.1 c	49 (NG) 0.1 c	168 2.0 a

ARI average reflective intensity
SE standard error of the mean
Effect of strain was significant according to ANOVA at a = 0.05.
Means with the same letter are not significantly different according to t-test a= 0.05.

DISCUSSION

There is a wealth of more than 50 years of literature on testing chemicals to control potato common scab. However, there are no effective chemicals registered to manage this disease, which already has a limited number of available control measures. Copper sulfate probably has the longest history of use of any antimicrobial agent to aid in plant disease control (Agrios, 1997). In the mid-1700s, copper sulfate was used in England to control wheat smut (Agrios, 1997). In 1885, Millardet, noticed an effect of a mixture farmers were using on their grapes to discourage pilferers, and determined that copper sulfate and hydrated lime was very effective in controlling downy mildew. This became known as the Bordeaux mixture and was used to manage diseases of many other plants. The Bordeaux mixture remains as one of the most widely used fungicides worldwide (Agrios, 1997). The Bordeaux mixture, fixed coppers and cupric hydroxide are also used to manage a broad spectrum of bacterial leaf spots, blights and other diseases caused by bacteria. In this study, copper sulfate was effective *in vitro* in inhibiting *S. scabies* strains only at 1,000 ppm. However, such a high concentration as this, if tested on potato plants inoculated with *S. scabies*, would most certainly lead to phytotoxicity and hence, reduce yield. In this study, the data obtained for the average area of growth determined by digital analysis were more precise in determining differences in concentrations of copper sulfate than ARI data, which were also determined by digital analysis. The average growth area data were more precise and statistical differences were more accurate than the ARI data.

In the late 1990s, a Mexican company applied for registration of an agricultural formulation of gentamicin with the U.S. Environmental Protection Agency (EPA). Currently, the formulation is used in Mexico and Central America on a variety of crops to control fire blight in field tests (McManus and Stockwell, 2001). Gentamicin is clinically very important in treating a wide variety of human infections. The EPA and Center for Disease Control questioned all antibiotic use on plants, and in October of 1999, the request for registration of gentamicin for use on crop plants in the U.S. was denied. Currently, the only antibiotics registered in the U.S. for use on plants are streptomycin and oxytetracycline (Agrios, 1997). In this study, gentamicin was effective at 10 ppm in complete inhibition of growth of the three *S. scabies* strains tested. Again, image analysis data using the average growth areas were more precise than the ARI data in detecting differences between the different concentrations of gentamicin tested. Because the registration of this antibiotic was not accepted, it is unknown how well this antibiotic would work in the soil, and it is not known if gentamicin could control the disease *in planta*, other methods of controlling the disease are needed.

Vancomycin, often referred to as the last resort antibiotic in medicine, is clinically one of the most important antibiotics in treating human infections caused by bacteria such as *Staphylococcus aureus* or *Clostridium difficile*. Produced by *Streptomyces orientalis*, vancomycin is only effective against gram-positive bacteria, such as *Streptococci*, *Corynebacteria*, *Clostridia*, *Listeria*, and *Bacillus* species. In this study, vancomycin was able to severely reduce growth of *S. scabies* at a low concentration and completely inhibited growth of all three *S. scabies* strains at a minimal concentration of 1.0 ppm. Like the data obtained from the other antimicrobial agents, average area as determined by

digital analysis were better indicators of differences found between the different concentrations of vancomycin tested rather than the ARI data. The fact that ARI values did not find differences in growth between 0 and 0.1 ppm vancomycin illustrates this notion.

Pentachloronitrobenzene (also known as PCNB, Blocker, Terraclor, Terraclor Super X, Quintozene and Tritisan) was effective in controlling common scab in many studies (Curwen, 1980; Nugent, 1956; Curwen, 1978; Davis *et al.*, 1974; Davis *et al.*, 1976; Fink, 1956: Menzies, 1957; Potter *et al.*, 1959: Hooker, 1954; and Houghland and Cash, 1954). PCNB treatments also led to phytotoxicity, were ineffective at low soil water potentials, and effectively decreased scab infections only with excessively high application rates. The long lasting soil fungicide is primarily used against *Rhizoctonia* and *Plasmodiophora* to control vegetable, turf, and ornamental diseases (Agrios, 1997). The results in this dissertation are consistent with results of other studies in that only the highest dose was able to inhibit growth of the three *S. scabies* strains tested. The lower concentrations were ineffective. Again, digital analyses were more accurate in determining differences between PCNB concentrations when the average area data were used. If tested *in planta*, the 1,000 ppm concentration (2,719 lbs/A or 3,045 kg/ha), which effectively inhibited growth of *S. scabies in vitro*, would most likely have phytotoxic effects and hence, negative effects on yield.

