Thaxtomin A to identify common scab resistance in potato and glucose conjugation as a mechanism of resistance
by Ivette Alicia Acuna

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
Potato scab is one of the most important diseases worldwide affecting market quality and increasing processor peel losses. Resistant varieties have been one of the best solutions to this disease; however, traditional potato breeding programs do not have good screening techniques that allow for early generation selection for scab resistance. The discovery of thaxtomin A (TA), produced by pathogenic Streptomyces scabies, and its important role in bacterial pathogenicity suggested new approaches in scab resistance screening techniques and the mechanism of resistance to thaxtomin A in potato tubers.

This research investigated the correlation between an optimized true potato seed (TPS)-TA resistance assay with common scab resistance of potato tubers as determined by tuber TA sensitivity and scab symptoms in field tests; and tested the hypothesis that glucosylation of TA is related to potato plant resistance to pathogenic S. scabies.

The TA seedling-screening assay was a useful tool for identifying scab resistant germplasm under homogeneous standard conditions. Doses of lμM of TA and an exposure time of 7 days allowed distinguishing between seedling progenies from cultivars that vary in resistance to scab. TA did not strictly select 100% resistant individuals, but it allowed approximately 30% a decrease in the size of the population to be tested under field conditions. Significant correlations were determined between a seedling index of damage by TA and tuber scab symptoms (R=0.63, P=0.0001; R=0.49, P=0.0001) and between tuber TA sensitivity and tuber scab symptoms (R=0.64, P=0.0001; R=0.75, P0.01). Scab resistant individuals were identified, which can be tested directly for commercial purposes, can serve as parents in breeding programs or as a source of resistance genes.

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THAXTOMIN A TO IDENTIFY COMMON SCAB RESISTANCE IN POTATO AND GLUCOSE CONJUGATION AS A MECHANISM OF RESISTANCE

by

Ivette Alicia Acuña

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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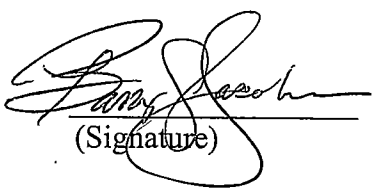
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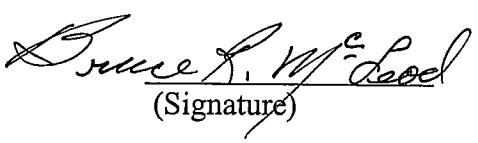
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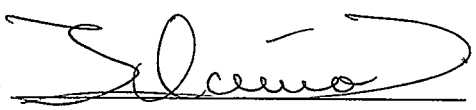
Signature 

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ABSTRACT

Potato scab is one of the most important diseases worldwide affecting market quality and increasing processor peel losses. Resistant varieties have been one of the best solutions to this disease; however, traditional potato breeding programs do not have good screening techniques that allow for early generation selection for scab resistance. The discovery of thaxtomin A (TA), produced by pathogenic *Streptomyces scabies*, and its important role in bacterial pathogenicity suggested new approaches in scab resistance screening techniques and the mechanism of resistance to thaxtomin A in potato tubers.

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CHAPTER 1

LITERATURE REVIEW

Thesis Preface

The potato (*Solanum tuberosum* L.) is one of the most important food crops in the world, exceeded only by wheat, maize and rice in total production. Hawkes (1994) comments that until the16th century potato was unknown to the people of Europe, Asia, Africa and North America, however in South America it was probably the most productive source of food for the communities of the high Andes and Southern Chile. Potato was cultivated and widely adapted in Colombia, Ecuador, Peru, Bolivia and the Araucarian Region of Chile before the Spanish arrived in South America.

Today, potato production and consumption occurs throughout the world. In 1998 in the United States 3.4 million ha of potatoes were harvested with an average yield of 39.9 ton/ha with a total production of 21 million tons. The per capita annual consumption of potatoes in the U.S. is about 65 kg as fresh and processed products. In addition, seed production acreage in the country was 62,370 ha in1998 (NPC, 1999).

Montana ranks 7th in potato seed production in the U.S. with 4,029 ha of seed certified in 1998. Montana is the main source of high quality seed for Washington and supplies seed to Idaho, Oregon, California, Minnesota, Wisconsin, Michigan and Canada. The total state production was 0.4 million ton with a yield of 33.6 ton/ha .The most important varieties produced in Montana are Russet Burbank, Russet Norkotah and Ranger Russet with 2,141 ha, 743 ha, and 510 ha, respectively (NPC, 1999).

The Potato (*Solanum tuberosum* L.)

The center of origin of potato cultivation may have been the Andes of southern Peru and northern Bolivia. Apparently, there were two introductions into Europe, one into Spain in c. 1570 and the second into England in c. 1590. These potatoes were the Andean form of the tetraploid potato *Solanum tuberosum* L. subsp. *andigena* Hawkes. A process of adaptation to long day conditions took place in Europe. Potatoes were spread into central and eastern Europe by the late 18th and early 19th centuries (Hawkes, 1992). Potatoes were introduced into North America from Bermuda in 1691 where they had been grown from an earlier importation from England in 1613 (Hawkes, 1994). Although Grun (1990) comments that after the arrival of late blight (*Phythophtora infestans*) in Europe in the 1840's, most of the spp. *andigena* clones grown there were killed resulting in the serious famine most notably in Ireland. Then, this author says, after the importation of Rough Purple Chili, a clone that came from ssp. *tuberosum* from Chile, the morphology of potatoes in Europe began to change to spp. *tuberosum*. This clone is a parent of a large proportion of North American and then European cultivars.

