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.

Glucosylation of TA by S. scabies occurs in vitro. The glucose conjugate of thaxtomin A from bacterial extracts was described as Thaxtomin A-β-di-0-gluosides (TAG). TAG was 6 times less toxic than TA in potato tubers. This research suggests a similar mechanism of TA detoxification in potato tubers. Resistant plants inoculated with 14C-TA were able to produce a higher amount of a radioactive metabolite with an Rf similar to TAG than susceptible ones. We found almost twice as much glucose transferase specific activity in resistant than in susceptible individuals. Glucose conjugation may be a mechanism of TA detoxification in potato and it appears to be related to scab resistance and susceptibility in potato plants.

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

by

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APPROVAL

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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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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 μmol/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).

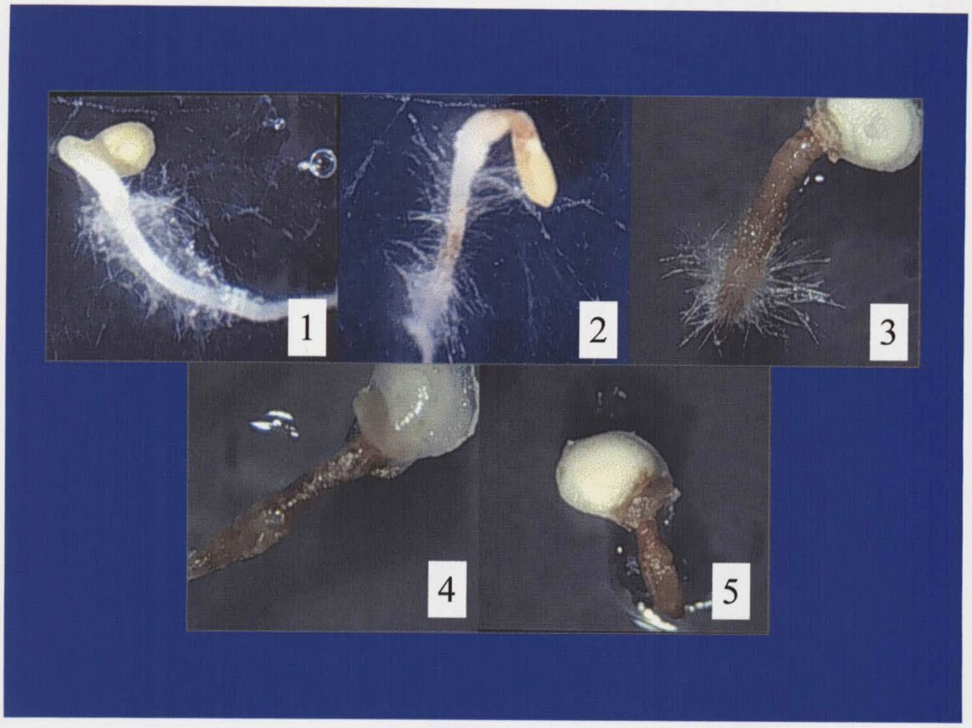

Figure 1. Index of Damage to the Radical of True Potato Seed Germinated on Agar Amended with Thaxtomin A. 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead.

Only germinated seed were evaluated. For statistical analysis an average index of damage was calculated for each experimental plot (petri dish) according to the following equation:

$$\text{Average Index of Damage} = \frac{\Sigma\ (\text{index of damage x \# of seedling per index of damage})}{\text{Total \# of seedling evaluated}}$$

Selected seedling toxin survivors and controls were rescued from the toxin media plates and placed in a Murashige and Skoog (1962) basal medium with Gamborg's vitamins (Sigma Chemical Co., St Louis, MO, M0404.). This in vitro medium was amended with sucrose (20g/L, Sigma), Kinetin (0.04%, Sigma), and Phytogel (2g/L, Sigma); the pH was adjusted to 5.7. Plantlets of the selected clones were multiplied and grown in a growth chamber at 20°C, 200 μmol/m^2/sec light intensity and 14 hrs light. These plantlets were used later for greenhouse minituber production and field experiments.

To determine the optimal toxin concentration and seedling exposure times, TPS from RxR, NxN, AtxAt and NkxNk were used. Thaxtomin A treatment concentrations tested in the media were the following: 50, 25, 15, 10, 5, 1, 0.1 and 0 (control) μM. The experimental plot was a petri dish where ten seeds per cross were plated with four replications per treatment. The plates were incubated up to 14 days. Index of damage and percentage of germination were recorded after 3, 5, 7, 10 and/or 12 days. After 14 days, the toxin survivor seedlings with indices of 0, 1, 2 and 3 were rescued and maintained and multiplied in vitro for field scab screening experiments. The data was statistically analyzed as a 4x10 factorial design for percentage and average index of damage for each evaluation date.

TPS from crosses from International Potato Center (CIP) were used selected with thaxtomin A as seedlings whose parents were of unknown scab susceptibility. The crosses selected were the following: Achira x Ccompis (cross 1), I-654 x Ccompis (cross 2), Aracy x Ccompis (cross 3), MF-II x Ccompis (cross 4), 4.1DI x Ccompis (cross 5), Ccompis x 4.1DI (cross 6), F-6 X Ccompis (cross 7), Yungay x Ccompis (cross 8). Controls were progenies from self-crosses of Ranger (RxR, cross 9) and Nooksack (NxN, cross10). Four levels of thaxtomin A were used in the media: 2, 1, 0.5 and 0 μM. The experimental plot was a petri dish with 10 seed. Four replications were established. Plates were incubated for 7 days and evaluated for index of damage and percentage of germination 3 and 6 days after plating. Some seedling survivors with damage index of 4, 3, 2 and 1 were rescued, multiplied in vitro and tested for scab resistance under field conditions. Statistical analysis was performed using a 10x4 factorial design for average index of damage and percentage of seed germination.

An experiment was also performed to test the effectiveness of thaxtomin A selection for scab resistance in contrast to selection in the absence of the toxin. Three crosses were tested, scab susceptible self-cross (RxR), scab resistant self-cross (NxN) and resistant x susceptible cross (RxN). Two levels of toxin were tested 1 and 0 μM (with and without toxin). Each treatment had 5 replications. Petri plates (50 seeds per plate) were incubated 7 days and scored for index of damage and percentage of seed germination. Toxin survivors with a damage index of 1, 2 and 3 were rescued, multiplied and maintained in vitro. Also, seedlings selected in the absence of the toxin were rescued in each treatment in equal numbers to those selected in the presence of the thaxtomin A.

These seedlings were used for field experiments for scab resistance evaluation. Statistical analysis was performed using a 3x2 factorial design for average index of damage and percentage of seedling with index of damage 1 and 2.

Tuber Slice Test

This assay, described by Loria et al. (1995), was used to confirm phytotoxicity of purified thaxtomin A and to test the toxin sensitivity of the selected resistant and susceptible clones. Plantlets about 1 month old from in vitro multiplication were transplanted to a 6 cm diameter container (Deep 606 Inserts, T.O. Plastics, Inc., Clearwater, MN.) with Sunshine Mix #1 (Sun Gro Horticulture Inc.) at the Plant Growth Center-MSU. The seedlings were maintained in a growth room at 18°C, 450 μmol/m^2/sec light intensity and 12 hrs light. After 2-3 weeks the plants were transplanted to a 20 cm diameter container (Belden Plastics, St. Paul, MN.) using the same soil mix, and kept in the growth chamber for minituber production (about 4-5 weeks). Minitubers were harvested immature and stored at 7°C for at least 8 weeks, to reduce nonspecific browning (Loria et al., 1995). Minitubers were surface sterilized with 0.5% NaOCl for 10 min, air-dried under sterile conditions, dipped in ethanol and flamed. Two cm diameter cores of pith tissue were removed aseptically with a cork borer, and sliced 0.5 cm thick. The slices were put in a 9 cm petri dish with wet Whatman #1 filter paper (Whatman Int., Maidstone, England). Thaxtomin A was dissolved in ethanol according to the concentration to be tested. Paper filter disks of 6mm diameter (Whatman #1) were immersed in this solution for 1 hr, air-dried and placed on the potato slice. Controls were immersed in ethanol. Ten μl of sterile distilled water were applied to each disk to facilitated toxin contact with the tissue. Plates were incubated at 24°C in the dark and evaluated after 3, 5 and 7 days. Evaluation was in the defined area under the paper filter disk using the followed index of necrosis: 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis. Statistical analysis was appropriate for a completely randomized design (CRD) was used.

Miniplot Field Experiments

Miniplot experiments were established to evaluate, under natural infested soil and field conditions, scab susceptibility of the tubers of plants selected from the seedling assays. Miniplots were carried out in 1997 and 1998 at the Horticultural Farm-MSU, Bozeman, MT.

Soil naturally infested with *S. scabies* was collected at the Tobol Farm Inc., Ronan, MT. This soil was mixed with vermiculite (1:2 v/v) using a cement mixer. In 1997, this soil mix was used to fill 280 plastic 19L containers (I.E.M. Plastics, Reidsville, NC.). Containers were buried in the field in 10 rows spaced 1.5 m apart and 0.5 m between containers in the row. In 1998, the soil mix was used to fill 860 plastic 9L containers (I.E.M. Plastics, Reidsville, NC.); that were buried in 10 rows 1.5 m apart with 1m between containers in the row.

Plantlets from in vitro multiplication were transplanted to small containers with a mixture of vermiculite and sand (1:1). These plants were grown under greenhouse conditions (20°C, 16 hr light) for 4 weeks when they were transplanted to the plastic pot in the field. The pot was the experimental plot. At harvest, minitubers were evaluated for tuber scab symptoms and yield. A scab index of damage was used to evaluate symptoms according to the percentage of tuber surface with scab. The scab index was as follows: 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95% tuber surface covered (Figure 2). This scab index was used to evaluate tubers in all the miniplots and field experiments in this research. An average scab index of damage was calculated per treatment according to number of tubers per index per plant as follows:

$$\text{Average Scab Index} = \frac{\Sigma\ (\text{scab index x \# tubers per index})}{\text{Total \# of tubers}}$$

Minituber samples were kept for field experiments and stored at 7°C until next the season.

In 1997, twenty-four surviving seedlings from the thaxtomin A concentration-time experiment were tested: ten from Ranger self-crosses, six from Nooksack self-crosses and eight from Atlantic self-crosses. Seed tubers of Ranger, Nooksack, Atlantic and Norkotah were used as controls in this experiment. The experiment was established in a CRD with 28 treatments (clones) with 10 replications. Five replications were transplanted on July 7 (Date 1) and the other 5 replications on July 11 (Date 2). Each pot was fertilized with 12 g of Osmocote ® (19-6-12) and irrigated with a sprinkler system twice a week. After 90 days, minitubers were harvested and evaluated for scab as previously described. Analysis of data was done for each date and both dates together.

In 1998, thirty-six seedlings that survived toxin treatments from CIP seed test were tested plus Ranger and Nooksack controls. Four from cross 1, 4 from cross 2, 3 from cross 3, 7 from cross 4, 1 from cross 5, 7 from cross 6, 2 from cross 7, 3 from cross 8, 4 from cross 9 and 1 from cross 10.

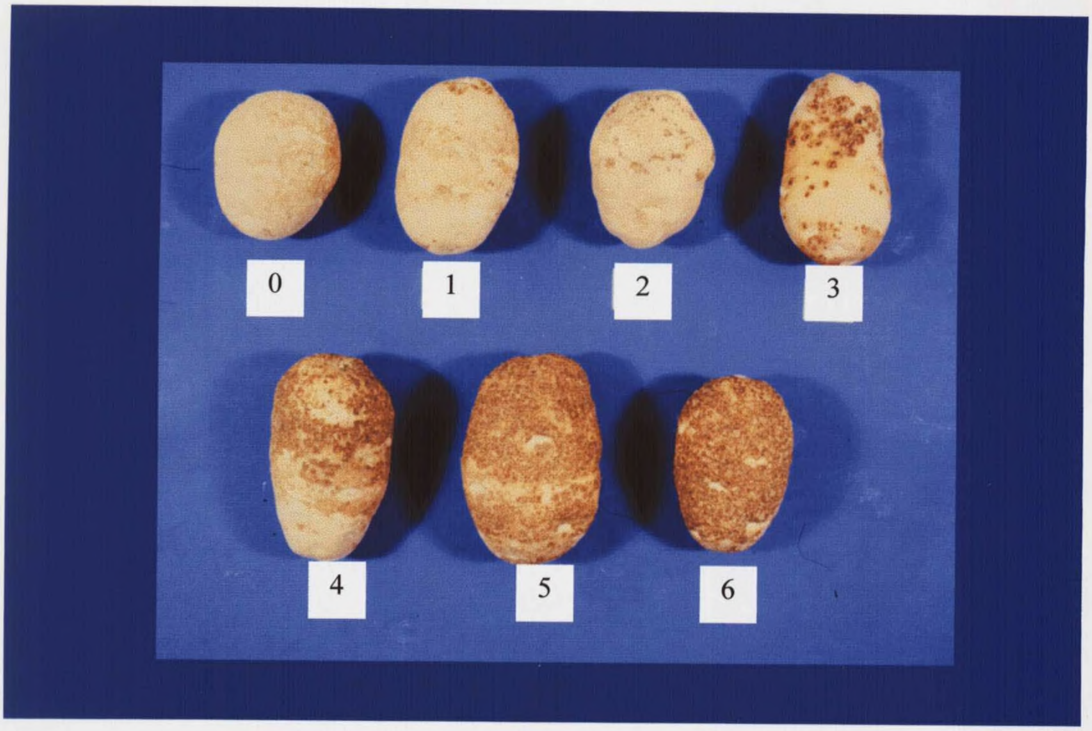

Figure 2. Scab Index on Potato Tubers According to the Percentage of Tuber Surface Covered with Scab Lesions
0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

Seedlings that showed an index of damage of 2 were used from crosses 1, 2, 3, 5, 7, 8, 9 and 10. But, seedlings with index of damage of 2, 3, and 4 were used from crosses 4 and 6. Plants were transplanted to containers in the field in a Complete Randomized Block Design (CRBD) with 38 treatments and 5 replications. Containers were fertilized with 6 g of Osmocote ® (19-6-12) and irrigated with a sprinkler system twice a week. After 90 days minitubers were harvested and evaluated for scab as previously described.

Also in 1998, one hundred and thirty two seedlings from the selection with and without thaxtomin A experiment were tested. These seedlings showed an index of damage of 1, 2 or 3. The following selections were tested: 21 from the Ranger self cross, 5 from the Nooksack self cross and 34 from the Nooksack x Ranger cross with toxin selection, and 20 from the Ranger self cross, 24 from the Nooksack self cross and 28 from the Nooksack x Ranger cross without toxin selection. In addition, Ranger and Nooksack parent plantlets were used as controls. The experiment was established in a CRBD with 134 treatments with 5 replications. Containers were fertilized with 6 g of Osmocote® (19-6-12) and irrigated with sprinkler system twice a week. After 90 days minitubers were harvested and evaluated. Statistical analysis was performed in a CRBD and in a factorial 2x3 (2 toxin levels x 3 crosses levels).

Field Experiments

Field experiments were performed in 1998 and 1999 at Tobol Farms, Ronan, MT. The soil is classified in the Ronan series (USDA-NRCS, 2000) that are characterized as very deep, well drained soils, formed in clay lacustrine and glaciolacustrine deposits. These are very fine soils, illitic, frigid, Typic Natrixeralfs with a typical pedon of Ronan silty clay loam, cultivated, with pH 8.0 and highly conducive to potato scab. The plot location had been heavily manured with dairy waste in previous years and tuber scab was very severe on susceptible potato cultivars in previous crops.

In 1998, the twenty-four clones and four controls tested in 1997 in miniplots were planted in the Ronan field plot, using as seed the minitubers harvested the previous season. The experiment was established in CRBD with 28 treatments and 4 replications. The experimental plot consisted of 5 plants in a row spaced 0.3 m in rows 0.9 m apart. After 120 days, plots were harvested and tubers evaluated for scab damage and yield. Harvested tubers were stored at 10°C to be used as seed the next season.

In 1999, three experiments were performed at the Ronan site. The first experiment consisted in the individuals selected from the "with and without" thaxtomin A selection experiment. Selections with average scab index similar or lower than Nooksack (resistant parent control) from the previous miniplot experiments were selected and planted in the field experiment. A total of 54 clones plus the Ranger and Nooksack controls were tested. The experiment was performed in a CRBD with 56 treatments and 3 replications, with 3 plants per experimental plot. Statistical analysis was performed in a CRBD and in a factorial 2x3 (2 toxin levels x 3 crosses (RxR, NxN, RxN)) according to the origin of the selection in the seedling assay. The second experiment was performed to test other clones with scab indices similar or lower than Nooksack from the same previous experiment; no replications were used because of a shortage of minitubers. Therefore, this experiment was established with 25 treatments and 1 replication, with the experimental plot being 4 plants. The third experiment consisted of the best 8 clones previously tested in 1998 field experiment. Clones were planted in a row of 30 plants each, with no replications. All experiments were harvest after 120 days and tubers were evaluated for scab symptoms and yield.