The three strains used in this study all responded similarly to the antibiotics at the five concentrations at which they were tested. Small differences were noted occasionally, but no apparent differences occurred. This suggests that it may be possible, when screening antimicrobial compounds against *S. scabies*, to gain accurate results by using a

small number of strains during the screening process. In this study, PDA worked well as the growth medium to which antibiotics were added. This is consistent with another study that screened antimicrobial agents against *S. scabies* using PDA as the screening medium (McIntosh and Bateman, 1979). Based on the results obtained by digital analysis for average area of growth and ARI, it was determined that the average growth area was a better predictor than ARI of differences in growth between the five concentrations of the four antimicrobial compounds tested against the three strains. Furthermore, using digital analysis to determine growth area is a model system that should work well for most organisms instead of measuring them with a caliper or any other manual means of obtaining growth measurements.

This study found that *S. scabies* was able to grow at high concentrations of two agriculturally important antimicrobial agents, copper sulfate and PCNB. Gentamicin and vancomycin, antibiotics targeted against gram-positive bacteria, were both effective in eliminating growth of *S. scabies* at low concentrations. The results from this study indicate that only very high concentrations of agriculturally important broad-spectrum microbicides are effective in eliminating growth of the scab causal agent. In addition, antibiotics targeted specifically toward gram-positive bacteria, were effective, but not at lower concentrations, in growth inhibition of *S. scabies*. More studies are needed, but it is likely that only very specific antibiotics targeted toward this group of organisms will be the only compounds that will effectively eliminate growth at concentrations less than 100 ppm. Also, it is important to sample soils to find strains of bacteria that can adapt to soils and produce scab inhibitory antibiotics in the rhizosphere soil. This may lead to the development of an effective biocontrol agent against plant pathogenic streptomycetes.

Furthermore, these antibiotics are not fit for use in agriculture. The results of this study imply that disease management methods other than chemical control must be investigated for potato common scab due to the pathogen's ability to grow well at high concentrations of broad-spectrum microbicides.

CONCLUDING REMARKS: SUMMARY OF THE DISSERTATION AND FUTURE RESEARCH

Summary of the Dissertation

Researchers, plant disease diagnosticians, extension agents, and growers rely upon rapid, reliable, and precise results from assays used for pathogen detection. Many of the current techniques used for plant pathogen detection involve extraction of nucleic acids following time-efficient nucleic acid detection methods (Louws *et al.*, 1999; Schaad and Frederick, 2002; and Schena *et al.*, 2004). Understanding the distribution, as well as quantity, of plant pathogens in a given unit area of land may enable risk reduction, site specific disease management strategies and profit maximization through decreased pesticide management costs and increased crop yields as well as increased marketability. Chapter I, "Evaluation of PCR-Based Detection of Pathogenic Streptomyces Species in Soil Populations of Michigan's Major Potato Production Counties and Assessment of Real Time PCR as a Method For Pathogen Quantification," examined the usefulness of two nucleic acid based detection methods for use in the potato-*Streptomyces* patho-system (Chapter I).

This study has provided evidence that supports a more time-effective technique than previously described methods for the detection of pathogens responsible for causing potato common scab. This method was tested in numerous locations in Michigan and was repeated for four years. PCR detection of *nec1*, a gene highly correlated with plant pathogenicity in the genus *Streptomyces* (Bukhalid and Loria, 1997; Bukhalid *et al.*, 1998; Bukhalid *et al.*, 2002; Healy *et al.*, 1999; Kreuze *et al.*, 1999; and Park *et al.*, 2003) was an effective method for the detection of pathogenic streptomycetes without

performing laborious and time-consuming pathogenicity tests on potato tubers (Chapter I). Furthermore, this study supports the findings of other studies that have found plant pathogenic *Streptomyces* species to be ubiquitously present in soil microorganism populations (Chapter I). Other researchers have provided evidence that the pathogens have even been detected in soils not previously cropped to potato (Loria, 2001 and Loria *et al.*, 1997).

In addition, the study also integrated quantification of pathogenic *Streptomyces* species directly from soil using quantitative real-time PCR for detailed results that may eventually be useful in cases where specific diagnostics of plant pathogens are required (Chapter I). Precise estimations of pathogen populations may become increasingly important in plant disease diagnostics and management, particularly for diseases with limited control measures and especially as the introduction of alien pathogens into new geographical areas, such as *Phakopsora pachyrhizi* (causal agent of soybean rust), becomes more prevalent and threatening to agricultural and horticultural plants as well as plants in natural ecosystems. However, the expense of quantitative real time PCR will likely negate its ability for frequent use as a diagnostic tool for the majority of plant diseases and its use may be limited to diseases such as soybean rust and sudden oak death. Because classical PCR can provide precise and accurate information, the quantitative techniques may not be used nearly as often for other plant pathogens until the costs are reduced.