Potato species belong to the large and very diversified genus *Solanum*, however only a small part of it has been cultivated. There are seven cultivated species of potato, occurring in a polyploid series with a base number of 12 and ranging from diploid to pentaploid. Several of them are fairly similar to each other. It is believed that in the evolution of cultivated potato four *Solanum* species were involved. Hawkes (1994) indicated that hybridization of S. *stenotomun* with the weedy species *S. sparsipilum* and

subsequent chromosome doubling produced the tetraploid *S. tuberosum* subsp. *andigena* in the central Andes. Ancient peoples carried this species into southern Chile, where it became adapted to long day length and evolved into spp. *tuberosum*. Probably, this adaptation occurred after it's crossing with *S. maglia*, a population present in Chile, adapted to the environmental conditions in the south (Grun, 1990).

In developing countries, as in Europe, potato production and consumption was influenced by the suitability of the environment for the crop, development of production and post-harvest systems that were appropriate for specific environment, and food habits and needs (Horton and Anderson, 1992).

Potato Diseases

The Compendium of Potato Diseases lists over 75 pathogens, including nematodes, fungi, bacteria, mycoplasmas, viruses and viroids (Hooker, 1981). Many of these diseases are able to alter the productivity or usefulness of the crop.

Potatoes are usually clonally propagated by planting potato seed pieces or whole tubers. This practice lends to itself the introduction of potato pathogens from one area to another and to the overwintering of pathogens in stored tubers that are used for propagation (Rich, 1983). This author comments that great efforts have been made to control potato diseases using effective fungicides and insecticides, improved cultural practices and resistant varieties. Systems of certification or registration have been established to minimize disease transmission problems in tubers and quarantines have been used to minimize spread of dangerous pathogens.

Common Scab of Potatoes

Common scab occurs in potatoes in almost every production area around the world. It is a major production problem that affects grade quality and market value (Hooker, 1981). When scabbed seed tubers are planted they are unlikely to be an important source of inoculum for progeny tubers (Lapwood, 1972; Adams and Hide, 1981) but if the disease is severe, plant vigor and yield may be decreased (Butler and Jones, 1949). Alternatively, Goto (1990) reported that while scab is a primarily soil-borne rather than seed-born disease, there is a high correlation between the incidence of disease on new progeny; and finally that the disease severity can be reduced by disinfections of seed tubers.

Disease Symptoms. Hooker (1981) describes the common scab symptoms as follows: tuber lesions of about 5-8 mm in diameter, irregular in shape and larger when infections coalesced. Affected tissue can turn light tan to brown. Symptoms of potato scab are variable, with two types being the most characteristics: shallow, corky, surface, or russet scab, and deep or pitted scab (Rich, 1993). Russet scab is a superficial corklike layer and pitted scab is an extension into the tuber of various depths up to 7 mm (Hooker, 1981). Infection of potato tubers usually occurs before or during enlargement of the tubers (Hooker and Page, 1960). Fellows (1926) demonstrated that tuber growth and scab disease were coincidental and that tubers, which were not growing, were not infected. Potato scab may originate in any place in the potato, but frequently occurring in lenticels (Adams, 1975; Lutman, 1913; Jones, 1931). Lutman (1913) described the development of

this disease due to the hypertrophy of the cells of the cork cambium. This author commented that this hypertrophy could be the result of the absorption of toxic substances produced in the growth of the parasitic organism in the exterior. In deep scab this condition is accompanied by a hyperplasia of that layer, due to its continuous regeneration from the outer cells of the starch parenchyma. Jones (1931) said that the newly divided cells are invaded by the bacteria producing cell collapse followed by lenticel enlargement. Then the cell meristem becomes less active, the last formed daughter cells cease to elongate and become suberized forming a barrier of wound cork that separates infected from healthy tissue. Further development is due to the organism ability to grow through incomplete suberized cells and infect the cells below. He adds that a new cork layer will form beneath the scab lesion. As a result of the development of successive cork layers an increasing amount of tuber tissue is excised and decays leading to the extension of the scab lesion. Goto (1990) comments that pitting or depression of the scab results from necrotic collapse of host cells by phytotoxins produced by the causal organism.

Causal Agent. The predominant causal agent of common scab is considered to be *Streptomyces scabies*, although other *Streptomyces* spp. can induce similar symptoms (Bonde and McIntyre, 1968; Doering-Saad et al., 1992; Faucher et al., 1992; Goyer et al., 1996; Loria et al., 1997; Millard and Burr, 1926). This pathogen has been classified in the Streptomycetes group in the order Actinomycetales (Goodfellow et al., 1983). They are bacteria that have the ability to form branching hypha at some stage of their development. Goodfellow and Cross (1983) described the Streptomycetes group as: "common

terrigenous Gram-positive sporoactinomycetes that are highly oxidative, form an extensive branching substrate and aerial mycelium and typically have a cell wall containing peptidoglycan with LL-DAP as the diamino acid and glycine as the cross-linking amino acid but no characteristic sugars". In Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974), members of the genus *Streptomyces* are described as "slender, coenocytic hypha, aerial mycelium forming chains of 3 to many spores 0.5-2.0 μm in diameter at maturity; small colonies discrete and lichenoid leathery or butyrous; initially smooth surface but later develop a weft of aerial mycelium; produce a wide variety of pigments; aerobes; utilize glucose, between others characteristics". The International *Streptomyces* Project (ISP) was organized in 1964 to evaluate taxonomic characters and descriptions of most of the available type strains (Goodfellow and Cross, 1983; Shirling and Gottlieb, 1966).