Estimation of Soil *Streptomyces* spp. Population

Soil samples from miniplots and field experiments were taken to estimate the potential inoculum of *Streptomyces* spp.in the soil. Isolation was done according to the techniques described by Loria and Davis (1989). Three soil samples from the root zone were collected randomly in the field or pots. Each sample was a combination of 10 subsamples. The soil was air dried overnight, 10 g of each sample was added to 90 ml of 0.1 % water agar (WA) (first dilution, 10^{-1}) and agitated on a shaker for 10 min. Four more 10-fold dilutions were done. 100ul of the 10^{-3}, 10^{-4}, 10^{-5} dilutions were spread on Nystatin, Polimyxin, Penicillin, Cycloheximide (NPPC) water agar medium (Loria and Davis, 1989). Plates were incubated at 28°C for 10 days. Colony forming units (CFU) were determined per gram of soil counting colonies with *S. scabies* characteristics (Loria and Davis, 1989).

Statistical Analysis

Statistical analyses were performed according to the experimental design of each experiment using Statistical Analysis System (SAS) software (SAS/STAT^{TM}, 1988). Data were analyzed by analysis of variance (ANOVA) and F test. Least significant difference (LSD) was calculated at α= 5% or 10% to compare differences between more than two treatments. Pearson correlation coefficients (Cody and Smith, 1985) were determined for both seedling index of damage and scab index, and necrosis in potato slice test and scab index.

Results

Thaxtomin A Production and Purification

Thaxtomin A produced and purified in our laboratory showed the same R_f as the standard in the five solvent systems used: A (0.3), B (0), C (0.91), D (0.57) and E (0.85). Spectrophotometer readings at 398, 343, 279, 249 and 220 nm, gave a similar spectral absorption curve for both the standard and our toxin sample. A standard curve was done with dilutions of a known quantity of the toxin using UV/vis spectrophotometer at 398 nm based in extinction coefficients determined by King et al (1992) (Figure 28, Appendix A). In addition, standard Thaxtomin A eluded a single peak at 20min in HPLC detection, as did our sample. Using dilutions of a known concentration of the toxin, a standard curve was established (Figure 29, Appendix A). These curves were used to quantify the toxin in our experiments. Thaxtomin A yield was estimated at about 4μg/ml of OMB. Thaxtomin A electrospray ionization mass spectroscopy showed an $[M+Na]^+$ at 461.4 yielding a molecular weight of approximately 438.4 (Figure 30, Appendix A). These data agree with King et al. (1992) results. Furthermore, NMR spectra (Figure 31, Appendix A) agree with the chemical structure described by Lawrence et al. (1990). Bioactivity assay of our thaxtomin on potato slices produced necrosis at 10 μM after 7 days of incubation, similar to the standard. Therefore, given the previous information, the thaxtomin A used in our experiments was pure and comparable to the standard supplied by Dr. King.

Thaxtomin A Concentration and Exposure Time Seedling Assay

Results of these experiments are shown in Tables 23, 24, 25 and 26 (Appendix B). Percentages of germination of TPS germinated on agar amended with thaxtomin A after 3, 5, 7 and 10 days of evaluation are shown in Table 1. The germination at 3 days was very low, except for Atlantic. At 5 days most of the seed had germinated, but Nooksack was still lower than the others. At 7 days all the progenies germinated in the 88-94% range. Due to the high differences in the germination rate over time, we tested the affect of gibberellic acid (GA) at 2000ppm on TPS in association with the thaxtomin A (data not shown). We concluded that there was no interaction of GA with the toxin, but early germination was enhanced for all varieties tested.

Table 1. Percentage of Germination of True Potato Seed from Self-crosses of Ranger, Atlantic, Nooksack and Norkotah after 3, 5, 7 and 10 Days Exposure to Thaxtomin A Across all Concentrations of 0 to 50 μM.

Self-crosses	Germination (%)			
	Exposure Time (days)			
	3	5	7	10
Ranger	27.1 c*	82.1 b*	88.6 b*	92.1 a*
Atlantic	75.3 a	91.5 a	94.2 a	92.7 a
Nooksack	4.3 d	75.7 c	88.0 b	90.5 a
Norkotah	37.5 b	88.2 a	94.1 a	94.4 a

(*) Numbers in a column followed by the same letter are not significant different at 5% LSD. Data from average of thaxtomin A dose treatments.

The affect of thaxtomin A on the percentage of germination of TPS is shown in Table 2. After 3 days of toxin exposure, the seed germination was reduced by concentrations higher than 10 μM; lower concentrations were statistically similar to the non-toxin controls. In addition, the ethanol control was not different from ethanol solvent at all the evaluation dates; therefore ethanol solvent at the concentration used (10% total in the media) did not affect germination. Previously, we tested higher concentrations of ethanol in the media and we detected toxicity on seed at concentrations higher than 10% (data not shown). At 5,7 and 10 days, seed germination at 25 μM of thaxtomin A was not different from the controls. However, the highest toxin concentration tested (50 μM) reduced the germination at all exposure periods tested.

Table 2. Percentage of Germination Across all cultivars of True Potato Seed from Ranger, Atlantic, Nooksack and Norkotah Self-crosses on Agar Amended with 0 to 50μM of Thaxtomin A after 3, 5, 7,and 10 Days of Exposure.

Thaxtomin A Levels (μM)	Percentage Germination (%) Exposure Time (days) 3	5	7	10
50	18.1 d*	67.5 c*	75.6 c*	76.9 c*
25	21.8 cd	75.0 bc	86.2 b	87.5 b
15	31.2 bc	84.4 ab	92.5 ab	93.7 ab
10	36.9 ab	84.4 ab	90.6 ab	91.8 ab
5	35.3 ab	84.0 ab	92.0 ab	94.0 ab
1	41.2 ab	87.5 a	93.7 ab	95.6 ab
0.5	48.1 a	84.4 ab	90.6 ab	93.1 ab
0.1	41.3 ab	91.2 a	97.5 a	96.9 ab
0(1)	49.3 a	93.7 a	96.2 ab	95.6 ab
0	40.9 ab	92.8 a	98.6 a	100.0 a

(1) Control with no toxin solvent (ethanol) in the media.

(*) Numbers in a column followed by the same letter are not significant different at 5% LSD. Data from average of self-crosses treatments.

Indices of damage of seedlings exposed to thaxtomin A from the 4 self-crosses tested by date of evaluation are shown in Table 3. In general, the seedling radical index of damage increased at times longer than 7 days exposure. However, the index of damage developed on self-cross progenies of Ranger and Nooksack were statistically different for all the dates of evaluation.

Table 3. Overall Average Index of Damage for True Potato Seed Seedlings from Self-crosses of Ranger, Atlantic, Nooksack and Norkotah at 5, 7, 10 and 12 Days Exposure to Thaxtomin A at Concentrations of 0 to 50 μM.

Self-crosses	Index of Damage(1)			
	Exposure Time (days)			
	5	7	10	12
Ranger	2.0 b*	2.2 b*	3.3 a*	3.3 a*
Atlantic	2.0 b	2.0 a	3.2 a	3.2 a
Norkotah	1.9 b	2.2 b	3.5 b	3.4 b
Nooksack	1.5 a	2.0 a	3.5 b	3.4 b

(1) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead.

(*) Numbers in a column followed by the same letter are not significant different at 5% LSD. Data from average of thaxtomin A concentration treatments.

The index of damage for TPS seedling germinated on agar with 0 to 50 μM of thaxtomin A by date is shown in Table 4. Non-toxin controls did not develop radical necrosis. In general, the index of damage increased with time of exposure to the toxin. After 5 and 7 days, 0.1 μM was similar to the control, while concentrations over 5μM after 5, 7, 10 and 12 days were not different from each other. Toxin concentrations of 1 μM after 5 and 7 days showed statistically significant differences compared to higher concentrations and controls.

Data in Table 5 indicate that the lowest concentration at which it is possible to see significant differences between Ranger (scab susceptible) and Nooksack (scab resistant) progenies are 1μM and 5μM at 5 and 7 days. In addition, combining this information with the analysis of interaction for self-cross-concentrations by date (Table 6), 1μM of thaxtomin A was identified as the lowest discriminatory concentration for use in seedling screening tests. This concentration provided discrimination between the progenies from resistant and susceptible potatoes tested. This can be clearly seen in the figures 3 and 4.

Table 4. Index of Damage Across Cultivars for True Potato Seed Seedlings from Ranger, Atlantic, Nooksack and Norkotha Self-crosses on Agar Amended with 0 to 50 μM of Thaxtomin A at 5, 7,10 and 12 Days of Exposure.

Thaxtomin A Levels (μM)	Index of Damage[1] Exposure Time (days) 5	7	10	12
50	2.6 d*	2.9 d*	5.0 e*	5.0 e*
25	2.6 d	3.0 d	5.0 e	5.0 e
15	2.7 d	2.9 d	5.0 e	5.0 e
10	2.7 d	2.9 d	4.9 e	4.9 e
5	2.4 c	3.0 d	4.9 e	4.9 e
1	1.2 b	1.9 c	3.1 d	3.1 d
0.5	1.1 a	1.3 b	2.3 c	2.3 c
0.1	1.0 a	1.0 a	1.3 b	1.1 b
0[2]	1.0 a	1.0 a	1.0 a	1.0 a
0	1.0 a	1.0 a	1.0 a	1.0 a

(1) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead

(2) Control with no toxin solvent (ethanol) in the media.

(*) Numbers in a column followed by the same letter are not significantly different at 5% LSD

Figures 3 and 4 show that 5 and 7 days are the points where susceptible parent progenies and resistant parent progenies can be discriminated. In addition, the percentage of germination at 7 days was higher than at 5 days (Figure 5), therefore 7 days maximizes the number of seedlings that can be screened and from which resistant individuals could be rescued. Furthermore, 1 and 5 μM (Figure 4) seems the best toxin concentration to discriminate differences between these two progenies. Because 1μM is adequate, this dose was chosen for further tests since it conserves available purified toxin. This is important since purified toxin preparation is a long intensive process.

Table 5. Index of Damage for True Potato Seed Seedlings from Self-crosses of Range(R) and Nooksack (N) Exposed to Thaxtomin A at Concentrations of 0 to 50 µM after 5, 7, 10 and 12 days, Analyses by Toxin Level.

ThaxtominA Level (µM)	Index of Damage(1)							
	Exposure Time 5 days		Exposure Time 7days		ExposureTime 10 days		Exposure Time 12 days	
	R	N	R	N	R	N	R	N
50	2.9b*	1.8a	2.9a*	2.8a	5.0a*	5.0a	5.0d*	5.0d
25	2.9c*	1.8a	3.1a*	3.0a	5.0a*	5.0a	5.0d*	5.0d
15	2.9b*	2.4a	3.0a*	2.9a	5.0a*	5.0a	5.0d*	5.0d
10	2.8b*	2.3a	3.0a*	2.9a	5.0a*	5.0a	5.0d*	5.0d
5	2.7bc*	1.6a	3.5b*	2.8a	4.9a*	4.8a	4.9d*	4.9d
1	1.6c*	1.0a	2.3c*	1.5a	3.3bc*	3.1bc	3.3c*	2.9c
0.5	1.2a*	1.0a	1.3a*	1.3a	1.9a*	2.8 b	2.0b*	2.7c
0.1	1.0a*	1.0a	1.0a*	1.0a	1.0a*	1.9 b	1.0a*	1.6b
0	1.0a*	1.0a	1.0a*	1.0a	1.0a*	1.0a	1.0a*	1.0a

(1) 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

(*) Numbers in a row per exposure time with same letter are not significantly different at 5% LSD

Table 6. Index of Damage for True Potato Seed Seedling from Self-crosses of Ranger (R) and Nooksack (N) Exposed to Thaxtomin A at Concentrations of 0 to 50µM at 5, 7, 10 and 12 days, Analysed by Progeny.

ThaxtominA Level (µM)	Index of Damage(1)							
	Exposure Time 5 days		Exposure Time 7days		ExposureTime 10 days		Exposure Time 12 days	
	R	N	R	N	R	N	R	N
50	2.9c*	1.8b*	2.9d*	2.8c*	5.0d*	5.0d*	5.0d*	5.0d*
25	2.9c	1.8b	3.1d	3.0c	5.0d	5.0d	5.0d	5.0d
15	2.9c	2.4c	3.0d	2.9c	5.0d	5.0d	5.0d	5.0d
10	2.8c	2.3c	3.0d	2.9c	5.0d	5.0d	5.0d	5.0d
5	2.7c	1.6b	3.5e	2.8c	4.9d	4.9d	4.9d	4.9d
1	1.6b	1.0a	2.3c	1.5b	3.4c	3.1c	3.2c	2.9c
0.5	1.2a	1.0a	1.3b	1.3b	1.9b	2.9c	2.0b	2.7c
0.1	1.0a	1.0a	1.0a	1.0a	1.0a	1.9b	1.0a	1.6b
0	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a

(1) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead

(*) Numbers in a column per exposure time with same letter are not significantly different at 5% LSD

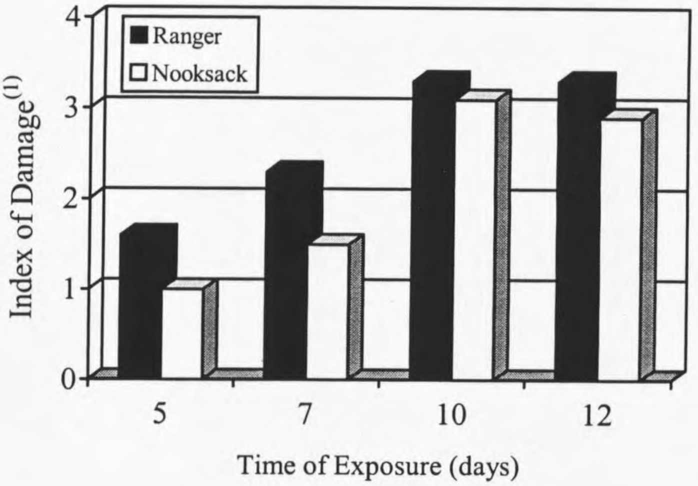

Figure 3. Index of Damage for True Potato Seed Seedling from Self-crosses of Ranger and Nooksack Germinated on Agar Amended with 1μM of Thaxtomin A after 5, 7, 10 and 12 Days of Exposure. (1) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead.

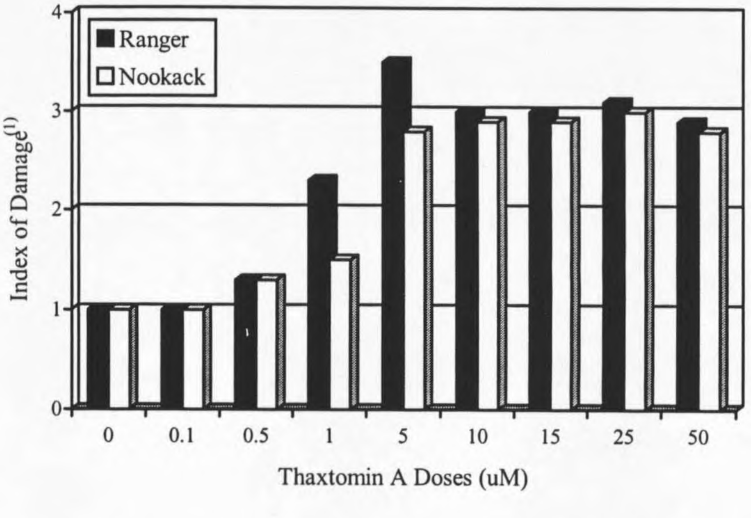

Figure 4. Index of Damage for True Potato Seed Seedlings from Self-crosses of Ranger and Nooksack Germinated on Agar Amended with 0 to 50μM of Thaxtomin A after 7 Days of Exposure. (1) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead.

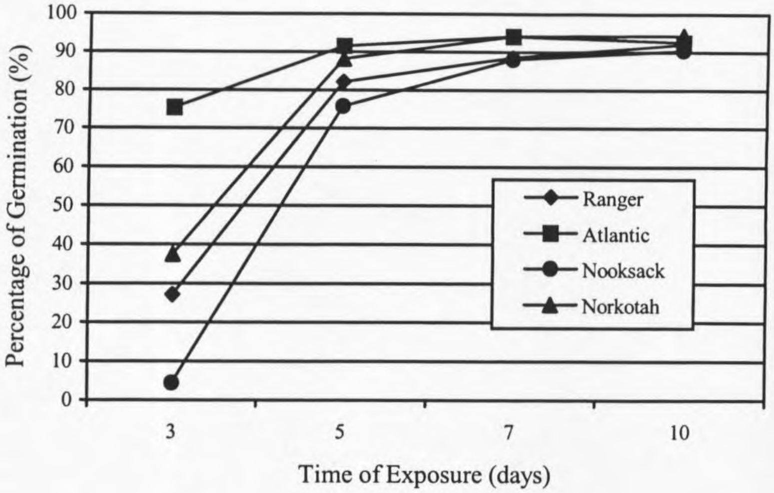

Figure 5. Percentage of Germination of True Potato Seed from Self-crosses of Ranger, Atlantic, Nooksack and Norkotah after 3, 5, 7 and 10 Days Exposure to Thaxtomin A at Concentrations Between 0 to 50 μM.
LSD=6.02 (3 days), 4.98 (5 days), 4.47 (7 days), 4.28 (10 days); α=0.05.