Chapter II, "Assessment of Cultivar Resistance and Soil Moisture Application as a Means to Manage Potato Common Scab," uniquely investigated the effects of an integration of cultivar resistance and soil moisture application on potato common scab.

This study provides evidence that cultivar resistance is the most effective method of managing potato common scab. This study, like other reports that span the literature for more than 80 years, found that soil moisture applied at or near field capacity was able to significantly reduce the severity of scab infections on susceptible cultivars. I also found that integrating cultivar resistance into a potato production regime might negate irrigating soils to such high soil moisture volumes (Chapter II).

Similar to other studies, I found that only the high soil moisture volumes were effective in reducing scab infections on susceptible cultivars (Chapter II). Soil moisture applied at lower percentages only slightly reduced scab infections on susceptible cultivars. In addition, my work supports the findings of other researchers who have concluded that soil moisture must be applied continuously during the critical susceptible period, and that allowing the soil to dry can lead to scab infections during the periods in which drier soil conditions were investigated.

The uniqueness of my research comes from examination of the results obtained from experiments in which the scab resistant cultivar, Liberator was used (Chapter II). This study provides direct evidence that supports the possibility that moderate to high levels of irrigation are probably not necessary to reduce scab infections when a cultivar with a high level of scab resistance is planted. This is increasingly important since irrigation that reduces scab infections on susceptible cultivars is not always highly efficient because of soil moisture evaporation, which is accelerated in warmer temperatures, as well as the water repellent nature of certain soils, which also increases as temperature increases. These factors and others often lead to irrigation of susceptible cultivars that is reached with an undesirable level of effectiveness. Also, as irrigation

becomes restricted in certain geographical areas and because limited water resources are an emerging problem worldwide, this may become a concern in the near future, especially as water usage peaks as a crucial issue, particularly in regions where water is extremely limited. Furthermore, water quality concerns arising from irrigation have been continually raised due to the leaching of dangerous pesticides and fertilizers into ground water that occurs with events of heavy irrigation. Clearly, continuously irrigating soils to high soil moisture volumes for potato common scab management may soon become a concept of the past as these issues surrounding water quality and water use are increasingly critical.

Chapter III, "Assessment of Four Compounds on Elicitation of the Systemic Acquired Resistance Response in Potato to Common Scab Infection," provides what may be the most detailed study to address the feasibility of systemic acquired resistance in potato to common scab (Chapter III). Chitosan and harpin, well-documented resistance activators, caused slight reductions of common scab infection as well as slight increases in harvested tubers free of scab infections for some of the treatment methods tested in the first trial. However, a lack of increased chitinase activity in the tubers indicated that the decrease in disease was not due to systemic acquired resistance (SAR). In other words, chitinase activities were not increased in the tubers that were sampled from the treatments that exhibited fewer scab infections (Chapter III). This suggested that SAR was not directly responsible for these differences and that other factors, such as reduced pathogen populations or patchy soil moisture may better explain these changes.

However, these results were not consistently reproduced in the two trials that followed. Chitosan and harpin treatments in the second and third trials did not support

result in reduced scab. There were no reductions in scab infections, no increases in the quantity of tubers free of scab infections, and systemic resistance was not induced in tuber tissue. Furthermore, some of the treatments responded more poorly than the untreated control to treatments with harpin, particularly for tubers that were categorized as having severe common scab infections (Chapter III). Formononetin and saponins, both natural plant-based compounds, were also tested in two of the trials as two different application methods. Neither compound significantly reduced scab infections or increased the numbers of scab free tubers. Chitinase activities were not increased in tissues treated with formononetin or saponins.

Although slight increases in tubers free of scab infection occurred in the first trial, increased chitinase activities were not correlated with these differences. Slight weather differences between the first, second, and third trials may explain some of the variability observed. It is likely that some of the treatments that responded well the first year were a result of weather conditions less favorable for scab development compared to the two following years as well as pathogen variability within a field. The lack of severe infections in the first trial compared to the following two trials supports this conclusion.

Chapter IV, "Evaluation of Four Antimicrobial Compounds on Growth Inhibition of Three *Streptomyces scabies* Strains *In Vitro*," provides one of the more extensive studies on antimicrobial compounds directly tested against *S. scabies*. Pentachloronitrobenzene (PCNB) and copper sulfate, pesticides with significant agricultural use, were barely effective in reducing growth at the all but the highest concentration tested. These slight reductions in growth that occurred from each ten-fold increase in concentration were difficult to detect with the unaided eye, however, the use

of computerized image analysis enabled detection of slight occasional reductions in growth as concentrations of the two were increased. PCNB and copper sulfate were only effective in completely inhibiting growth of the three *S. scabies* strains tested when the concentrations were excessively high at 1,000 ppm (Chapter IV).