The first to isolate and identify a causal organism from scabby potatoes was Thaxter (1892, cited by Goyer et al, 1996), who named it *Oospora scabies* and described it as having gray spores borne in spiral chains and producing a blackish-brown discoloration in media. Later, this causal agent was renamed *Actinomyces scabies* by Güsow (1914), since the organism was found not to be a fungus, but a filamentous bacterium in the Actinomycetes group. Later, Millard and Burr (1926) studied and described 24 strains of Actinomyces isolated from potato scab and other sources. They found that only 11 of them were able to produce scab, but only one appeared to be identical to Thaxter's description. Waksman and Henrici (1948) proposed *Streptomyces scabies* as a name for this pathogen causing potato scab. Recently, Lambert and Loria

(1989a) proposed the revival of the name *Streptomyces scabies* for the predominant species causing common scab of potato. They proposed the following characteristics for this species: smooth grey spores borne in spiral chains, production of melanin, utilization of L-arabinose, D-fructose, D-glucose, D-mannitol, rhamnose, sucrose, D-xylose and raffinose and sensitivity to streptomycin and to acidic pH. Other species also are described producing potato scab symptoms. For example, *Streptomyces acidiscabies* was described by Lambert and Loria (1989b) as causing a scab disease of potato in soils with pH values below 5.2. This organism was first isolated by Bonde and McIntyre (1968) and later by Manzer et al. (1977). The scab symptoms caused by this organism are indistinguishable from the symptoms of common scab caused by *S. scabies*. However, in culture, *S. acidiscabies* has flexuous spore chains, a growth medium-dependent spore mass with color ranging from white to red, and a red or yellow, pH-sensitive diffusible pigment rather than melanin. It grows on agar at pH 4.0, does not use raffinose and tolerates higher concentrations of crystal violet, thallium acetate, streptomycin, oleandomycin and penicillin G than *S. scabies* (Lambert and Loria, 1989b). *S. acidiscabies* is acid tolerant rather than an acidophilic organism and it is not limited to acid soils (Faucher et al., 1992; Lambert and Loria, 1989b). Recently, a new species of bacteria, *Streptomyces turgidiscabies*, was described causing potato scab in eastern Hokkaido, Japan (Miyajima et al., 1998). This organism produces erumpent lesions on potato, has flexuous spore chains, grey mass color, cylindrical and smooth spores. This bacterium does not produce melanin or other diffusible pigments, does not grow on agar media at pH 4.0 or 37°C, utilizes raffinose and inulin as carbon sources and is sensitive to

streptomycin, penicillin G, polimyxin B and thallium acetate (Miyajima et al., 1998). Also, *S. caviscabies* (Goyer et al., 1996), *S. diastatochromogenes*, *S. resistomycificus*, *S. atroolivaceus* and *S. lydicus* (Archuleta and Easton, 1981) have been identified as possible causal agents of deep-pitted scab in potatoes. Furthermore, Doering-Saad et al. (1992) classified the potato scab inducing organism in three phenotypic groups, whose spore chain morphology ranges from spiral to flexuous to retinaculum-apertum to rectus and spore ornamentation from smooth to warty to spiny.

Bacterial Pathogenicity Factors. The production of melanoid pigments is widespread in actinomycetes, especially in the genus *Streptomyces,* due to the presence of the enzyme tyrosinase (Baumann et al.1976). Gregory and coworkers (1961,1964a, 1964b) found a positive correlation between the capacity for melanin production and pathogenicity. They postulated that an extra chromosomal localization of the genes, in a plasmid, is responsible for melanin formation.

In addition, Courteau and Beaulieu (cited by Goyer et al., 1996) found that *S. scabies* strains causing deep-pitted scab presented a high proteolytic activity. In addition, Faucher et al. (1995) found high cellulolytic activity in these strains but no proteolyctic or cellulolytic activity in isolates from scab with shallow or raised lesions. Pectinase activity has been described in pathogenic and non-pathogenic *Streptomyces*, thus, it has not been associated with pathogenicity (Spooner and Hammerschidt, 1989; Faucher et al., 1995).

Cutin and suberin are the waxy polyesters that cover the external portions of plants, e.g. potato tuber skins, and form a barrier against moisture loss and pathogen

invaders (Kollattukudy, 1980; Kollattukudy and Agrawall, 1974). McQueen and Schottel (1987) characterized a novel extracellular esterase from pathogenic *S. scabies* not produced by non-pathogenic strains. The expression of the esterase gene from *S. scabies* is regulated by zinc as a cofactor and by levels of esterase mRNA (Babcock et al., 1992; Hale et al., 1992; Raymer et al, 1990). This enzyme is thought to be involved in breaking down the waxy polyester suberin compound that covers the tubers during the infection process (Green et al, 1992). However, Fett et al. (1994, cited by Goyer et al., 1996) found a pathogenic strain of *S. acidiscabies* and a saprophytic strain of *S. badius* that produced esterase on media with cutin.

King et al., (1989) isolated and characterized a family of phytotoxins capable of inducing scab-like lesions on immature potato tubers. They isolated these toxins from cultured potato tubers infected with S. *scabies*. Later, the two major toxins were designated thaxtomin A and thaxtomin B and characterized as unique 4-nitroindol-3-yl containing 2.5-dioxopiperazines (Lawrence et al., 1990; King et al., 1992). These toxins were named in honor of Roland Thaxter, the American plant pathologist who first identified the causal organism of common scab (King and Lawrence, 1996). Thaxtomin A was determined to be the predominant phytotoxin associated with both *S. scabies* and *S. acidiscabies* (King et al., 1992). Later, King and Lawrence (1996) characterized new thaxtomin A analogues generated in vitro by *S. scabies*. Loria and her coworkers (1995) described the ability of *S. scabies* to produce thaxtomins in different media, the best being oatmeal broth. Babcock et al. (1993), found that the optimal temperature to produce the toxins in vitro was 28°C and that the toxin production was repressed by addition of