CIP Seed Test

Table 7 shows the results of this experiment where TPS progenies from 10 crosses of different *Solanum tuberosum* selections were tested for index of damage after exposure to 0 to 2 μM of thaxtomin A for 6 days. At toxin concentrations of 0.5, 1 and 2 μM it was possible to observe a significant difference between self-crosses of Ranger and Nooksack. At the 0.5 μM dose statistically significant differences were discerned but were not discriminatory in practice due to the low index of damage observed, while concentrations of 1 and 2 μM constantly produced visible but variable damage in these seedling tests. Also, all of the crosses tested seem to be very tolerant to the toxin, except cross # 6 at 1 μM. The range of symptoms developed for cross # 4 and # 6, allowed us to rescue seedlings with different indices of damage to be tested in the miniplot experiment.

Table 7. Index of Damage for True Potato Seed Seedling from 10 Crosses of Potato Supplied by The International Potato Center Germinated on Agar Amended with 0 to 2 μM of Thaxtomin A After 6 Days of Exposure.

Crosses	Index of Damage [1]			
	Thaxtomin A Concentrations (μM)			
	0	0.5	1	2
1. Achira x Ccompis	1.0 a*	1.1 cd*	2.0 c*	2.2 cde*
2. I-654 xCcompis	1.0 a	1.5 b	2.1 c	2.2 de
3. Aracy x Ccompis	1.0 a	1.2 c	2.1 c	2.8 b
4. MF-II x Ccompis	1.0 a	1.0 d	2.3 bc	3.0 b
5. 4.1DI x Ccompis	1.0 a	1.0 d	2.0 c	2.2 cde
6. Ccompis x 4.1DI	1.0 a	1.5 b	2.5 ab	2.3 cde
7. F-6 X Ccompis	1.0 a	1.3 c	2.0 c	2.6 bcd
8. Yungay x Ccompis	1.0 a	1.7 b	2.0 c	2.0 e
9. Ranger self cross	1.0 a	1.9 a	2.6 a	4.0 a
10. Nooksack self cross	1.0 a	1.1 cd	2.0 c	2.7 bc

(1) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead.

(*) Numbers in a column followed by the same letter are not significantly different at 5% LSD

Seedling Selection with and without Thaxtomin A

Our hypothesis was that seedlings with index of damage of 1 and 2 were less sensitive to the toxin, therefore the most resistant to scab. Seedlings with these scores were rescued and used in miniplot experiments. In addition, seedlings with higher indices were also rescued. The results of the seedling tests are shown in Table 8. Confirming previous results, there was a significant difference in the index of damage of seedling progenies from the resistant cross and the susceptible cross. In addition, the percentage of seedlings with indices of damage 1 and 2, considered resistant, was higher in Nooksack as expected. Moreover, the cross Nooksack x Ranger was not statistically different from Nooksack self-cross for index of damage and percentage of seedlings with indexes 1 and

2. This could be because of the simple heritability of scab resistance, the high combining ability of Nooksack or the cytoplasmic heritability of resistance (Lauer and Edie, 1963; Pfeffer and Effemert, 1985; Zadina, 1985). However, we did not perform heritability studies to confirm this. Seedlings with indices of damage 1, 2 and 3 were rescued to be multiplied in vitro. Thirty-four seedlings were rescued from Ranger self-cross, 69 from Nooksack self cross and 51 from the Nooksack x Ranger cross. The same number of seedlings per cross from non-toxin selection treatments were randomly selected and multiplied in vitro.

Table 8. Index of Damage and Percentage of Seedlings with Index 1 and 2 of TPS Seedling from Self-crosses of Ranger and Nooksack and Nooksack x Ranger Cross Germinated in Agar Amended with 1 and 0 μM of Thaxtomin A for 7 days

Cross	Level of Thaxtomin A (μM)	Average Index of Damage⁽¹⁾	% Seedling with Indices 1 and 2
Ranger self-cross	1	3.6 a*	10 c*
Ranger self-cross	0	1.0 c	100 a
Nookasck self-cross	1	2.9 b	33 b
Nookasck self cross	0	1.0 c	100 a
Nooksack x Ranger	1	3.1 b	26 b
Nooksack x Ranger	0	1.0 c	100 a

(1) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead.

(*) Numbers in a column followed by the same letter are not significantly different at 5% LDS.

Miniplot Field Experiments

Miniplot experiments were performed in 1997 and 1998 to test the toxin seedling sensitivity and scab reaction in infested field soils. Individual selections tested were from TPS- toxin experiments. Results of soil sampling from field and container to estimate the population of *Streptomyces* spp. were as follows: Ronan field soil 2.6 x 10^6 cfu/gr soil; and 1:2 soil-vermiculite miniplot mixture 1.9 x 10^6 cfu/g soilmix.

In 1997, 24 rescued clones from the seedling assay testing thaxtomin A concentration x time were field tested in miniplots. These seedlings were rescued after 14 days at the end of the seed experiment. The labeling of the clones was as follows: Self-cross (Ranger-R, Nooksack-No, Atlantic-At); toxin concentration in which the TPS was germinated (0.1, 0.5,1 µM); rescued plant number (1, 2, 3, ...etc.). Results from this experiment are shown in Table 9. This table shows the scab index of the harvested minitubers after 90 days growing at field conditions for the 2 dates of transplanting to the field. Planting date influenced tuber and scab development. Statistical differences between date 1 and date 2 were identified for scab index and weight of tubers per plant, with the July 11 planting having smaller tubers (Table 10). Significant differences were detected between the scab susceptible Ranger parent control and the scab resistant Nooksack in date 1 and the total for date 1 and 2, but not for date 2. An explanation for this is because date 2 minitubers escaped to favorable conditions for disease development. This is shown for the low scab index for the cultivar Ranger (Table 9). The coefficient of variation (CV) for date 1 was 40.0 while the CV for date 2 was 55.7. In most cases, the scab indices of the toxin-selected individuals were lower than the parent cultivar in the date 1 trial. Thus, toxin selection appears to be a promising tool for scab resistance selection, considering that the selected seedling came from selection of TPS exposed to 0.1, 0.5 and 1µM of the toxin. These progenies were tested 2 more seasons in the field.

Table 9. Scab Index on Minitubers of Clones Tolerant to Thaxtomin A In Vitro, Grown Under Field Conditions and 2 Dates of Plantlets Transplanting. 1997, Bozeman Miniplots.

Clones	Scab Index[1]		
	Date 1(July 7)	Date 2 (July 11)	Total[2]
Ranger	3.1 a*	1.4 abcdef*	2.3 a*
R0.1-1	1.3 defghi	0.4 efg	0.9 efghijk
R0.1-2	0.2 i	0.0 g	0.1 k
R0.1-3	2.7 ab	1.5 abcdef	2.1 ab
R0.1-4	2.4 abc	1.5 abcdef	1.9 abc
R0.1-5	2.0 abcd	1.7 abcd	1.9 abc
R0.1-6	0.4 ghi	0.5 efg	0.4 hijk
R0.5-1	1.2 cdefghi	1.2 abcdefg	1.2 cdefgh
R0.5-2	0.5 fghi	0.3 fg	0.4 hijk
R0.5-3	1.6 bcdefg	1.5 abcdef	1.5 abcdef
R0.5-4	1.7 bcdef	2.0 ab	1.8 abcd
Nooksack	1.5 bcdefgh	0.5 defg	1.0 defghij
No0.1-1	1.2 cdefghi	1.1 abcdefg	1.2 cdefghi
No0.1-2	0.4 ghi	0.3 efg	0.4 ijk
No0.1-3	1.8 bcde	2.0 abcdefg	1.5 abcdef
No0.5-2	1.3 cdefghi	0.4 efg	0.8 efghijk
No0.5-3	2.4 abc	1.4 abcdef	1.9 abc
No1-1	0.3 hi	0.0 g	0.1 k
Atlantic	1.8 bcde	1.8 abc	1.8 abcd
At0.5-1	0.2 i	0.3 fg	0.2 jk
At0.5-2	1.1 defghi	0.9 bcdefg	1.1 defghij
At0.5-4	0.7 efghi	0.8 bcdefg	0.8 fghijk
At0.5-5	0.5 fghi	0.8 bcdefg	0.6 ghijk
At1-1	2.2 abcd	1.1 abcdefg	1.6 abcde
At1-5	2.4 abc	2.2 a	2.3 a
At1-8	2.0 abcd	1.6 abcde	1.9 abcd
At1-9	0.6 efghi	0.3 efg	0.5 ghijk
Norkotah	2.0 abcd	0.7 cdefg	1.3 bcdefg

(*) Numbers in a column followed by the same letter are not significantly different at 5% LSD.

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) Data from combination of 5 repetitions from date 1 and 5 from date 2.

Table 10. Overall Scab Index and Weight of Tubers of Thaxtomin A Tolerant Clones at 2 Dates of Transplanting.

Transplanting Date	Scab Index (1)	Weight (g/plant)
July 7	1.4 a*	239.9 a*
July11	1.0 b	106.3 b

(*) Numbers in a column followed by the same letter are not significantly different at 5% LSD.

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6>95%.

In 1998, two miniplot experiments were done. These involved: 1) testing scab resistance of the clones selected from the CIP seed test, and 2) testing scab resistance of thaxtomin A selected and non-selected individuals.

The soil mixture was the same as used the previous year; but stored over winter in a bulk polyethylene container in the field. A soil sample was taken at planting to estimate *Streptomyces* spp. present. Streptomycetes population was 1.64 x 10^6 cfu/g soil, which was similar to the 1997 population.

The field screening of selected individuals from the CIP seedling test involved 36 survivors exposed to 1μM thaxtomin A that were multiplied and maintained in vitro. The plant coding label was as follows: Cross number (1,2,3,4,5,6, 7, 8, R or N); Concentrations (1); Repetition (1,2,3,or 4); Plant number (1 to 10); Seedling index of damage (1, 2, 3, 4 or 5). Ranger and Nooksack parents were tested as controls. The tuber scab resistance of these clones is given in Table 11, although not all the tested selections produced tubers under the conditions at our experimental site. The scab resistance was very variable with a CV of 51.3. Moreover, this experiment did not show statistical differences for Ranger and Nooksack controls. But, all tubers showed a very low scab index, which probably means that the conditions for scab development were not favorable. Nevertheless, most of the clones developed indices similar to Nooksack, and the Ranger index was higher than Nooksack on average. A Pearson correlation was estimated for seedling index of damage and tuber scab index for some of these clones (Figure 6). This correlation was R= 0.63 and p=0.0001. This indicates that generally lower seedling index scores gave lower field scab index scores.

Table 11. Scab Index on Tubers of Clones Selected with 1μM of Thaxtomin A from Crosses from CIP TPS. 1998, Bozeman Miniplots.

Selected Clones	Scab Index(1)(2)
11162	2.1 bc *
11322	1.7 bcdef
11272	1.0 bcdef
11422	1.9 bcde
21122	2.3 b
21232	1.7 bcdef
21322	1.1 bcdef
21442	2.0 bcd
31222	1.0 bcdef
31332	ND
31422	1.7 bcdef
51342	0.0 f
71432	ND
71322	ND
81112	0.6 bcdef
81232	0.9 bcdef
81432	ND
R1122	0.8 bcdef
R1272	ND
R1322	1.0 bcdef
R1432	1.6 bcdef
N1432	0.3 def
41132	0.4 cdef
41322	4.0 a
41232	1.0 bcdef
41173	0.5 cdef
414103	0.3 def
41243	ND
41424	0.9 bcdef
61412	0.3 def
61212	0.6 bcdef
61283	0.6 bcdef
61224	0.2 ef
614104	0.3 def
61474	0.3 cdef
61265	1.9 bcde
Ranger	1.9 bcde
Nooksack	0.7 bcdef

(*) Numbers in a column followed by the same letter are not significantly different at 5% LSD.

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) ND= no data

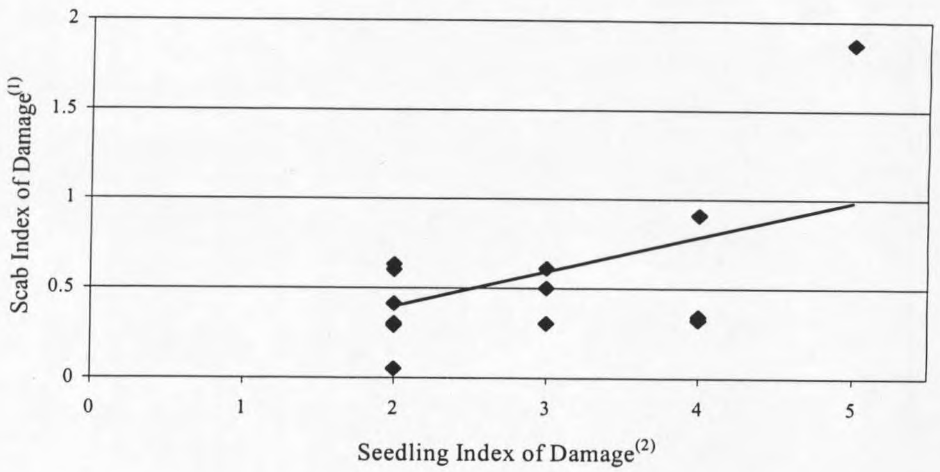

Figure 6. Pearson Correlation for Seedling Index of Damage and Tuber Scab Index of Clones from CIP True Potato Seed Selected with Thaxtomin A. R= 0.63, P=0.0001.

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5=Radical dead.

The miniplot test involving thaxtomin A selected and non-selected TPS involved 132 individuals. The plant coding label used for these selections is the following: Cross (Ranger=RR, Nooksack=NN, Nooksack x Ranger=RN), Toxin selection (C= non-toxin selected), Repetition (1,2,3,4 or 5), Plant number (1 to 50). Results of this experiment per cross are found in tables 27, 28 and 29 (Appendix B). This experiment did not show statistical differences between Ranger and Nooksack controls and the scab index was very low. Again, data suggests that conditions were not favorable for tuber scab development. However, Ranger had a higher scab index than Nooksack. Analyzing the effect of thaxtomin A using contrast analysis in the selection for scab resistance per cross (Figure 7), demonstrated that the average scab index of toxin tolerant selective from RR and RN progenies were statistically different from the control non-toxin selections with

P= 0.08 and 0.05, respectively. On the contrary, NN was not different from NNC. This can be explained considering that Nooksack a random selection of Nooksack self-progeny cross would be expected to have a higher level of scab resistance than RxR or RxN.

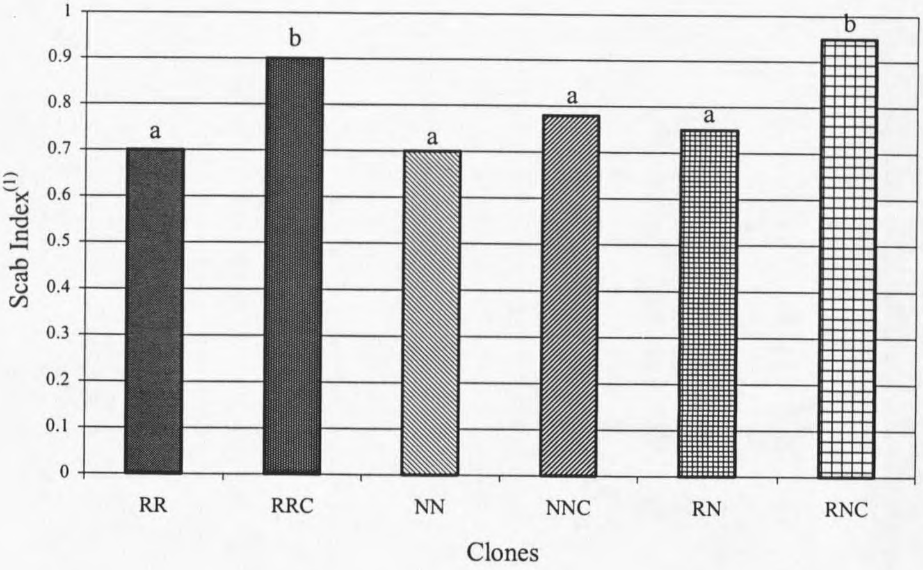

Figure 7. Average Scab Index for Clones Selected with and without (C) Thaxtomin A from Ranger Self-crosses (RR), Nooksack Selfcrosses (NN) and Nooksack x Ranger (RN). 1998, Bozeman, Miniplots.