This study also evaluated the effects of two clinically important antibiotics, gentamicin and vancomyin, both targeted against gram-positive bacteria, on growth inhibition of *S. scabies*. These antibiotics were effective in inhibiting growth of the three tested strains of *S. scabies* at relatively low concentrations. The three strains were able to grow well at the lower concentrations tested; however, the concentrations that were effective in inhibiting growth of *S. scabies* were much lower than the two other compounds tested (Chapter IV).

The results from this study indicate that broad-spectrum antimicrobial compounds, such as PCNB and copper sulfate, were not highly effective in inhibiting growth of *S. scabies* at concentrations less than 1,000 ppm. Furthermore, their use *in planta* would likely lead to phytotoxicity at such high concentrations. In addition, because the two antibiotics, gentamicin and vancomyin, were effective in inhibiting the growth of *S. scabies* at the lower concentrations tested, it is possible that only specific antimicrobial compounds targeted toward this group of organisms will be effective in managing scab. Furthermore, it is possible to speculate that broad-spectrum antimicrobial compounds will only be effective in reducing or inhibiting growth of *S. scabies* at concentrations that are too high to be feasible in terms of economics, environmental quality and plant toxicity. The image analyses used in this study indicated that for *Streptomyces* species the area of growth was a better and more precise indicator

of small responses to ten-fold increases in antimicrobial compound concentrations than the average reflective intensity measurements. The results of this study indicate that methods other than chemical control should be researched to manage this potato disease.

Future Research

The results from this research probe further examination regarding pathogen detection and disease management in the potato-*Streptomyces* pathosystem. Among the numerous future analyses that are of critical priority in potato scab research include the following list. These issues are probably the highest priority necessities for understanding and controlling this disease and must include the questions listed below:

- Perform a more comprehensive examination of the mechanism(s) of pathogenicity in the genus *Streptomyces*. More research in needed on *nec1*, the gene closely associated with pathogenicity. The gene products of *nec1* need to be more extensively evaluated and its precise identification and function must be fully described to better understand its role in pathogenicity. Genetic comparisons of pathogenic *Streptomyces* species having and lacking this gene should be conducted since it is documented that most, but not all, of the pathogenic streptomycetes contain this gene. These comparisons between strains with and without *nec1* may provide useful insight into the missing link to the *Streptomyces* spp. pathogenicity factor. Primers and probes can then eventually be developed that will enable detection of all, not just the majority, of pathogenic *Streptomyces* species.

- Assess the mechanism(s) underlying the effective control of potato common scab by irrigation. Experiments are needed that determine if the management of this disease is due to a biological factor, an abiotic factor, or both. The biological components of scab reduction via irrigation include possibilities of documented evidence of competitive exclusion at the lenticels and immature stomata as well antibiotics produced from other soil dwelling microbes. Also, the reduction of populations due to the change in the physical soil environment needs to be examined. A more comprehensive understanding of the mechanism(s) involved will enable maximized control of potato common scab via irrigation as well as locations where it may not be effective based on soil microorganism populations and/or soil texture. This may become more important as water resources become increasingly limited.
- Analyze cultivars with high levels of resistance to scab. Since this study indicated that scab resistance is more effective in managing the disease than irrigation, more extensive studies using cultivar resistance are needed. Highly scab resistant cultivars should be assessed under different irrigation regimes in regions where they are to be grown. This may allow for regional irrigation optimization under ideal experimental conditions performed on land owned by universities, rather than growers. This will provide evidence to support the findings of this study that indicate irrigation is needed in scab resistant cultivars only to sustain growth and yield, not minimize scab infection while reducing the reliance upon and use of irrigation.

- Compare combinations of resistance activators, moisture application, and biocontrol agents to determine if synergistic effects are possible. Because induced resistance must be incorporated with other disease management methods, it is possible that combining resistance activators with other control measures, such as irrigation and biocontrol agents may lead to enhanced protection against infection caused by *Streptomyces* spp. Completely randomized, factorial experiments that reduce scab infections may provide insight into combinations of methods that may be useful to growers. Furthermore, numerous cultivars should be tested in these trials since cultivars with the same level of resistance may respond differently to inducing agents. As environmental quality and human health concerns are constantly escalating from agricultural pesticide usage, this will become an increasingly important possibility.

New research investigating the mechanism(s) of pathogenicity in the genus *Streptomyces*, the mechanism(s) responsible for control of common scab via irrigation, and the reduced reliance upon irrigation via cultivar resistance, and the combination of resistance activators with other disease management methods may enable better understanding of the host-pathogen interaction, as well as provide valuable insight into new and exciting ways to manage potato common scab, which remains one of the least adequately controlled of all potato diseases.

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