0.5 % glucose in the media and by tryptophan and tyrosine amendments. Thaxtomin production has been suggested to be responsible for pathogenicity since a positive correlation has been described between thaxtomin A production and pathogenicity among *Streptomyces* strains that infect potatoes (King et al., 1991; King et al., 1996; King et al., 1992). In addition, all *Streptomyces* strains that are pathogenic on potato produce thaxtomin in vitro, and all *Streptomyces* that produce thaxtomin in vitro are pathogenic in potato (King et al, 1991; Loria et al., 1995). Kinkel et al. (1998) determined that only pathogenic *Streptomyces* spp. produce thaxtomin A in culture and its production is positively correlated with the percentage of tuber surface infected but not with the number of lesions per tubers. Leiner et al. (1996) demonstrated that thaxtomins are involved in the development of symptoms on the roots of a wide variety of others plant hosts. Moreover, Delserone et al. (1991) determined a positive correlation between *S. scabies* resistance and thaxtomin A sensitivity in different potato cultivars.

More recently, Bukhaild and Loria (1997) described the nec1 gene, which is adjacent to the open reading frame, ORFtnp. They indicated that nec1 could transform non-pathogenic/non-thaxtomin producing *Streptomyces* spp. into pathogenic species. They cloned nec1 into *Streptomyces lividans*, a non-pathogenic species, resulting in the ability of *S. lividans* to necrotize and colonize potato tuber slices and produce scab like symptoms on potato minitubers. These transformed bacteria were not able to produce thaxtomin A. In this same paper they report that nec1 and ORFtnp are physically linked in *Streptomyces* strains that are pathogenic on potato and that produce the phytotoxin thaxtomin A. Bukhalid et al. (1998) proposed that nec1 and ORFtnp have been moved

horizontally from *S. scabies* to *S. acidiscabies* and *S. turgidiscabies* and that nec1 is involved in pathogenicity and physically linked to the thaxtomin A biosynthetic genes.

Epidemiology and Control. Common scab disease is most common in light well-drained soils, alkaline or slightly acid in reaction and of low humus content (Keinath and Loria, 1989; McGregor and Wilson, 1966; McGregor and Wilson, 1964). Disease incidence and severity increase as soil pH increases from 5.0 to 7.5, although "acid scab" has been described in certain soil with a pH lower than 5.0 (Manzer et al., 1977; Rich, 1983) Temperature and moisture also play important roles in the epidemiology of this disease, mainly due to microbial antagonism (Adams and Lapwood, 1978; Davis et al., 1976; Davis et al., 1974; Lapwood, 1966; Lewis, 1970).

Management of potato scab has been partially accomplished by several means. For example, applications of nutrients to soil that affect the pH have been widely studied. Davis et al. (1974) demonstrated that both elemental sulfur and gypsum reduce scab, due mainly to a reduction of soil pH, which occurs when S is oxidized. Keinath and Loria (1989) comment that the causal agent of common scab (*S. scabies*) is affected directly by pH. For example, *S. scabies* growth is inhibited in vitro at pH 4.9 to 5.2 (Waksman, 1922), although other potato pathogenic *Streptomyces* spp. are able to grow at pH 4.8 (Loria et al., 1986). Addition of calcium carbonate has been found to increase the incidence and severity of scab, proportionally to the increase in soil pH (Blodgett and Cowan, 1935; Goto, 1985; Odland and Albritton, 1950). In addition, an increase of available calcium in the soil, results in higher calcium level in the tuber, which increases susceptibility to *S. scabies* (Horsfall et al, 1954). According to Davis et al. (1976), the

severity of scab is correlated with calcium concentration in the peeling of the tubers. These authors also found that scab severity increases as the Ca: K ratio increases in potato tubers. McGregor and Wilson (1964, 1966) studied the effect of manganese on the development of potato scab, and found that the manganese treatment increased the average tuber weight, markedly reduced the incidence of scab and that the development of scab is controlled by the amount of available manganese in the soil. Furthermore, Mortvedt et al. (1963,1961) indicated that direct manganese toxicity to *Streptomyces* could be involved in the scab suppression.

There are many references to the influence of soil moisture and its effects on potato scab (Adams and Lapwood, 1978; Davis et al., 1974; Davis et al., 1976; Lapwood, 1966; Lapwood and Adams, 1975; Lewis, 1970). An increase in soil water tends to cause a decrease in scab incidence. Irrigation is a practice highly recommended by experts to practically manage potato scab (Hooker, 1981; Pavlista, 1997; Powelson et al., 1993; Rich, 1983). Davis et al. (1976) suggested that mean moisture depletion to –0.65 bars approximates the minimum soil moisture required for scab control. One factor that causes scab reduction might be the increase in antagonistic bacterial population caused by the increase in water potential (Adams and Lapwood, 1978; Lapwood, 1966; Lewis, 1970). Soil moisture greatly influences the microflora on the tuber surface. These authors found an extensive development of actinomycetes under dry conditions, but they were almost absent in wet conditions. However, they demonstrated that soil moisture appears not to affect the pathogen directly, since it grows well in wet sterile soil and could infect tubers under wet sterile conditions. They concluded that some form of microbial antagonism is

the most likely explanation for scab control in wet soils. Research by Lapwood and Adams (1975) showed that true nonpathogenic bacteria colonized the lenticel area more quickly in wet soil than *S. scabies*, therefore protecting them from pathogen attack. Labruyere (1971, cited by Bruelhl (1987)), explained that in dry soils true bacteria could not move rapidly to the developing lenticels, and *S. scabies*, which has filamentous characteristics, would have the advantage in colonization.