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

Figure 8 shows the percentage of plants per cross selected with and without toxin that were statistically similar to or lower to the resistant Nooksack parent control for scab index. In this figure it can be clearly seen that clones from RR with toxin selection produced a higher percentage of plants with scab symptoms similar to the resistant parent Nooksack, than the non-toxin selection. On the other hand, no differences were detected for NN and RN, though the last had a percentage of resistant plants slightly higher with toxin selection. Even though thaxtomin A seems to be an important factor related to scab disease susceptibility/resistance, it is not the only one. Our results show that not all the individuals tolerant to the toxin were resistant to scab, but selection in the presence of toxin provides a larger percentage of resistant individuals than random selection. The 1998 data demonstrate the hazard of field selection where disease development is highly influenced by environment.

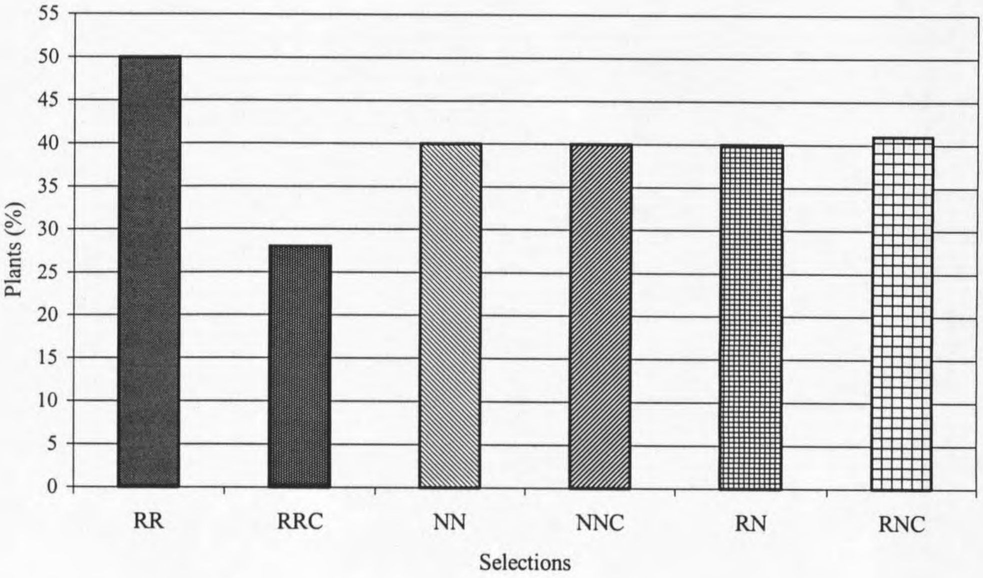

Figure 8. Percentage of Plants with Tuber Scab Scores Similar to or Lower than Nooksack Selected from Progenies of Ranger Self-crosses (RR), Nooksack Self-crosses (NN) and Nooksack x Ranger Cross, either with or without (C) Thaxtomin A. 1998, Bozeman, Miniplots.

Pearson Correlation for seedling index of damage and tuber scab index (Figure 9) for some of the progenies selected with thaxtomin A in the 3 crosses revealed a positive correlation with R=0.49 and P=0.001. This result suggests that thaxtomin A selection at seedling stage is a useful tool for developing tuber scab resistance.

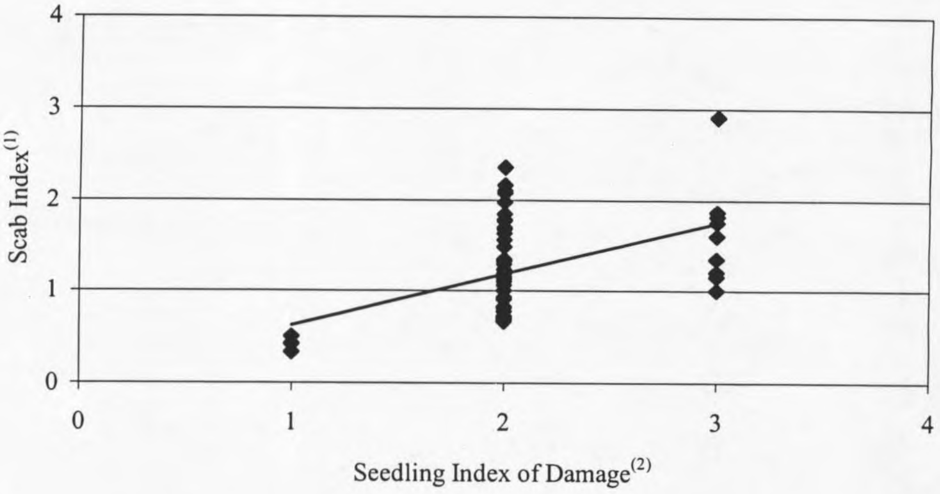

Figure 9. Pearson Correlation for Seedling Index of Damage and Tuber Scab Index for Clones Selected with Thaxtomin A from Ranger and Nooksack Self-crosses and Nooksack x Ranger Cross. R=0.49 and P= 0.0001

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) 1= Radical healthy, 2=Radical with necrotic flecks, 3=Radical with large necrotic areas, 4= Radical with large necrotic areas and deformed, 5=Radical dead.

Field Experiments

Field experiments were done in 1998 and 1999 at Tobol Farms Inc., Ronan, MT. Soil samples were taken at planting and tuber initiation to estimate *Streptomyces* spp. population. At planting and tuberization the populations in the root zone were estimated at 2.36 x 10^6 cfu/g soil and 3.23 x 10^6 cfu/g soil, respectively.

In 1998, the 24 individuals selected from thaxtomin A, previously tested at Bozeman in miniplots, were evaluated under field conditions at Ronan for scab resistance as a second year trial. Results of this test are presented in Figure 10. A wide range of scab indices was found (0.5 - 6.0). In addition, statistical differences were detected between Ranger and Nooksack parents, although not all of the toxin-selected clones were resistant to scab. On the whole, most of the toxin selected clones developed a lower scab index than the parental controls. The best 8 clones were tested again, as third year trial, in 1999 in the same field.

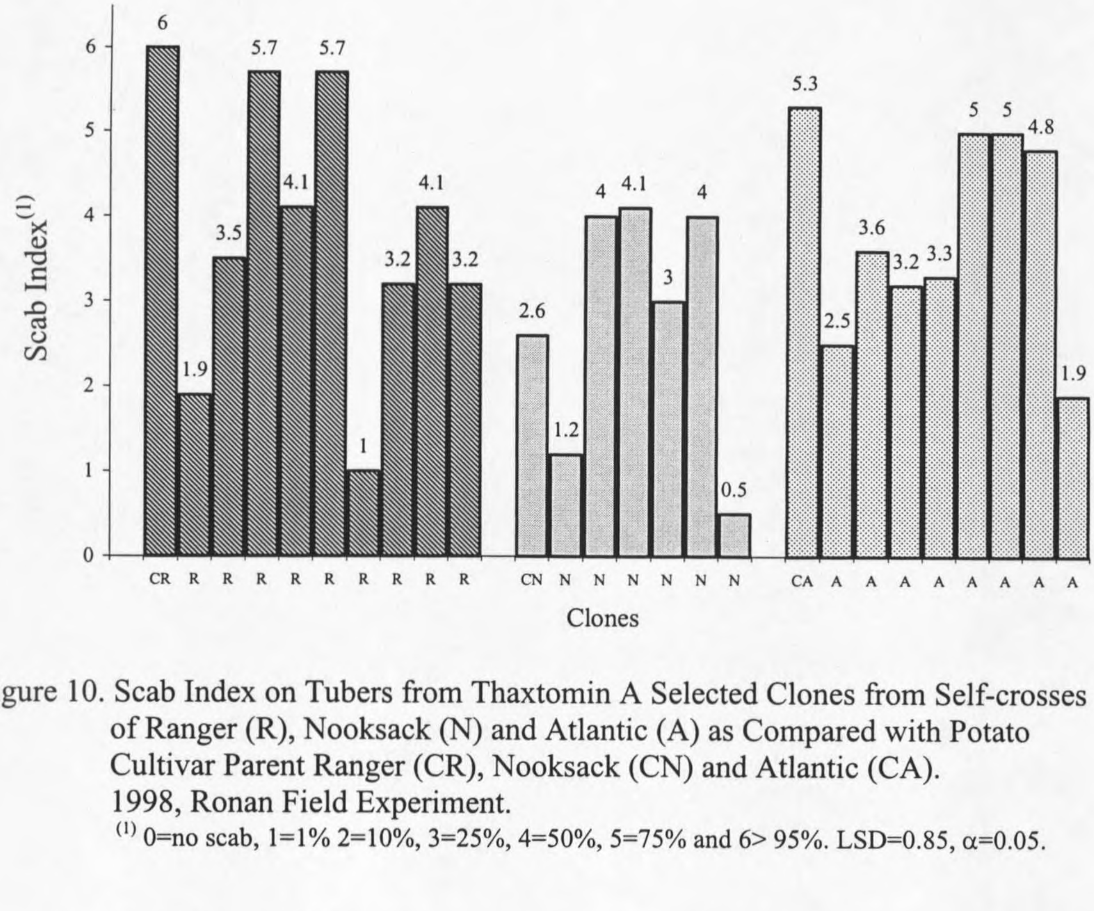

Figure 10. Scab Index on Tubers from Thaxtomin A Selected Clones from Self-crosses of Ranger (R), Nooksack (N) and Atlantic (A) as Compared with Potato Cultivar Parent Ranger (CR), Nooksack (CN) and Atlantic (CA). 1998, Ronan Field Experiment.

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%. LSD=0.85, α=0.05.

In 1999, three field experiments were set up at Ronan. The first and second experiments involved the best toxin and non-toxin selected clones (scab index score similar or lower than Nooksack) in the 1998. The third experiment tested the most resistant individuals from 1998 Ronan field plots.

The results of the first and second experiments are shown in Table 30 and 31 (Appendix B). The scab indices in these experiments were lower than expected ranging from 0.65 to 4.47. However, statistical differences were detected between Nooksack and Ranger parent cultivars at 10% LSD. In addition, a contrast analysis for the first and second experiments showed no statistical differences between progenies selected with thaxtomin A and non-selected, for each of the crosses (Table 12). This result was expected due to a previous selection in 1998 where only the clones with low index of damage in miniplots were selected for 1999 field tests. All the clones developed an average scab index lower than the Ranger parent control. Although, clones selected the first year for resistance showed higher indices this second year (Table 27, 28, 29, 30, and 31; Appendix B), an estimation of the percentage of selected clones with indices similar or lower than Nooksack over the total tested under field conditions after two years of selection is shown in Figure 11. By using the toxin to select for scab resistance in the seedling stage, the number of resistant individuals in the population was improved by about 30% for Ranger and Nooksack self-crosses. Some of these individuals are shown in Figure 12 compared to parent controls.

In tuber slice tests to determine the thaxtomin A sensitivity (Table13) for some of these clones, statistical differences were detected between Ranger and Nooksack parent controls (LSD test, p=0.05) as was expected. Additionally, almost all of the clones tested in this assay had significantly less necrosis than the Ranger susceptible parent (p=0.05). In a Pearson correlation analysis between tuber scab index and index of necrosis in tuber slices (Figure 13) the correlation coefficient was R=0.64 and P=0.001.Thus, the tuber slice test to evaluate thaxtomin A sensitivity is a good test to measure scab resistance.

Table 12. Average Scab Index for Clones that were either Selected (TA) and Not-selected (non-TA) with Thaxtomin A from Ranger and Nooksack Self- Crosses and Nooksack x Ranger Crosses, and Ranger Parent Control and Nooksack Parent Control, after 2 Years of Field Selection. 1999, Ronan Field Experiment.

	Scab Index (1)	
Clones**/Cultivar	TA selected	Non-TA selected
Ranger Self-crosses	2.0 a*	1.5 a
Nooksack Self-crosses	2.0 a*	2.1 a
Nooksack x Ranger Cross	2.0 a*	2.4 a
Parent Control	Ranger	Nooksack
	4.5 a*	2.6 b

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

* Numbers in a row with the same letter are not significantly different at 10%LDS.

** Miniplot selected for low scab index in 1998

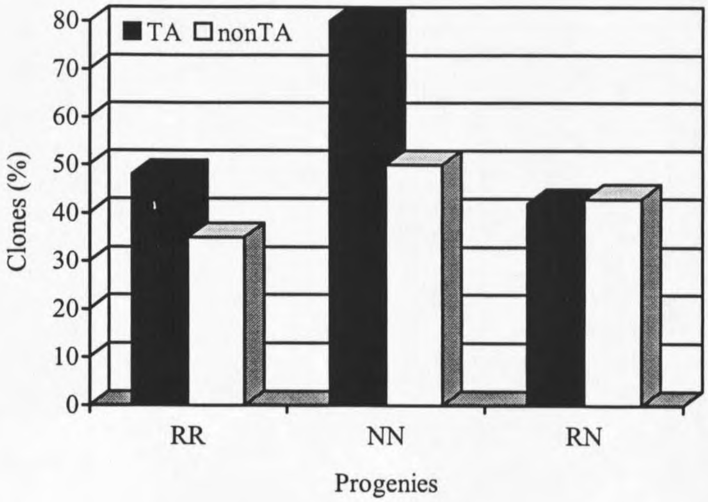

Figure 11. Percentage of Clones with Scab Index Similar or Lower than Nooksack Selected from Self-crosses of Ranger (RR) and Nooksack (NN) and Nooksack x Ranger Cross (RN), either with (TA) and without (nonTA) Thaxtomin A, after Two Years of Field Selection. 1999, Ronan Field Experiment.

Figure 12. Scab Resistant Clones Selected with Thaxtomin A at the Seedling Stage from Self-crosses of Ranger (RR119) and Nooksack (NN21). Parent varieties Ranger and Nooksack in the top row, selected clones in the bottom row.

Table 13. Index of Necrosis on Tuber Slices of Clones Selected from Ranger (RR) and Nooksack (NN) Self-crosses and Nooksack x Ranger (RN) Cross either with or without (C) Thaxtomin A, Exposed to 50μM Thaxtomin A for 7 Days.

Selected Clones	Scab Index [1]
RR17	3.0 b
RR110	1.8 ghi
RR119	1.9 fgh
RR322	1.6 hi
RR335	3.0 b
RR46	2.0 efg
RR418	1.9 fgh
RR55	2.6 cd
NN140	1.0 k
RN13	0.9 k
RN132	2.0 efg
RN144	2.7 bc
RN224	1.9 fgh
RN246	1.7 ghi
RN248	1.9 fgh
RN39	1.0 k
RRC28	1.7 ghi
RRC41	3.0 b
RRC44	1.9 fgh
RRC52	1.4 ij
NNC25	1.7 ghi
NNC36	2.1 ef
NNC37	1.6 hi
NNC39	1.0 k
NNC52	2.0 efg
RNC22	3.9 a
RNC24	1.1 jk
RNC33	2.3 de
RNC34	1.7 ghi
RNC36	2.9 bc
RNC51	3.0 b
Ranger	3.9 a
Nooksack	2.0 efg

(*) Numbers in a column followed by the same letter are not significantly different at 5% LSD

[1] 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis

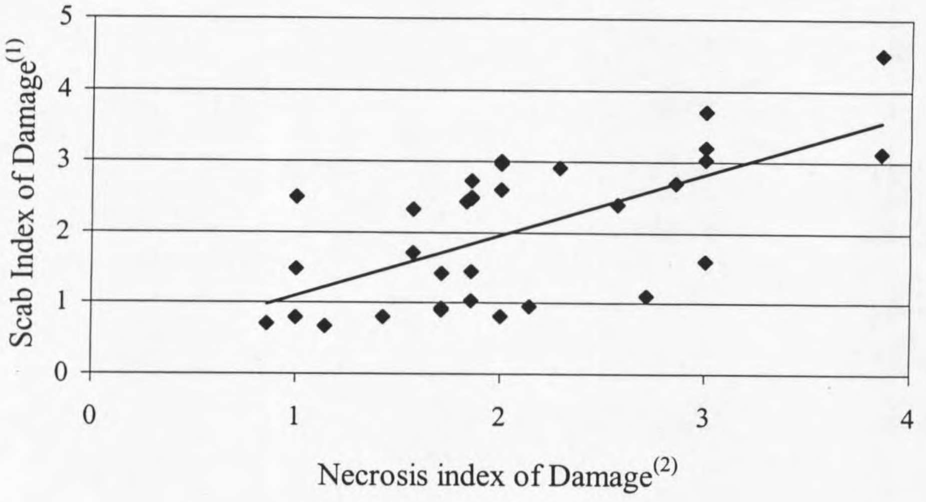

Figure 13. Pearson Correlation between Tuber Scab Index and Index of Necrosis in Tubers Slices Tested for Thaxtomin A Sensitivity. R=0.64, P=0.0001

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis

Results from the third field experiment are shown in Table 14, including the three years of field evaluation for these clones. During these 3 trial years, consistent differences were identified between Ranger and Nooksack varieties for scab severity. In addition, all the selected clones during the 3 years of evaluation, had scores lower than Ranger, especially the 8 selected clones tested 3 times. These last clones are highly resistant to scab. Some of these highly scab resistant selections from RxR cross are shown in Figure 14, as compared to the Ranger parent control.