Biological control is another of the alternatives for control of potato scab. Menzies (1959) demonstrated a scab-suppressing factor in several soils, which was transferable soil to soil and destroyed when steamed. He attributed this effect to a biological factor. Lorang et al. (1995) identified scab inducing and suppressive *Streptomyces* species. They isolated *S. diastatochromogenes* and *S. albogriseolus* from soil that had become suppressive. In addition, Liu et al. (1995) described the control of potato scab by the use of antagonistic *Streptomyces* strains under field conditions. The biological factor responsible for the scab suppression was attributed to antibiosis (Liu, 1992).

Other alternatives listed by Powelson et al. (1993) include: soil incorporation of green manure, avoidance of animal manure, rotation with non susceptible crops like alfalfa, rye and soybean, and use of scab free seed potatoes. Most authorities indicate that integration of several practices is required for a successful management.

Breeding for Scab Resistance

Many studies have been done in the inheritance of resistance to scab. For example, Lauer and Eide (1963) explained it on a single-gene basis, with a duplex level

necessary for effective resistance. Zadina (1958, cited by Wastie, 1994) indicated that the inheritance of resistance is due to a maternal (cytoplasmic) influence. Other authors, including Cipar and Lawrence (1972), thought that more than one gene pair is needed for the expression of resistance. Pfeffer and Effemert (1985, cited by Wastie, 1994) said that resistance is inherited polygenetically, and that identifying genotypes with high general combining ability, GCA, for intercrossing would produce progenies with a high proportion of resistant individuals. Alam (1972) and Murphy (1995) indicated that scab resistance most likely involves two loci, with resistance conditioned at one locus by a dominant allele and at second locus by homozygous recessive alleles.

Golmirzaie et al. (1994) noted the principal advantages of using true potato seed, (TPS), over seed tubers in potato production including reduction of seed cost, area required and flexibility of planting time. The major source of genetic variation is generated by sexual reproduction and currently TPS production provides the most commonly used method of incorporating new genetic material. TPS is the primary source of genetic manipulation for breeders of this clonally propagated crop. The seedlings from TPS are each genetically unique (Caligari, 1992). In addition, if any desirable combination of variation from sexual segregation is found in TPS, it can be stabilized through vegetative propagation (Caligary, 1992). At the same time, the development of new molecular based technologies allows us to identify a given gene for resistance and transfer it rapidly to commercial varieties (Belknap, 1994) without crossing. One last advantage of using TPS in screening for disease resistance or other traits is that it reduces the number of seedling that are retained and grown in the greenhouse and planted for

field evaluation. Plaisted et al. (1984) said that there is interest in screening for scab resistance in the seedling stage. However, many breeding programs did not adopt this system mainly because of the need for large volumes of inoculum, the difficulty in testing the virulence of the *S. scabies* isolate used infecting seedling, the wide gradation between resistant and susceptible symptoms, and the relative ease of field exposure to scab of the first clonal generation.

The greatest difficulty in field screening is the difference in scab severity between different years of evaluation (Clark et al., 1938). Haynes et al. (1997) found a significant genotype x environment interaction for both the surface area covered with scab and the type of lesion on potato tubers.

Loria et al. (1994) said that many breeding programs emphasize scab resistance, but there are few highly resistant cultivars, and these still develop scab under favorable conditions. Many sources of resistance have been identified, in both breeding material and wild species (Wastie, 1994). Hosaka et al. (2000) evaluated scab resistance in wild diploid tuber-bearing *Solanum* species. They found that some of the ancestral species of the cultivated potatoes such as *S. bukasovii*, *S. canasense* and *S. multidissectum*, produced resistant clones.

Toxins and Plant Pathogens

In plant pathology, substances described as toxins are usually microbial metabolites that are harmful to plants at very low concentration (Graniti, 1991). Toxins have an important role in the development of symptoms in plant diseases and possess

some unusual and special chemical and biological properties. Some affect only those plant species or cultivars within a species that are similarly affected by the pathogen producing them (Host Specific Toxins), others have host specificity that is different from the organism that produces them (Non-host Specific Toxins) and some compounds that have no specificity at all (Strobel, 1982).

Host specific toxins are usually low molecular weight secondary metabolites with diverse structures that act as agents of virulence or pathogenicity (Walton, 1996; Walton and Panaccione, 1993). Most known host specific toxins are produced by fungal species of *Alternaria* and *Cochliobolus*, although *Phyllosticta*, *Periconia* and *Pyrenophora* also have been reported to produce this type of toxin (Kohmoto and Otani, 1991; Strobel, 1982; Walton, 1996). Usually, mutants of the pathogen lacking the genes for toxin production are non-virulent (Sheffer and Livingston, 1984)

Many known bacterial phytotoxins are non-host specific (Graniti, 1991; Walton, 1996). Mitchell (1991) said that bacterial toxins are secondary metabolic products that play beneficial roles in allowing organisms to occupy various ecological niches. Durbin (1991) described bacterial toxins as producing a range of symptoms including: chlorosis, necrosis, watersoaking, growth abnormalities or wilting. With few exceptions, bacterial toxins increase disease severity (Yoder, 1980).

The Use of Toxins in Breeding

Traditional breeding programs suffer from several problems when they searching for resistance to diseases, such as the lack of genetic variability, the high cost of time and space required for screening plant populations, and the lack of good field screening

methods. The use of selection techniques at the cellular level based on toxins and associated biochemical markers of resistance and genetic engineering for single genes affecting host parasite interaction has proven to be a practical alternative to screening using the pathogen (Buiatti and Ingram, 1991).

Purified toxins or culture filtrates have been used for screening where a correlation between toxin tolerance and resistance to pathogens exists (Durbin, 1981). Buiatti and Ingram (1991) commented that toxins or culture filtrates have been used primarily for early screening of segregating populations in traditional breeding programs or for selection in vitro of tolerant cells with successive regeneration of resistant plants.

The most commonly used tests to select for resistance using toxins are root growth, leaf necrosis and chlorosis, protoplast and cell survival, cell aggregate growth, and ion leakage from leaves, cotyledons and calli (Yoder, 1981). In addition, Durbin (1981) indicated that screening for resistance using toxins requires large populations, implying that the assay needs to be able to handle large numbers, be simple, rapid and significantly differential. He added that it is also necessary to initially use the lowest toxin concentrations that provide identification of resistant individuals to minimize plant growth inhibition while selecting for resistance individuals.

The first attempt to screen for resistance using toxins was in oat using crude preparations of HV-toxin, a host specific toxin produced by *Dreshslera (Helminthosporium) victoriae* (Wheeler and Luke, 1955). They germinated seed, drenched the seedlings with toxin preparation, selected the normal appearing plants, and then inoculated with the pathogen. After 1 month 92% of the selected plants were disease

free. Later, this method was used to select grain sorghum for resistance to PC-toxin, a toxin produced by *Periconia circinata* (Schertz and Tai, 1969). In 1972, Byther and Steiner sprayed seedling leaves of sugarcane with helminthosporoside, a toxin produced by *D. sacchari* to select for resistance to eye spot disease. Today, many studies are reported in the literature that involve toxins such as ACR-toxin I and ACT-toxin Ib from *A. alternata* in citrus (Kohmoto et al, 1991); AT-toxin from *A. alternata* in tobacco (Ishida and Kumashiro, 1988); AAL-toxin from *A. alternata* in tomato (Clouse and Gilchrist, 1987); HM-toxin from *C. heterostrophus* in maize (Earle et al., 1978); PM toxin from *Phyllosticta maydis* in maize (Kono, 1989); cercosporin from *Cercospora beticola* in sugar beet (Moser et al., 1990); cercosporin from *C. oryzae* in rice (Batchvarova et al, 1992); roridin from *Myrothecium roridum* in muskmelons (Mackay and Ng, 1994); toxins from *Pyrenophora teres* in barley (Sharma, 1984); toxins from *P. tritici-repentis* in wheat (Tomas and Bockus, 1987); filtrates from *Xanthomonas campestris* pv. *pruni* in peach (Hammerschlag, 1988). Buiatti and Ingram (1991) comment that in many cases toxin tolerance is not necessarily sufficient by itself for resistance to pathogens due to interactions between passive and active defense mechanisms. He suggested that toxins, mainly those that are non-host specific, act primarily by inhibiting active defense processes.

Mechanism of Resistance to Toxins in Plants

Little is known about specific targets of toxin action and detoxification mechanisms. However, several sites of toxin action have been described These include effects on cell membrane permeability producing ionic imbalance, leakage of

electrolytes, enzyme inhibition or stimulation, and increases in ethylene production or respiration (Goodman et al., 1986). Sensitivity to toxins is also correlated with the ability of host membrane proteins to bind toxin. This is the case for HS- toxin (Bournival et al., 1994; Kenfield and Strobel, 1981; Strobel and Hess, 1974) and HV- toxin (Wolpert and Macko, 1989). Thus, insensitivity of cultivars to toxins can be explained by inactivation of the toxin or self-repair mechanisms. Meeley and Walton (1991) and Johal and Briggs (1992) explained that the dominant allele of HM1 gene in maize controls both race-specific resistance to the fungus *C. carbonum* race 1 and the expression of the NADPH-dependent HC-toxin reductase, which inactivates the HC-toxin. This enzyme is present only in resistant cultivars. On the contrary, Hv-1 is the gene that confers susceptibility to *D. victoriae* in oat and is genetically dominant; therefore, Walton (1996) concluded that HV-1 probably affects an activation reaction that produces a biologically active product from a protoxin. Tabtoxin, a toxin produced by *Pseudomonas tabaci,* induces chlorosis in plants. The hydrolysis of this toxin by peptidases produces tabtoxinine β-lactam, an inhibitor of glutamine synthetase. As a consequence, an accumulation of ammonia occurs in cells. Tabtoxin does not inhibit this enzyme (Goodman et al., 1986; Strobel, 1982; Durbin, 1991). It has been described that *Cunninghamella elegans* is able to glucose-conjugation the flavones produced by *Psiadia arabica* producing two glucose conjugates with glucosilation of phenolic hydroxyl groups, as a detoxification mechanism (Milanova et al, 1995; Ibrahim et al, 1997). Park et al. (1994) reported the metabolism of maculosin, a phytotoxin produced by *Alternaria alternata*, by spotted knapweed. They found that the toxin was converted to three polar compounds. One of them was a neutral metabolite

identified as maculosin-β-O-glucoside. This glucoside was not toxic to spotted knapweed or other plants tested. Later, Strobel and Hess (1997) described the glucosylation of leucinostatin A, a toxin produced by the endophyte *Acremonium* spp. The host plant, European yew, is immune to the fungal toxin due to the presence of an enzyme that transfers glucosyl residues to leucinostatin A, reducing the peptide's bioactivity.

Hypotheses and General Objectives

Potato scab is an important disease worldwide causing losses due to reduced quality and processing yield. Many attempts to control this disease have been tested, mainly agronomic and cultural practices; none of them have been highly effective when used alone. Today only a few commercial cultivars are available with high level of resistance. The discovery of thaxtomins and the demonstration of their important role in *S. scabies* pathogenicity suggested two hypotheses: 1) Thaxtomin A can be used to select common scab resistant germplasm using true TPS; and 2) The interaction between thaxtomin A and potato is different in scab resistant and susceptible plants due to thaxtomin A detoxification.