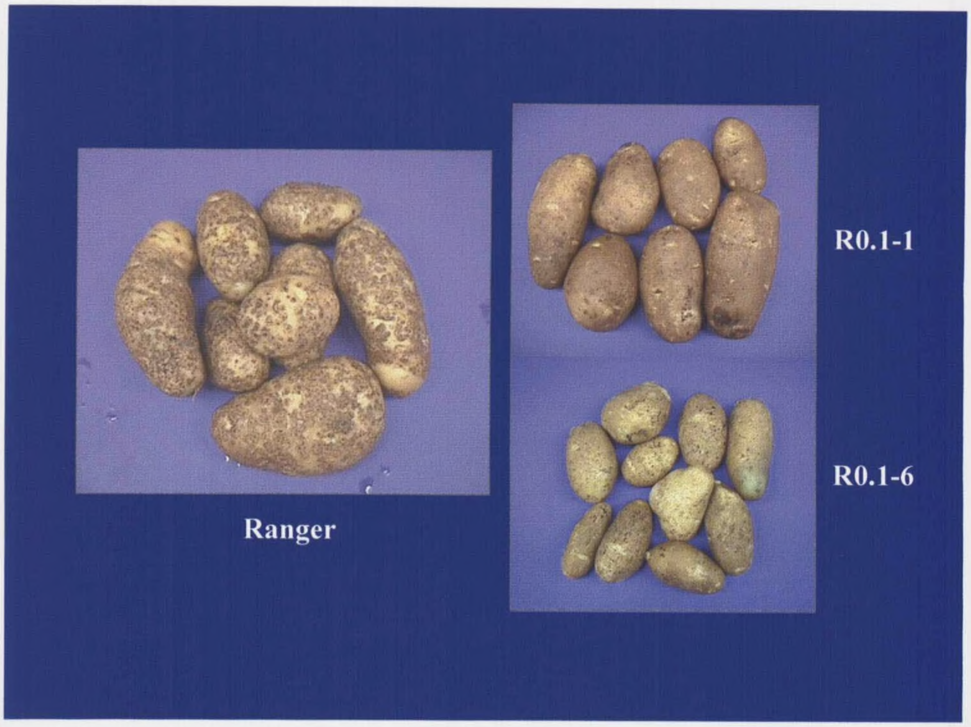

Figure 14. Tubers from Scab Resistant Progenies from Self-crosses of Ranger, Selected with Thaxtomin A During Germination of True Potato Seed and Produced in the Field Naturally Infested with *Streptomyces scabies*.

Table 14. Scab Index of Clones Selected with Thaxtomin A from Self-crosses of Ranger (R), Nooksack (N) and Atlantic (At). Three Years of Field Trials.

Clone/Cultivar	Scab Index (1)		
	1997 Bozeman Miniplot	1998 Ronan Field	1999 Ronan Field
Ranger	3.1 a*	6.0 a*	4.5**
R0.1-1	1.3 defghi	1.9 jk	1.4
R0.1-2	0.2 i	3.5 fgh	1.6
R0.1-3	2.7 ab	5.8 ab	ND
R0.1-4	2.4 abc	4.1 efg	ND
R0.1-5	2.0 abcd	5.8 ab	1.6
R0.1-6	0.4 ghi	1.0 l	0.7
R0.5-1	1.2 cdefghi	3.3 hi	ND
R0.5-2	0.5 fghi	ND	ND
R0.5-3	1.6 bcdefg	4.1 efg	ND
R0.5-4	1.7 bcdef	3.3 hi	ND
Nooksack	1.5 bcdefgh	2.6 ij	2.6
No0.1-1	1.2 cdefghi	1.3 kl	1.3
No0.1-2	0.4 ghi	4.3 def	ND
No0.1-3	1.8 bcde	4.1 efg	ND
No0.5-2	1.3 cdefghi	3.0 i	ND
No0.5-3	2.4 abc	4.9 cde	ND
No1-1	0.3 hi	0.5 l	0.1
Atlantic	1.8 bcde	5.4 abc	ND
At0.5-1	0.2 i	2.5 ij	0.9
At0.5-2	1.1 defghi	3.6 fgh	ND
At0.5-4	0.7 efghi	3.3 hi	ND
At0.5-5	0.5 fghi	3.3 ghi	ND
At1-1	2.2 abcd	5.5 abc	ND
At1-5	2.4 abc	5.0 bcd	ND
At1-8	2.0 abcd	4.9 cde	ND
At1-9	0.6 efghi	1.9 jk	2.0
Norkotah	2.0 abcd	6.0 a	ND

(*) Numbers in a column followed by the same letter are not significantly different at 5% LSD

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6=100%.

(**) ND= no data

It is possible to detect the toxin tolerance of these clones using a tuber slice test (Table 15). The Pearson correlation between necrosis development in tuber slices exposed to thaxtomin A and scab symptomatology in tubers grown in natural infested soil (Figure 15) was R=0.75 (P=0.01). This result confirms the previous correlation estimated for the other experiments.

In conclusion, there is a good correlation between index of damage for seedling radicals and tuber scab index, and tuber slice index of necrosis and tuber scab index.

Table 15. Index of Necrosis on Tuber Slices of Thaxtomin A Selected Clones of Ranger (RR), Nooksack (NN) and Atlantic Self-crosses. Slices were exposed to 50 μM of Thaxtomin A for 5 and 7 Days.

Clone/Cultivar	Index of Necrosis (1)	
	Exposure Time	
	5 days	7 days
Ranger	2.8 a*	3.7 a
R0.5-1	2.2 b	2.4 bc
R0.1-1	1.9 bc	2.0 cd
R0.1-2	0.8 d	2.2 bc
R0.1-6	0.8 d	1.6 de
Nooksack	1.7 c	2.5 bc
No0.1-1	0.9 d	1.4 de
No1-1	0.5 d	1.3 de
Atlantic	2.2 b	2.9 b
At0.5-1	2.0 bc	2.3 bc
At1-9	0.6 d	1.0 e

(*) Numbers in a column followed by the same letter are not significantly different at 10% LSD

(1) 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis

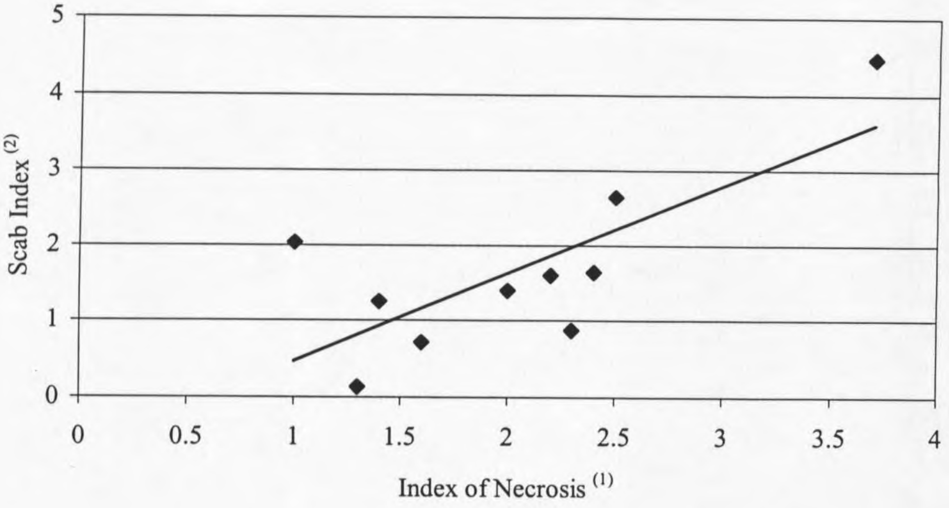

Figure 15. Pearson Correlation between Scab Index and Index of Necrosis in Tubers Tested for Thaxtomin A Sensitivity. R=0.75, P=0.01

[1] 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

[2] 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis

Discussion

Traditional potato breeding programs do not have good screening techniques that allow for early generation or in vitro selection for scab resistance. Results of this thesis research using Ranger Russet and Nooksack self crosses demonstrated the utility of thaxtomin A-seedling screening as a tool for early generation selection for scab resistance.

My research focus was to evaluate and optimize a screening assay using TPS and to determine if sensitivity to thaxtomin A correlates with tuber scab resistance. In these experiments, I determined the optimal concentration for this assay to be between 1 and 5 μM of thaxtomin A as determined by the ability to discriminate between progenies of

Ranger self-cross (susceptible x susceptible) and Nooksack self-cross (resistant x resistant). Data suggests that at 7 days of exposure to the toxin at the 1μM concentration allowed to distinguish between progenies of these two cultivars. These results were confirmed in multiple seedling tests at 7 days of exposure using 1 μM of thaxtomin A. Higher or lower concentrations or longer exposure time does not allow for efficient discrimination. It would be helpful to evaluate crosses from a wider range of cultivars varying in resistance before using this selection tool.

It is important to notice that Nooksak, although generally considerer very resistant to scab, developed scab symptoms under Ronan soil conditions, where individual Nooksack tubers had scores as high as 5. This confirmed the variability of field-testing for evaluation for scab resistance, since cultivars apparently resistant to scab under some conditions are susceptible under others. Work by Haynes et al., (1997) describes significant genotype x environment interactions for both the surface area covered with scab and the type of lesion on potato tubers. The results of the miniplot test also demonstrated the variability of field screening.

Scab reaction was highly variable among the progeny from the same cross. Each seed from TPS families is genetically unique (Caligari, 1992; Golmirzaie et al., 1994). Thus, seedling screening allowed for individual selection and not for population selection. In these tests I determined the index of damage for selected individuals, and used the advantage of potato clonal reproduction that makes easier multiplication of individuals identified as resistant.

Although the miniplot tests did not have optimal conditions for scab symptom development, they allowed for a first clonal generation screening and selection. Transplants in miniplot as well as field experiments with seed tubers allowed to test the scab susceptibility of the parent cultivars, which were always used as controls. While, all the selected clones that were tolerant to thaxtomin A at the seedling stage (Index 0,1,2) were not resistant to scab in the field, most of the thaxtomin selected individuals developed less severe disease than the parents. It seems that thaxtomin A is an important factor in scab development but not the only one. In the literature other factors are described such *S. scabies* esterase activity (Babcock et al., 1992) and tuber sugar content that affect scab development (Goto, 1981). Future research might focus on combining some of these factors with thaxtomin A as a tool for screening.

After 3 years of field trial selection I identified individual clones highly resistant to scab under very conducive conditions for disease development. Examples are self-crosses selections R0.1-1, R0.1-2, R0.1-5, R0.1-6, No0.1-1, No1-1, At0.5-1 and At1-9. While parents such as Ranger are susceptible to scab, selfing of this cultivar and screening TPS progenies using thaxtomin A, allowed for identification of germplasm with resistance to scab in the field.

While thaxtomin A does not strictly select 100% resistant individuals, it does allow a decrease in the range of population to be tested under field conditions. The data demonstrated that it is possible to obtain 30% more of resistant individuals using toxin selection compared to selection without toxin. This is a great advantage for breeders since traditional breeding for scab resistance is performed at clonal generation under field conditions, with the consequences of testing large populations under the variability of field conditions. Moreover, TPS level selection can be done under highly reproducible conditions in a timely manner, where as the level of *S. scabies* inoculum and virulence are very difficult to standardize (Plaisted et al., 1984).

In addition, I have demonstrated a positive correlation between radical seedling sensitivity to thaxtomin A and tuber scab resistance. The correlation coefficients were R=0.63, P=0.0001; R=0.49, P=0.0001) when I compared the index of damage in seed germinated on agar with the toxin and the scab index in tubers grown under field conditions. Furthermore, there was a positive correlation between tuber scab resistance and tuber thaxtomin A sensitivity (R=0.64, P=0.0001; R=0.75, P0.01) evaluated as index of necrosis in the potato slice test for our selected clones.

One of the problems using the toxin for screening is the lack of availability of this compound. I produced my own toxin for these experiments, since thaxtomin A is not commercially sold; this may be a problem for breeders who desire to use this technique. However, only very small amounts are required to set up the test. Moreover, breeding programs can use thaxtomin A to identify highly resistant clones to be used as parents to improve scab resistance in the breeding population.

The selected resistant clones identified in our experiments have not yet been tested for agronomic or processing quality, but if these characteristics are not competitive with commercial potato cultivars, they can be used as parents in a breeding program. Moreover, with molecular techniques, the genes for resistance can be identified in these lines and transferred rapidly to commercial varieties (Belknap, 1994). Thaxtomin A can be used to identify genes for scab resistance in wild Solanum populations that are normally very difficult to evaluate under field conditions.

In conclusion, thaxtomin A seedling screening assay is a useful tool to identify scab resistant germplasm under homogeneous standard conditions, allows at least a 1/3 reduction the population to be tested under field conditions and it can be used to identify highly scab resistant clones to be tested for commercial purposes, or serve as sources of resistance genes in breeding programs.

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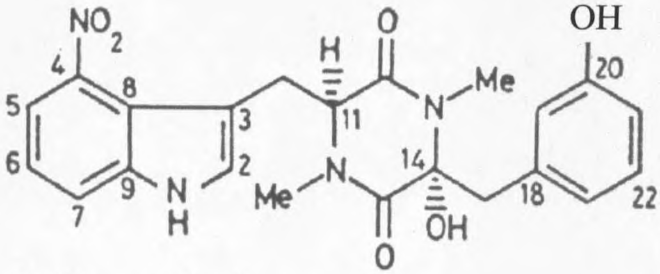
CHAPTER 3

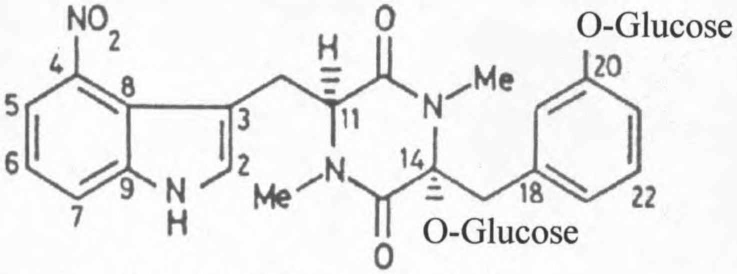
GLUCOSE CONJUGATION AS A MECHANISM OF RESISTANCE TO THAXTOMIN A IN POTATOES

Introduction

Thaxtomin A is a phytotoxin produced by *Streptomyces scabies*, causal agent of common scab disease in potato, and has an important role in the disease development (Lawrence et al., 1990; King et al., 1989; King et al., 1991;Loria et al., 1995). Thaxtomin A reproduces typical symptoms of the common scab disease (King et al., 1992). In addition, a positive correlation has been demonstrated between the pathogenicity of various *S. scabies* isolates and their ability to produce the phytotoxin (King et al., 1991; Kinkel et al., 1998; Loria et al., 1995). Non-pathogenic strains do not produce thaxtomins (Goyer et al., 1998; King et al., 1991). Thaxtomins commonly occur as a family of phytotoxins and include thaxtomin A and thaxtomin B. These toxins have been characterized as unique 4-nitroindol-3-yl containing 2,5-dioxopiperazine (King, et al., 1989; King and Lawrence, 1995; Lawrence, 1990). Thaxtomin A has been described as the most abundant of these toxins with a molecular formula of $C_{22}H_{22}N_4O_6$ with a M.W. of 438.1708 (King et al., 1992). King et al. (1992) determined that the 4-nitrotryptophan and phenylalanine groups are required for phytotoxicity. Specifically, the phenyl portion of the phenylalanine and the nitro group in the indol ring of tryptophan are necessary structural requirements for phytotoxicity (Figure 16A).



A. Thaxtomin A



B. Thaxtomin A-β-di-O-glucoside

(Adapted from King et al., 1992)

Figure 16. Structure of Thaxtomin A and Thaxtomin A-β-di-O-glucoside

Potato cultivars vary in their resistance to scab (PAA, 1998), however only a very few cultivars are highly resistant to this disease. Delserone et al. (1991) showed positive correlation between susceptibility of potato cultivars to *S. scabies* and sensititivity to thaxtomin A. In our laboratory, we have tested the use of thaxtomin A for screening true potato seed for scab resistance and have demonstrated a variable reaction to toxin associated with scab resistance (Acuña, 1999). Nevertheless, nothing is known about the biochemistry of scab resistance (Loria et al, 1997).