To demonstrate the first hypothesis I focused on the following objectives: 1) to develop an optimized discriminant scab resistant screening assay for TPS using thaxtomin A and 2) to investigated the correlation between an optimized TPS-Thaxtomin A resistance assay for common scab resistance with tuber sensitivity to thaxtomin A and scab susceptibility in field tests in naturally infested soils.

To investigate the second hypothesis, I focused on the potato tuber-thaxtomin A interaction in scab resistant and susceptible potato plants. My objective was to demonstrate that glucosylation of thaxtomin A is related to potato plant resistance to pathogenic *S. scabies*. To evaluate this theory I produced ^{14}C labeled thaxomin A in vitro, tested toxin metabolism in scab resistant and susceptible potato cultivars, and identified and characterized their metabolites.

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CHAPTER 2

THE USE OF THAXTOMIN A FOR SCREENING POTATO GERMPLASM FOR RESISTANCE TO COMMON SCAB

Introduction

Common scab is of worldwide importance causing losses to both fresh market and processing growers by reducing market quality and increasing processor peel losses. This disease is caused by *Streptomyces scabies* (Lambert and Loria, 1989a), *S. acidiscabies* (Lambert and Loria, 1989b) and other *Streptomyces* spp. (Hooker, 1981). Loria (1994) describes scab lesions on potato tubers as superficial, erumpent, or pitted, due to the infection through the lenticels of immature tubers with lesions expanding as the tubers grow.

S. scabies (Thax.) Lambert and Loria and *S. acidiscabies* Lambert and Loria, are the most important causal agents of potato scab. Loria (1994) states that not only is the resistance of potato cultivars to the two pathogens the same, but also the host range and symptomatology. Therefore, this author suggests that these species share common determinants for pathogenicity on potatoes.

King et al. (1989) were able to isolate and characterize two active phytotoxic components from extracts of tissue infected with *S. scabies*. They designated these pathotoxins as thaxtomin A and thaxtomin B. These thaxtomins were able to produce symptoms typical of common scab disease when they were placed on aseptically cultured minitubers (Lawrence et al., 1990). Later, King et al. (1991) demonstrated a positive

correlation of thaxtomin A production with the pathogenicity of *S. scabies* isolates from scab infected potato tubers. Positive correlation between pathogenicity and thaxtomin production has been demonstrated in all strains studied of *S. scabies*, *S. acidiscabies* and *S. ipomoeae*. Thaxtomin A has been described as the most abundant of these toxins (Lawrence et al., 1990; King et al., 1991; King et al., 1992). Loria et al. (1995) described the differential production of thaxtomin by pathogenic *Streptomyces* species in vitro and the absence of production in non-pathogenic strains. Kinkel et al. (1998) found that thaxtomin A production in vitro was positively correlated with the percentage of tuber surface affected, but was not correlated with the number of lesions produced by an isolate. They suggested that the amount of thaxtomin A produced by an isolate determined the degree of lesion expansion but it is not predictive of lesion initiation. However, studies done in Canada by Goyer et al (1998) using mutants of pathogenic *S. scabies* altered in thaxtomin A production, showed no correlation between virulence and toxin production, however mutants that were not able to produce the toxin, were non pathogenic.

Hooker (1949) tested resistance to necrosis of seedlings from open pollinated seed of a number of potato cultivars resistant and susceptible to potato scab in soil infested with *S. scabies*. He did not find differences between progenies under his conditions. Later, Leiner et al. (1996) evaluated the pathogenicity of *S. scabies* on seedlings of diverse crop species and tested the hypothesis that thaxtomin A is involved in disease development. They confirmed that *S. scabies* is indeed pathogenic on seedlings of a variety of monocot and dicot crops. They did not test the toxin on true potato seed. These

authors indicated that the lesion type may be partially determined by the concentration of thaxtomin A and other thaxtomins in the infection site, with low concentrations of the toxin producing erumpent lesions and high concentrations resulting in cell death and pitted lesions.

Pathogen toxins have been used in other crops for resistance screening with interesting results (Buiatti and Ingram, 1991). Loria (1994) first suggested the possible application for the thaxtomins in the identification of scab resistant potato germplasm. She and her group developed an assay in which thaxtomin A was applied to immature tuber periderm and demonstrated a strong correlation between thaxtomin sensitivity and the susceptibility of cultivars to *S. scabies*. Previous work done by Delserone et al. (1991) indicated a strong correlation between thaxtomin sensitivity and the susceptibility of two-week-old tubers from cultivars differing in levels of scab resistance. Susceptible cultivars developed extensive necrosis, while resistant cultivars developed only necrotic flecks around the lenticels.

Traditionally, screening for scab resistance is done under field conditions in later clonal generations. There are several advantages of using TPS in screening for disease resistance including: sexual reproduction represents the greatest genetic diversity (Caligari, 1992); identified genes can be rapidly transferred to commercial cultivars without crossing through new molecular technologies (Belknap, 1994) or resistant individuals can be multiplied vegetatively; and lastly; reduced population sizes will need to be tested in the field. However, many breeding programs have not adopted this system because of the large volume of inoculum needed, variability in the virulence of *S. scabies*

isolates, variability in seedling infection and symptom development that makes evaluation for resistance and susceptibility difficult, and exposure to scab of the first clonal generation (Plaisted et al., 1984).

Due to the absence of efficient methods to select for resistance to potato scab, the importance of this disease to potato improvement programs, the advantages previously discussed for using early generation TPS selection in breeding, and the role of thaxtomin A in pathogenicity, evaluation of the use of thaxtomin A as a scab resistance screening tool using TPS was logical. This research focuses on the development of an optimized discriminant scab resistant screening assay for TPS using thaxtomin A. In addition, we investigated the correlation between an optimized TPS-Thaxtomin A resistance assay with common scab resistance of potato tubers as determined by potato slice exposed to the toxin and field tests in naturally infested soils.

Materials and Methods

Thaxtomin A Production and Purification

S. scabies strain 87/22, provided by Dr. Rosemary Loria, Cornell University, was used for thaxtomin A production. A scab lesion on potato was the origin of this isolate and Loria et al. (1995) described its high pathogenicity and thaxtomin A production. This strain was stored as a spore suspension in glycerol (20%) at –20 and –40°C (Hopwood et al., 1985). To prepare the bacterial inoculum, stored spores were streaked onto oatmeal agar (OMA) (Loria et al., 1995) plates and incubated for 10 days at 28°C. Washed spores from these plates were used to inoculate oatmeal broth (OMB) (Loria et al., 1995). Five

hundred ml Erlenmeyer flasks with 200ml of OMB were inoculated with a spore suspension (about 10^8 spores per flask) and incubated on a rotary shaker at 150-180 rpm at 28°C for 10 days.

The methods used for extraction, purification and identification of thaxtomin A were done as described by King et al, (1992) and Loria et al. (1995). A pure thaxtomin A sample, provided by Dr. R.R. King, New Brunswick, Canada, was used as a standard for our toxin production and purification. After the liquid culture was filtered through cheesecloth, filtrates were extracted twice with chloroform in ½ volume proportion. The organic phase was dried by flash evaporation, and then the crude extract was stored in the dark at 4°C. This crude extract was dissolved in methanol and loaded on EM Science silica gel 60 F254 thin layer chromatography (TLC) plates and run in chloroform and methanol (9:1). The yellow band co-migrating with the pure standard thaxtomin A was eluted from the silica and dissolved in methanol. This sample was rechromatographed on Whatman KC18F reversed phase silica gel 60 A TLC and run in acetone and water (3:2). Final thaxtomin A purification was carried by successive passage in TLC on silica gel 60 in the following solvent systems: A) chloroform:methanol (9:1), B) chloroform: acetonitrile (9:1) and C) ethyl acetate:isopropanol (9:5) and on reversed phase C18 in D) acetone:water (3:2) and E) isocratic methanol solvent systems.

Identification and quantification of thaxtomin A was done by electrospray ionization mass spectroscopy, H-NMR spectra, high pressure liquid chromatography (HPLC) and spectrometry relative to the pure standard thaxtomin A. Mass spectroscopy and H-NMR were performed by Department of Chemistry and Biochemistry at Montana

State University. Mass spectroscopy was done in methanol and water and acetic acid (50:50:1) solvents. The sample was injected with a spray flow of 2µl/min and a spray voltage of 2.2 KV via the loop injection method. An NMR spectrum was done in a Bruker DRX-500 instrument with 64 scans, while the sample was dissolved in deuterated methanol. HPLC was done using a Waters 600E System controller and Waters 441 absorbance detector spectrophotometer (Millipore Waters, Milford, MA) with a Microsorb-MV (Microsorb, Woburn, MA) C8 column 5 µm 100A of 22 cm. The sample was eluted with a 25-50% acetonitrile gradient over 20 min. and monitored at A_{380}. A standard curve was made with serial dilutions of the standard thaxtomin A. Ultraviolet and visible spectroscopy analyses were done in a Beckman (Beckman Instruments, Inc., Fullerton, CA) UV/vis DU-50 spectrophotometer using the extinction coefficients determined by King et al. (1992). A standard curve was performed with serial dilutions and read at A_{398}.

Potato Cultivars and TPS Crosses

The potatoes cultivars used in this research were commercial cultivars with differing resistance to common scab according to the descriptions of American Potato Varieties (PAA, 1998). The cultivars used were as follows: Ranger Russet (Pavek et al., 1992), Russet Norkotah (Johansen et al., 1988), Atlantic and Nooksack (Hoyman and Holland, 1974) that are susceptible, tolerant, tolerant and resistant to common scab, respectively (PPA, 1998). These cultivars were used as parents for TPS progenies and as controls in field experiments and potato slice tests.

TPS from self-crosses of Ranger (RxR), Norkotah (NkxNk), Atlantic (AtxAt) and Nooksack (NxN) and crosses of Nooksack x Ranger (RxN) were provided by Drs. J. Pavek and D. Corsini, USDA/ARS, located at the University of Idaho Research and Extension Center, Aberdeen, Idaho. In addition, TPS from crosses from several *S. tuberosum* selections were provided by Dr. Oscar Hidalgo from International Potato Center, Lima, Peru.

Seedling Assay

Seedlings were exposed to thaxtomin A at different concentrations to evaluate their susceptibility to the toxin. Seeds were surface disinfected in a solution of 0.5% NaOCl for 10 min, air-dried, treated overnight with 2000ug/ml of gibberelic acid, air dried and plated according to the respective treatment.

The growth medium used was 1.5% water agar (WA) (Difco Lab, Detroit, MI.) amended with thaxtomin A. Thaxtomin A was dissolved in an amount of ethyl alcohol no greater than 10% of the total medium and incorporated into the WA after autoclaving in an amount necessary for the required final toxin concentration. Controls with no toxin were amended only with the appropriate amount of ethyl alcohol. Five ml of the medium was added to 60x15 mm plastic petri dishes (VWR Scientific, West Chester, PA.).

Seeds were plated on the media, using sterile technique, and incubated in a growth chamber at 20°C, 200 $\mu mol/m^2/sec$ light intensity and 14 hrs light. Each seed was evaluated after 3, 5, 7, 10, 12 and/or 14 days according to the following index of damage to the radical: 1=Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4=Radical deformed with large necrotic areas and 5=Radical dead (Figure 1).