Engelhardt et al. (1999) described metabolism of fungal products in plants exemplified by oxidation, reduction, hydrolysis, methylation, glucosylation and the formation of cell wall bound residues for compounds such as zearalenone, deoxynivalenol and ochratoxin A. Bacterial toxins like tabtoxin, produced by *Pseudomonas syringae* pv *tabaci*, have been shown to be hydrolyzed in plants to a more active compound, tabtoxinine-β–lactam. (Anzai et al., 1989; Thomas and Durbin, 1985). Imazethapyr, an herbicide, is metabolized in tolerant corn through oxidative hydroxylation and further glucosylation in some cases (Mallipudi et al., 1994). *Cunninghamella elegans* is able to glucose-conjugate the flavones of *Psiadia arabica,* producing two glucose conjugates with glucosylation of phenolic hydroxyl groups as a detoxification mechanism (Ibrahim et al, 1997; Milanova et al, 1995). Park et al. (1994) showed that maculosin, a phytotoxin produced by *Alternaria alternata*, on spotted knapweed is metabolized to other products. The reaction to maculosin is very variable in different cultivars of the plant. Detached leaves of the plant were punctured and labeled toxin, applied. 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 in 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. These authors commented than this toxin produces necrotic symptoms in many non-host plants and is toxic to many fungi. They suggest that leucinostatin A contributes to the defense of the host when attacked by other organisms. 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.

Thaxtomin A plays an important role in bacterial pathogenicity and scab disease development in potato, however little is known about the mechanism of scab resistance. Moreover, there seems to be no explanation for the relative selectivity expressed by thaxtomin A in potato. Therefore, my research focused on the interaction of *S. scabies* and thaxtomin A with scab resistant and susceptible potatoes. I tested the hypothesis that glucosylation of thaxtomin A is related to the resistance of potato to the pathogen *S. scabies.*

Materials and Methods

Bacterial Culture and Fermentation

S. scabies strain 87/22, provided by Dr. Rosemary Loria, Cornell University, was used for thaxtomin A and Thaxtomin A-glucoside 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). Cultures were incubated at 28°C on a rotatory shaker at 150-180 rpm.

Chromatography

Thin layer chromatography (TLC) were done on EM Science silica gel 60 F254 plates using the following solvent systems: A) chloroform:methanol (9:1 v/v), B) chloroform:acetonitrile (9:1 v/v), C) ethyl acetate:isopropanol (9:5 v/v), D) chloroform: methanol:ammonium hydroxide (6:2.5:0.1 v/v), E) chloroform:methanol:acetic acid (14:6:1v/v), F) n-butanol:acetic acid:water (4:1:2 v/v), G) n-butanol:methanol:acetic acid (4:1:4 v/v) and on Whatman KC18F reversed phase silica gel 60 A plates using H) acetone:water (3:4 v/v) and I) isocratic methanol solvent systems.

Paper chromatography was done using n-butanol:acetic acetic acid:water (4:1:5 v/v); and ethyl acetate:pyridine:water (8:2:1v/v) solvent systems. The method described by Trevelyan et al. (1950) using $AgNO_3$ was used to detect sugars on the chromatograms. Mannose, galactose and glucose were used as reference compounds.

Spectroscopy and Chemical Analysis

Electrospray ionization mass spectroscopy and Nuclear Magnetic Resonance (H-NMR) were performed by Department of Chemistry and Biochemistry at Montana State University. Mass spectroscopy was done on methanol and water and acetic acid (50:50:1) solvents. Samples were injected with a spray flow of 2µl/min, a spray voltage of 2.2 KV via the loop injection method. NMR spectra were done in a Bruker DRX-500 instrument with 64 scans, samples were dissolved in deuterated methanol. High Pressure Liquid Chromatography (HPLC) was done using a Waters 600E System controller and Waters 441 absorbance detector spectrophotometer (Millipore Waters, Milford, MA) with a Microsorb-MV 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_{245}. A standard curve was made with serial dilutions for the standard thaxtomin A. Ultraviolet and visible spectroscopy analysis were done in a Beckman UV/vis DU-50 spectrophotometer using the extinction coefficients determined by King et al. (1992). A standard curve was performed for thaxtomin A with serial dilutions and read at $A_{398.}$ Carbohydrate analysis was performed by Complex Carbohydrate Research Center, The University of Georgia, Athens, Georgia. Samples were hydrolyzed with 1M methanolic-HCl for 16 hrs. at 80°C . The released sugars were derivatized with Tri-Sil and samples were run on GC using a Supelco column. Myo-inositol was added (20 µg) as an internal standard.

Production, Purification and Characterization of Thaxtomin A and Thaxtomin A-β-di-O-glucoside

Thaxtomin A production, extraction, purification and identification were done according to the methodology described by King et al, (1992) and Loria et al. (1995), with some modifications. Erlenmeyer flasks of 500ml with 200ml of OMB were inoculated with a spore suspension (about 10^8 spore per flask) and incubated for 10 days. After, the fermentation, the liquid culture was filtered through cheesecloth, extraction was done twice with ½ volume of n-butanol, instead of chloroform. 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 subjected to TLC in solvent system A. Pure thaxtomin A (King et al., 1992) was provided by Dr. R.R. King, New Brunswick, Canada. Yellow bands co-migrating with standard thaxtomin A and thaxtomin A-β-di-O-glucoside R_fs were eluted, flash dried and rechromatographed in solvent system H. An alternative method for purification was to pass the crude extract first through a reversed phase C18 vertical column chromatography (octodecyl-funcionalized silica gel, Aldrich Chemical Company, Inc., Milwaukee, WI.) using methanol as a solvent, collecting the yellow liquids, followed by TLC in solvent system A. Two bands co-migrating close to thaxtomin A-glucoside were detected and designated TAG-T1 and TAG-T2.

Final thaxtomin A purification was carried out by successive passage in solvent systems A, B, C, H and I. Identification of thaxtomin A and TAG-T1 was done by electrospray ionization mass spectroscopy, H-NMR spectra, HPLC and spectroscopy relative to the pure standard thaxtomin A. Thaxtomin A-glucosides were tested for purity by TLC in A, D, E, F, and G solvent systems. Carbohydrate analysis, acid hydrolysis and bioactivity assay were performed to characterize thaxtomin A glucosides (TAG-T1 and TAG-T2).

Acid hydrolysis was performed in trifluroacetic acid (TFA) and water (1:3). The glucosides TAG-T1 (0.11mg) and TAG-T2 (0.135mg) were mixed independently with 100μl of TFA and 300μl of water, incubated overnight at 110°C in a sealed vial. The reaction was stopped after 15 hrs and the residue air-dried.

Bioactivity assay was determined using the tuber slice test (Loria et al., 1995). Tubers of Ranger potato cultivar were used for these assays and filter paper disks of 1 cm of diameter were saturated with the compound to be tested. The treatments were thaxtomin A, TAG-T1 and TAG-T2, in concentrations of 0, 0.1, 1, 10, 50,100, 200 and 300 μM with 4 repetitions. Necrosis was measure after 3 and 10 days. Experiments were in a factorial block design of concentrations X compounds. Statistical analysis was performed using Statistical Analysis System (SAS) (SAS/STAT, 1998) software.

Determination of Thaxtomin A Production and *S. scabies* Growing Curves

Erlenmeyer flasks of 250 ml with 100 ml oatmeal broth (OMB) each were inoculated with 100μl of bacterial spore suspension (1.5 x 10^6 cfu/ml) and incubated for 11 days. Extraction of cells and thaxtomin A was done daily on days 2 through 11. Samples were centrifuged at 10,000 x g for 10 min in a Sorvall RC2-B (Ivan Sorvall, Inc.) to separate cells from broth. Cells were rinsed with methanol and centrifuged again, followed by drying at 60°C for 48 hrs. Then the dry weight was determined and growth a curve estimated. The thaxtomin A production curve was developed by extracting the broth twice with chloroform in ½ volume relation. Samples were flash evaporated and quantified for thaxtomin A by HPLC.

Effects of Amino Acids in Thaxtomin A Production

This experiment was done to evaluate the effects of phenylalanine, tryptophan and tyrosine as amendments to OMB on thaxtomin A production. Erlenmeyer flasks of 250 ml with 100 ml OMB were amended with the amino acids at 2.5, 1, 0.5 and 0 mM. before autoclaving. Each flask was inoculated with a 100µl of bacterial spore suspension with 6.8 x 10^8 cfu/ml and incubated 10 days. Broth was filtered through cheesecloth and extracted twice with chloroform, flash evaporated and resuspended in 1ml of methanol. Thaxtomin A was quantified using HPLC.

Compound Radioactivity Determination

Radioactivity of respective compounds was determined in a Packard Tri-carb Liquid Scintillation Spectrometer model 3320 (Packard Instrument Co., Spokane, WA.). Samples were dissolved in 1 ml methanol and 10 ml of Aquasol (DuPont, Universal LSD Cocktail, Cat No.NEF-934) was added prior to counting. Readings were corrected to dpm by quench correction methods. Autoradiography was performed using film 20.3 x 25.4 (Kodak X-OMAT, XAR-2, F-5763, Sigma Chemical Co. St Louis, MO.); plates were exposed for several weeks, according to each case.

Radiolabeling Thaxtomin A

Radiolabeled Thaxtomin A was produced for use in metabolism experiments in scab resistant and susceptible potatoes. ^{14}C-thaxtomin A was produced as follows: Four 500 ml flasks with 250ml of OMB were inoculated with 200µl (4.2 x 10^8 cfu/ml) of bacterial spore suspension and incubated for 6 days. Then, the culture was amended with 50µCi of L-Phenylalanine-UL-^{14}C, incubated until day 10 and extracted as previously described. The sample was flash evaporated and resuspended in methanol. Purification was carried out in a C18 reverse phase column in methanol solvent followed by TLC in the A solvent system. Radiochemical purity was tested in a two-dimensional TLC in A and C solvent systems followed by autoradiography. Bioactivity was tested using the potato slice test. ^{14}C-thaxtomin A was quantified by spectroscopy and specific activity determined by liquid scintillation counting methods.

^{14}C-thaxtomin A and ^{14}C-thaxtomin A glucoside production curves were estimated as follows: 2L flask with 1L OMB was inoculated with 200 μl of bacterial spore suspension (4.2 x 10^8 cfu/ml) and incubated for 20 days. At day 6, 50 μCi of L-Phenylalanine-UL-^{14}C was added to the medium. Samples (100ml) of cultures were taken at days 8, 9, 11, 13, 15 and 20. These samples were extracted twice with ½ volume of n-butanol, flash evaporated and resuspended in methanol. TLC was performed in solvent system A. Yellow bands co-migrating with standard thaxtomin A and thaxtomin A-glucoside R_f were eluted from the silica. Further purifications were performed in solvent system H. Specific activity was determined by liquid scintillation counting and autoradiography used to determine radiochemical purity.

Plant Material

Potato cultivars and clones used in this research were Ranger Russet (Pavek et al., 1992) a scab susceptible cultivar; Nooksack (Hoyman and Holland, 1974) a scab resistant cultivar, Atlantic (PAA, 1998), moderately susceptible to scab; and thaxtomin A seedling selected individual clones R0.1-1, R0.1-2, R0.1-6 and At1-9 previously characterized as resistant in potato slice and field tests.

Plant materials were maintained and multiplied in vitro in a Murashige and Skoog (1962) basal medium with Gamborg's vitamins (Sigma, M0404). This in vitro medium was amended with sucrose (20g/L, Sigma, S9378), Kinetin (0.04%, Sigma K3378), and

Phytogel (2g/L, Sigma P-8169); the pH was adjusted to 5.7. Plantlets were grown in a growth chamber at 20°C, 200 µmol/m^2/sec light intensity and 14 hrs light. Plantlets about 1 month old from in vitro multiplication were transplanted to 6 cm diameter containers (Deep 606 Inserts, T.O. Plastics, Inc., Clearwater, MN.) with Sunshine Mix #1 (Sun Gro Horticulture Inc.) at the Plant Growth Center-Montana State University. The seedlings were maintained in a growth room at 18°C, 450 µmol/m^2/sec light intensity and 12 hrs light. After 2-3 weeks the plants were transplanted to a 20 cm diameter container (Belden Plastics, St. Paul, MN.) in the same soil mix, and kept in the growth chamber for minituber production (about 4-5 weeks). Microtubers were produced in vitro in a medium provided by the Seed Potato Certification Laboratory, Montana State University, and maintained in a growth chamber at 20°C in the dark for more than 4 weeks.

Tuber Slice Test

This assay was used to confirm phytotoxicity of purified thaxtomin A, ^{14}C-thaxtomin A and thaxtomin A-glucosides and to test the toxin sensitivity of resistant and susceptible plant material. Minitubers produced under growth room conditions from in vitro multiplication were harvested immature and stored at 7°C for at least 8 weeks, to reduce nonspecific browning (Loria et al., 1995). Tubers were surface sterilized with 0.5% NaOCl for 10 min, air-dried in sterile conditions, dipped in ethanol and flamed. A core of pith tissue was removed aseptically with a cork bore of 2 cm diameter, and cut in slices of 0.5 cm thick. The slices were put in 9 cm petri dishes with wet filter paper Whatman #1 (Whatman Int., Maidstone, England). Thaxtomin A, ^{14}C-thaxtomin A or thaxtomin A-glucosides were dissolved in ethanol according to the concentration to be tested. Filter paper disks of 6mm diameter (Whatman #1) were immersed in this solution for 1 hr, air-dried and placed on the potato slice. Controls were immersed in an ethanol solution. Ten μl of sterile distilled water were applied to each disk. Plates were incubated at 24°C in the dark and evaluated after 3, 5 and 7 days. Evaluation was in the defined area under the filter paper disk using the following index of necrosis: 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis.

Determination of Penetration of Thaxtomin A in Microtubers

This test was done using microtubers of Atlantic about 4 weeks old. Ten microtubers of the same size were selected from a single container. The microtubers were left attached to the plantlet. Then, 27 μl of ^{14}C-Thaxtomin A (10mM) in 50% ethanol (1:1 v/v) with a specific activity of 3190 dpm/μmol was applied over the microtuber. Controls were 11.5 μl of 2mM solution of thaxtomin A in 50% ethanol and 27 μl of 50% ethanol. Tubers were kept in the dark at 20°C for 7 days. The microtubers were harvested, cut in half and frozen. One half was autoradiographed at –20°C for 3 weeks. The other half was peeled in 4 layers (peel, cork, xylem and medulla) and the radioactivity of each layer was determined in a liquid scintillation spectrometer after being macerated with 1ml of tissue solubilizer (Beckman, No 580691, Fullerton, CA).

Thaxtomin A–β-di-O-glucoside Production in Scab Resistant and Susceptible Potatoes

This experiment was performed to determine if thaxtomin A is converted to its glucoside in potato tubers and to compare this conversion in scab resistant and susceptible potatoes. Microtubers of Atlantic, At1-9, Ranger, R0.1-1, R0.1-6 and Nooksack were used in this experiment. A solution of ^{14}C thaxtomin A of 100μM in 50% ethanol with specific activity of 30269 dpm/μmol was prepared. Ten microtubers of each line were dipped in this toxin solution for 5 min. Controls were dipped in 50% ethanol solution. Then, they were air dried and stored in a moist chamber in the dark at 20°C for 5 days. The microtubers were then macerated in 2ml methanol, filtered through a double layer of filter paper (Whatman #4) with vacuum, rinsed with methanol and air-dried. Samples were recovered in water and extracted twice with n-butanol ½ volume. Aqueous and organic phases were flash evaporated and chromatographed in solvent system A. Compounds co-migrating with thaxtomin A and TAG-T1 standards were taken and radioactivity determined. TLC plates from the crude extract and from aqueous and organic phases were autoradiographed for 4 month.

A second experiment was done using minitubers of Ranger, R0.1-1, R0.1-6, and R0.1-2. Five minitubers of each cultivar/clones were used and 2 of them were boiled for 5 min. to inactivate metabolic enzymes.. Minitubers were surface sterilized with 0.5% NaOCl for 10 min and air-dried. Minitubers were injected to a depth of 3mm with 100μl of 100uM solution of ^{14}C thaxtomin A with specific activity of 30269 dpm/μmol. Each minituber was injected 10 times and each time 1/10 of the total liquid was delivered. Minitubers were incubated in the dark in moist chamber at 20°C for 4 days. Extraction, separation and reading were as previously described.

Enzyme Preparation and Assay

Ranger and Nooksack minitubers were used in this experiment, about 40 g per cultivar. Minitubers were ground in ice cold 100 ml of 10mM TRIS buffer pH 7.5 containing 1 g of polyvinylpyrrolidone and 0.1 g of dithiothreitol using a Sorvall Omni-Mixer homogenizer (Ivan Sorvall, Inc.) for 1 min at top speed. Samples were centrifuged at 10,000 x g for 5 min to separate debris in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc.). The supernatant was filtered through four layers of cheesecloth. The filtrate was precipitated with cold acetone, -20°C, in equal volume, mixed slowly and allowed to stand for 10 min. Solutions were then filtered through single layer of filter paper (Whatman #1) with vacuum; the precipitate that collected on the filter paper was washed with acetone and allowed to air dry. The resultant acetone powder extract was stored at – 20°C. Total protein in samples was estimated according to the Lowry's method for protein determination (Lowry et al., 1951). Fifty mg of acetone powder was reconstituted in 10ml of 10mM TRIS buffer pH 7.4.

The standard reaction mixture for the assay contained: 0.4 ml enzyme solution (3.9 mg Ranger protein and 4.0 mg Nooksack protein), 2 μCi UDP glucose-1- ^{3}H (Sigma, U-1756) and 100 μg of thaxtomin A (dissolved in ethylene glycol monomethyl ether). Treatments were the following: 1=Ranger enzyme plus thaxtomin A plus glucose, 2=Ranger enzyme plus glucose, 3=Ranger boiled enzyme plus thaxtomin A plus glucose, 4=Ranger enzyme plus thaxtomin A, 5=Nooksack enzyme plus thaxtomin A plus glucose, 6=Nooksack enzyme plus glucose, 7=Nooksack boiled enzyme plus thaxtomin A plus glucose and 8=Nooksack enzyme plus thaxtomin A. Reaction mixtures were incubated for 6 hrs at 23°C, then the reaction was stopped by adding 0.5 ml of methanol. Samples were air dried and stored at –20°C. Samples were dissolved in 100μl of methanol and chromatographed (TLC) in solvent system A. Standard thaxtomin A was used as reference. Four bands below standard thaxtomin A R_f (0.21, 0.19, 0.13, 0.07) were scrapped out and the radioactivity of these samples was determined in liquid scintillation spectrometer.

Results

Production, Purification and Characterization of Thaxtomin A and Thaxtomin A-β-di-O-glucoside

Thaxtomin A produced and purified in our lab showed the same R_f as the standard in the solvent systems A (0.3), B (0), C (0.91), H (0.57) and I (0.85). Spectrophotometer readings at 398, 343, 279, 249 and 220 nm, gave a similar spectral curve for standard and our toxin sample. A standard curve was made with dilutions of a known quantity of the toxin using UV/vis spectrophotometer at 398 nm based on extinction coefficients determined by King et al. (1992) (Figure 28, Appendix A). In addition, standard Thaxtomin A eluted as a single peak at 20min in HPLC, as did our sample. Using dilutions of a known concentration of the toxin, a standard curve was established (Figure 29, Appendix A). These curves were used to quantify the toxin in our experiments. Thaxtomin A yield was estimated at about 4μg/ml of OMB. Thaxtomin A electrospray ionization mass spectroscopy showed an $[M+Na]^+$ at 461.4 yielding a molecular weight of 438.4 (Figure 30, Appendix A). These data agree with King et al. (1992). Further, NMR spectra (Figure 31, Appendix A) agree with the chemical structure described by Lawrence et al. (1990). Bioactivity assay of our thaxtomin A on potato slices produced necrosis at 10 μM after 7 days of incubation, similar to the standard.

S. scabies produced two glucoside compounds, TAG-T1 and TAG-T2. The R_f of TAG-T1 was 0.21 and TAG-T2 was 0.19 in solvent system A. Glucosides gave a single spot in each of the TLC solvents systems A, D, E, F and G. TAG-T1 electrospray ionization mass spectroscopy (Figure 17) reveled an $[M+K]^+$ at 804 and an $[M+Na+K]^{+2}$ at 413 yielding a molecular weight of 762. This mass is the equivalent of two anhydro hexose units linked to thaxtomin A MW=438 (Figure C, Appendix A). TAG-T1 H-NMR spectroscopy (Figure 18) showed a thaxtomin A spectrum with the addition of a chemical shift of glucosyl proton at 5.3-5.4 with a chemical coupling consistent with a β-linkage. Carbohydrate analysis detected glucose as the major carbohydrate residue at 73.3 % per mole residue for TAG-T1 and 89.6% per mole residue for TAG-T2. Furthermore, glucoside acid hydrolysis in trifluoroacetic acid (Figure 19) yielded a compound with the same chromatographic mobility as glucose with an $R_{mannose}$ of 0.91 and 0.84 in n-butanol and acetic acid and water (4:1:5) and ethyl acetate and pyridine and water (8:2:1) solvent systems, respectively.

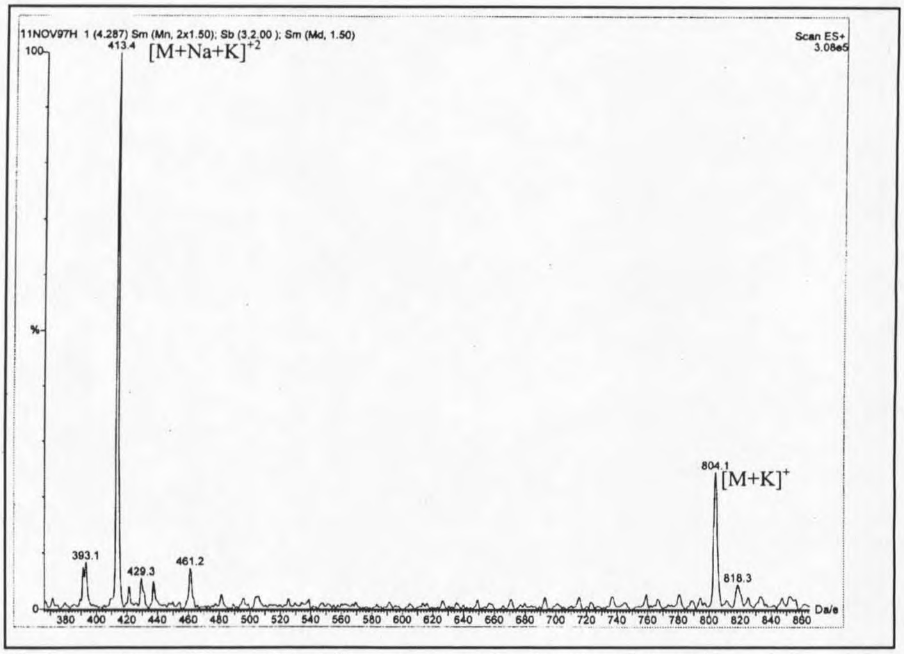
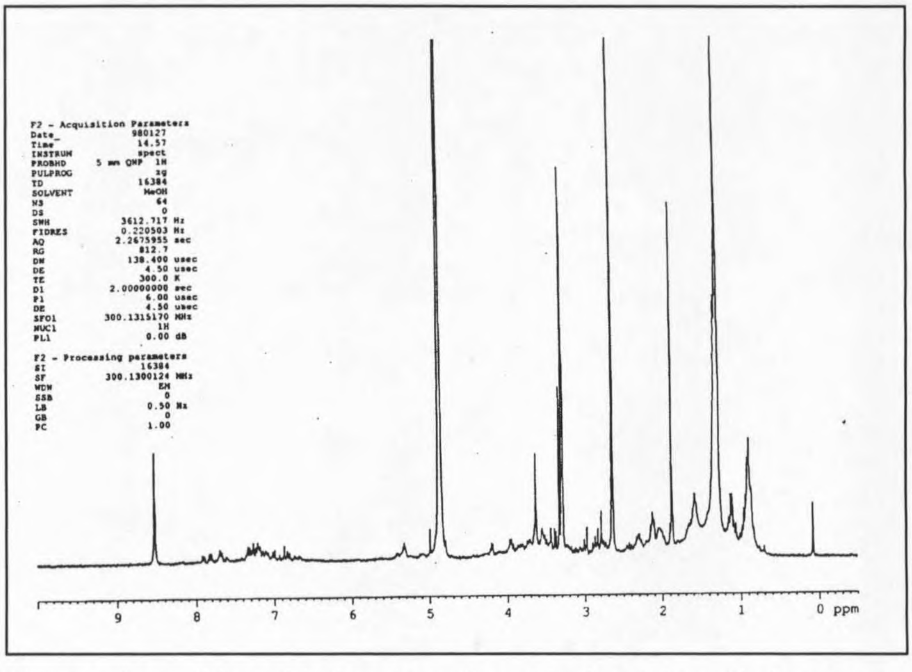


Figure 17. Thaxtomin A-Glucoside Electrospray Ionization Mass Spectroscopy. $[M+Na+K]^{+2}$ at 413.4 and $[M+K]^{+}$ at 804.1, yielding a MW of 762.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TE               300.0 K
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DE                4.50 usec
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NUC1                1H
PL1               0.00 dB

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Figure 18. Thaxtomin A- Glucoside Spectrum from Nuclear Magnetic Resonance Spectroscopy (H-NMR)

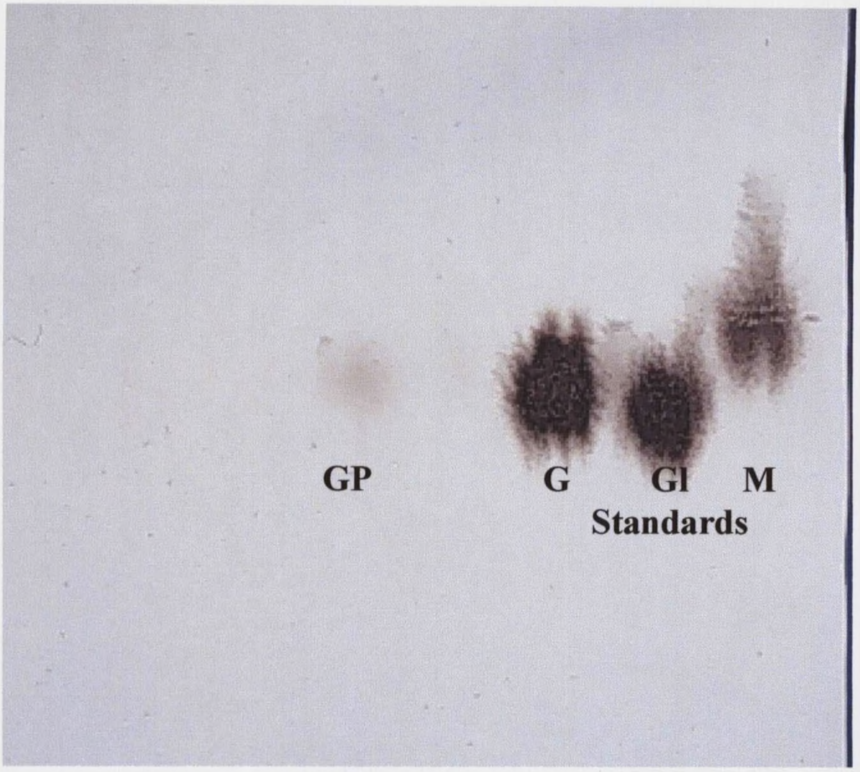


Figure 19. Paper Chromatography of the Products of Acid Hydrolysis of Thaxtomin A-β-di-O-glucoside Run in n-butanol and Acetic Acid and Water (4:1:5) GP=Glucose Product and G=Glucose, Gl=Galactose, M=Mannose Standard Controls. GP $R_{mannose}$=0.91.

Bioactivity assay in potato slices (Table 16) demonstrated that thaxtomin A caused necrosis in potato slices test at 10uM at 10 days, but not the glucosides at the same molarity. Additionally, all concentrations tested produced necrosis statistically different between thaxtomin A and the glucosides at 3 and 10 days of exposure, being comparable only at concentrations of 300μM after exposure of 10 days. Furthermore, TAG-T1 produced 6 times less necrosis than that produced by thaxtomin A at 50 μM, the standard concentration used for these tests. In addition, TAG-T2 produced less necrosis than TAG-T1 in all levels tested.

Table 16. Index of Necrosis in Potato Tuber Slices Exposed to Thaxtomin A and Thaxtomin A-glucosides (TAG-T1 and TAG-T2) in Concentrations of 0 to 300μM at 3 and 10 days.

Compound	Thaxtomin A Concentration (μM)							
	0	0.1	1	10	50	100	200	300
	Index of Necrosis (1) at 3 days							
Thaxtomin A	0 f *	0 f	0 f	0 f	3.0 b	3.7 a	4.0 a	4.0 a
TAG-T1	0 f	0 f	0 f	0 f	0.5 def	1.0 dc	2.8 b	3.0 b
TAG-T2	0 f	0 f	0 f	0 f	0 f	0.3 ef	0.8 cde	1.25 c
	Index of Necrosis (1) at 10 days							
Thaxtomin A	0 g*	0 g	0 g	0.3 fg	3.8 a	4.0 a	4.0 a	4.0 a
TAG-T1	0 g	0 g	0 g	0 g	0.5 ef	1.3 c	2.8 b	4.0 a
TAG-T2	0 g	0 g	0 g	0 g	0 g	0.3 fg	0.8 de	1.0 dc

(1) 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis

(*) Numbers in and across columns per exposure time followed by the same letters are not significantly different at 5% LSD.

Thaxtomin A Production Curve and *S. scabies* Growth

The maximum cell growth occurred between 3 and 4 days after OMB media inoculation with *S. scabies* spore suspension (Figure 20), decreasing after that constantly until day 11. However, thaxtomin A maximum production occurred after 8 days, decreasing after 10 days. This suggests that thaxtomin A is a secondary metabolite that is produced in the lag phase of bacterial growth, and it appears to be metabolized to other compounds after 10 days of culture. Therefore, for our research purposes, I decided to extract thaxtomin A at10 days after inoculation to obtain the highest yield.

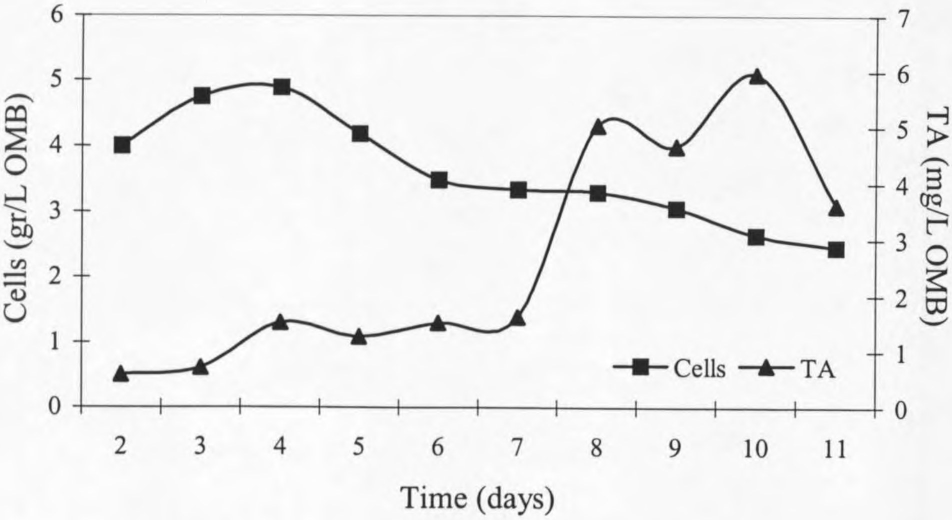


Figure 20. Growth Curve and Thaxtomin A (TA) Production by *Streptomyces scabies* Strain 87/22 Growing in Oat Meal Broth (OMB) for 11 Days at 28°C.

Effects of the Amino Acids Phenylalanine, Tryptophan and Tyrosine on Thaxtomin A Production

Table 17 shows the results of thaxtomin A production in OMB amended with these 3 amino acids after 10 days of culture. Amino acid amendments negatively affected the production of thaxtomin A, under the conditions tested. However, at the 2.5 mM concentration of phenylalanine or 0.5 mM of tryptophan, thaxtomin A yields were similar to the non-amended control.

Table 17. Thaxtomin A Production in OMB Amended with 3 Different Amino Acids after 10 Days of Culture at 28°C in Shake Culture.

Amino acids/Concentration	Thaxtomin A (mg/L OMB) (1) 2.5 mM	1.0 mM	0.5 mM	0.0 mM
Phenylalanine	3.8	1.2	1.2	
Tryptophan	2.1	2.9	4.1	
Tyrosine	2.2	1.7	1.9	
No amino acids				4.0

(1)Yield estimated based in HPLC reading standard curve per liter of Oat Meal Broth (OMB).

Radiolabeled Thaxtomin A

Thaxtomin A was produced using L-phenylalanine-UL-^{14}C as amendment at 6 days after inoculation, just prior to the exponential phase of thaxtomin A production. ^{14}C-thaxtomin A with a specific activity of 30,269 dpm/μmol was produced. ^{14}C-thaxtomin A purity was tested in a two dimensional TLC followed by autoradiography. One spot with the same location, size and shape appeared as the authentic toxin (Figure 21). Bioactivity of this labeled toxin was tested in potato slices with similar results to the standard thaxtomin A.

Crude extract samples from cultures were taken from the ^{14}C labeling experiments at days 8 to 11 and were chromatographed (TLC) and autoradiographed (Figure 22). The result shows that *S. scabies* is able to metabolize phenylalanine into thaxtomin A, thaxtomin A-glucosides and other products. In addition, thaxtomin A production increased after day 8, and still was detected at day 20. Furthermore, toxin glucosides bands were detectable at day 9. Liquid scintillation counting of these samples (Figure 23) showed a maximum toxin label and toxin-glucoside label at 9 days, with a very similar pattern for the two compounds.

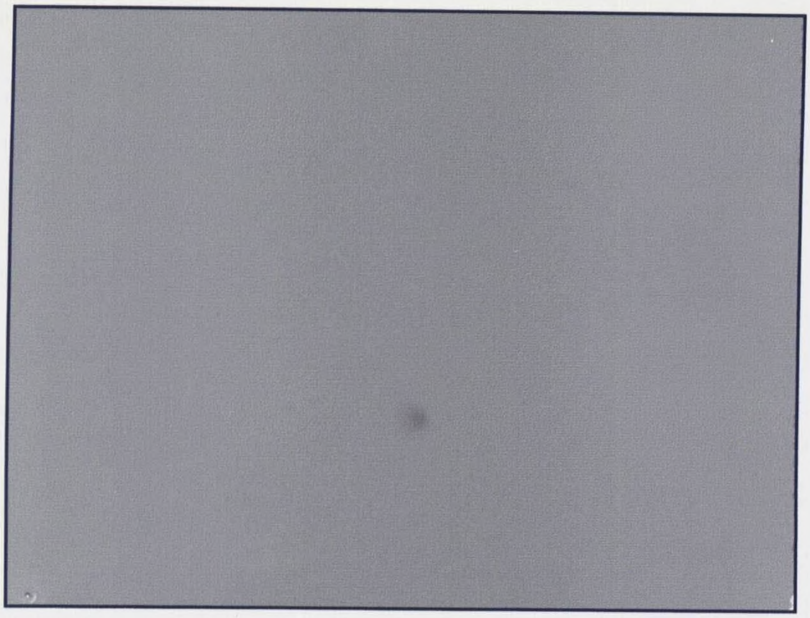


Figure 21. Autoradiograph of a Two Dimensional Thin Layer Chromatography of ^{14}C-Thaxtomin A run in Chloroform and Methanol (9:1) and Ethyl Acetate and Isopropanol (9:5) Solvent Systems.

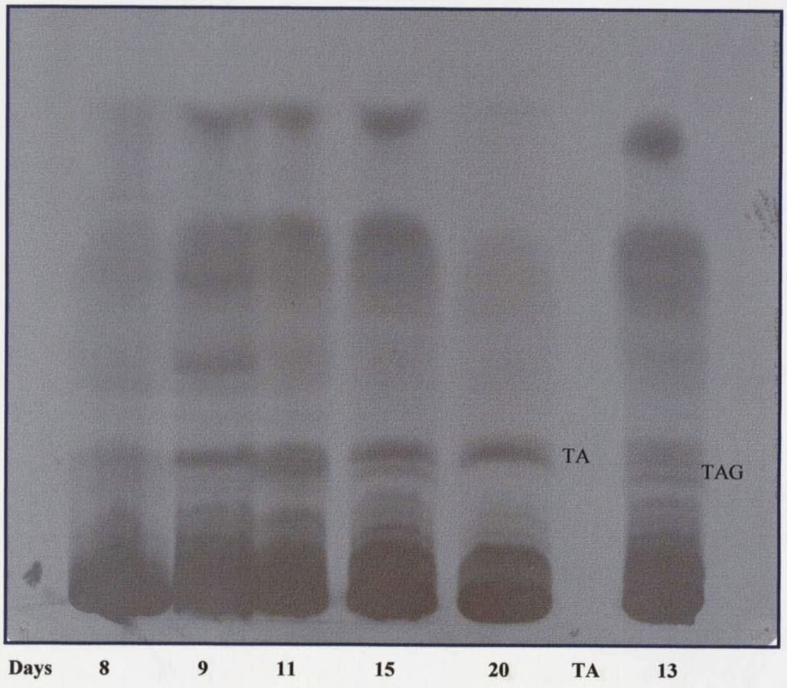


Figure 22. Autoradiograph of Thin Layer Chromatography of Crude Extracts of Culture of *S. scabies* Amended with L-Phenylalanine-UL-^{14}C at 8 to 20 Days. TA=thaxtomin A, TAG= Thaxtomin A-Glucoside.

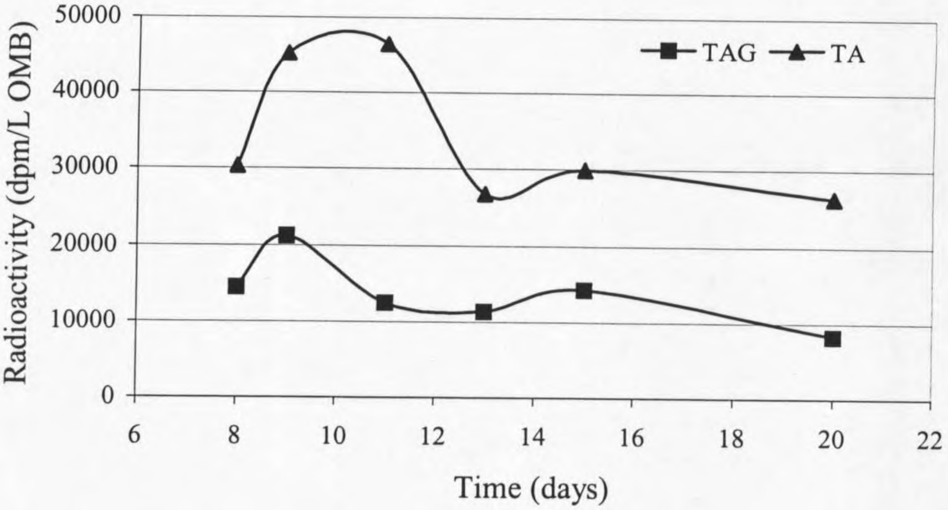


Figure 23. ^{14}C-Thaxtomin A (TA) and. ^{14}C-Thaxtomin A-Glucoside (TAG) Production Curves as Read in Liquid Scintillation Spectrometer and Produced by *Streptomyces scabies* Strain 87/22 in Oat Meal Broth (OMB) Amended with L-Phenylalanine-UL-^{14}C Growing at 28°C.

Thaxtomin A Tissue Penetration

When microtubers of the cultivar Atlantic were surface treated with ^{14}C-thaxtomin A, necrosis was observed below the spot where the toxin was applied (Figure 24). Radioactivity was measured in the peel, cork, xylem and medulla tissue under the application zone. Radioactivity was detected mainly in the outer layers of the microtubers (Table 18). It suggests that thaxtomin A in an infection process probably does not go very deep in the tissue, explaining the characteristic superficial symptom of scab.

Table 18. Radioactivity Detected in Four Layer of Microtubers of Atlantic Surface Treated with ^{14}C-Thaxtomin A.

Microtuber Layer	Radioactivity (dpm/g sample)
Peel	305.2
Cork	123.5
Xylem	19.8
Medulla	28.8

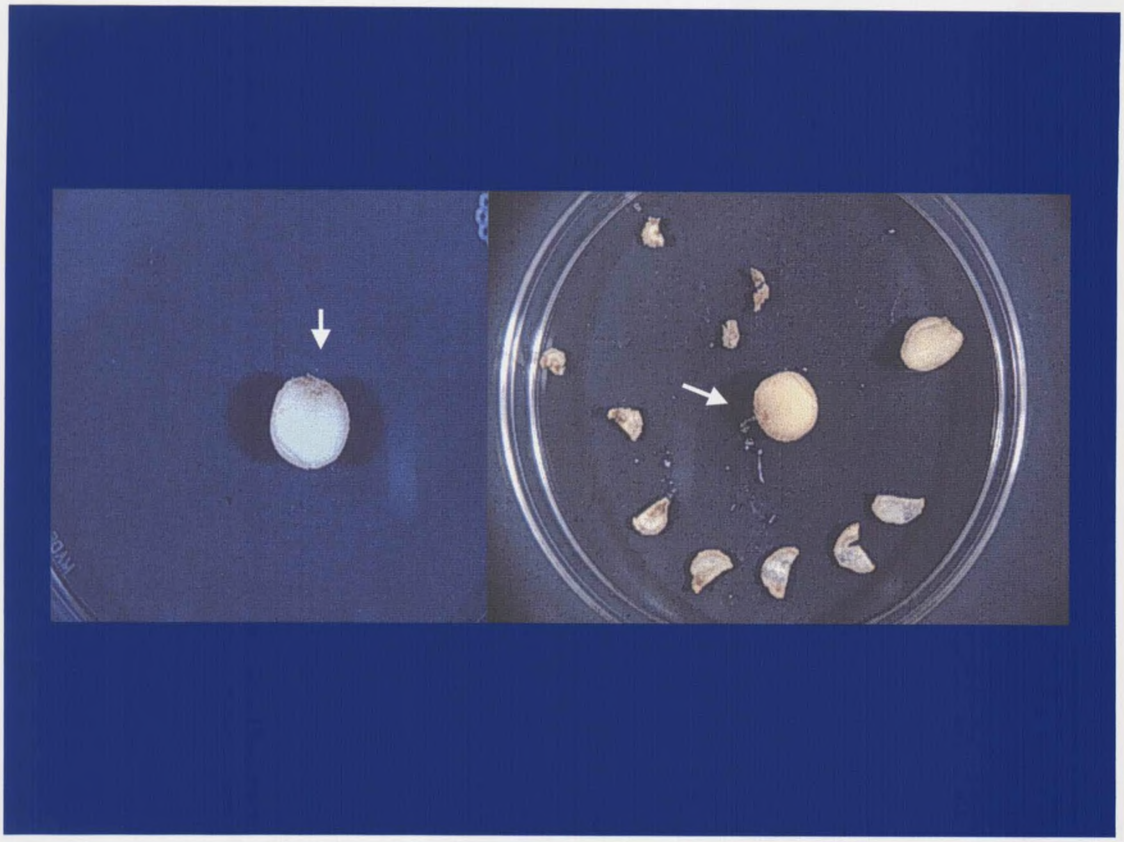


Figure 24. Atlantic Microtubers Treated with ^{14}C-Thaxtomin A. Arrows indicate the toxin application area and necrosis caused by the toxin.

Thaxtomin A-β-di-O-glucoside Production in Scab Resistant and Susceptible Potatoes

Sensitivity to thaxtomin A of the cultivars and selected clones used in this experiment is shown in Table 19. They were tested in the tubers slice assay, with Ranger being the most sensitive. It is also the most susceptible to scab.

Table 19. Index of Necrosis on Potato Tuber Slices Exposed to 50μM of Thaxtomin A for 7 Days.

Cultivar or Clone	Index of Necrosis (1)
Ranger	3.7 a*
R0.1-1	2.0 cd
R0.1-2	2.2 bc
R0.1-6	1.6 de
Nooksack	2.5 bc
Atlantic	2.9 b
At1-9	1.0 e

(*) Numbers in a column followed by the same letter are not significant different at 10% LSD

(1) 0=No necrosis, 1=Few brown flecks, 2=Brown flecks in determined necrotic area, 3=Brown necrosis and 4=Brown black necrosis

Figure 25 shows an autoradiograph of a TLC plate from crude extract of the organic phase n-butanol extraction of microtubers dipped in ^{14}C-thaxtomin A solution. Two radioactive bands can be distinguished; the first at the thaxtomin A R_f band, that co-migrated with standard thaxtomin A, and a second at the same R_f (0.21) as the bacterial thaxtomin A-glucoside (TAG-T1). These bands were present in all of the samples. Quantification of the glucoside bands, in the liquid scintillation spectrometer (Table 20), revealed undetectable quantities in the scab susceptible Ranger cultivar extract but significant amounts in samples from tolerant and resistant varieties and lines R0.1-1, R0.1-6 and At1-9. These lines were selected with thaxtomin A as resistant to scab in the seedling stage.

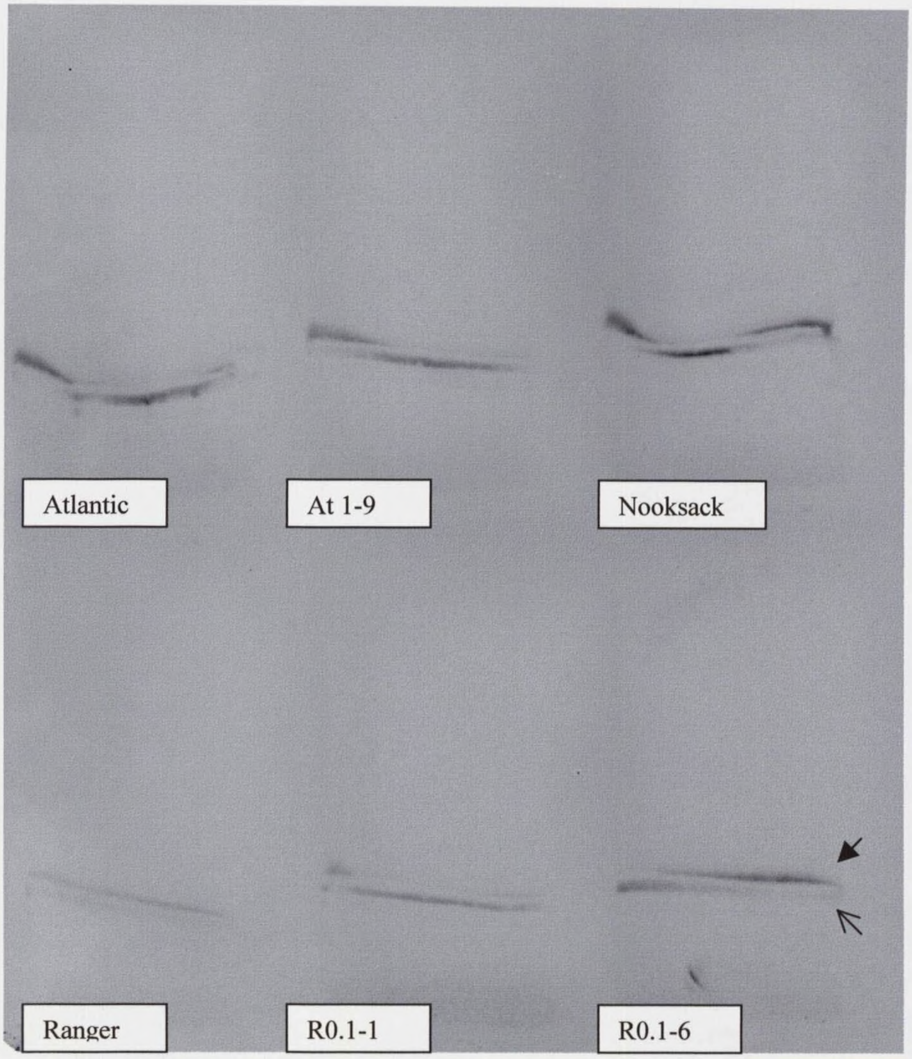


Figure 25. Autoradiograph of Organic Phase n-butanol Extracts Separated in TLC from Extraction of Atlantic, At1-9, Nooksack, Ranger, R0.1-1 and R0.1-6 Microtubers Treated with ^{14}C-Thaxtomin A.
➔ = Thaxtomin A, ⇾ = Thaxtomin A-glucoside.

The toxin selected lines showed evidence of more thaxtomin-glucoside than their respective parents. Radioactive bands were not detected in the aqueous phase extract of these samples.

Table 20. Thaxtomin A-glucoside Radioactivity in Potato Microtuber Extracts from Cultivars with Different Levels of Susceptibility to Scab Inoculated with ^{14}C-Thaxtomin A.

Cultivar or Clone	Radioactivity (dpm/g sample)
Ranger (S)(1)	0
R0.1-1 (R)	19.2
R0.1-6 (R)	14.3
Atlantic (T)	20.6
At 1-9 (R)	48.3
Nooksack (R)	35.9

(1) S=Scab Susceptible, T= Scab Tolerant, R=Scab Resistant

Extracts of minitubers injected with labeled thaxtomin A of scab resistant and susceptible varieties showed similar results (Table 21) for Ranger and three of its toxin selected lines. Ranger reveled radioactivity levels of TAG-T1 R_f, lower than lines more resistant to scab. Figure 26 shows that line R0.1-1, resistant to scab, developed a stronger TAG-T1 band than Ranger in TLC observed under UV. In addition, it can be seen that while many bands are apparent, the only bands with radioactivity were thaxtomin A and TAG bands. Controls were boiled minitubers injected with labeled thaxtomin A. TLC showed some bands in these controls, thaxtomin A and others, but not glucosides. The glucosides band in the controls showed lower dpm counts than non-boiled treatments.

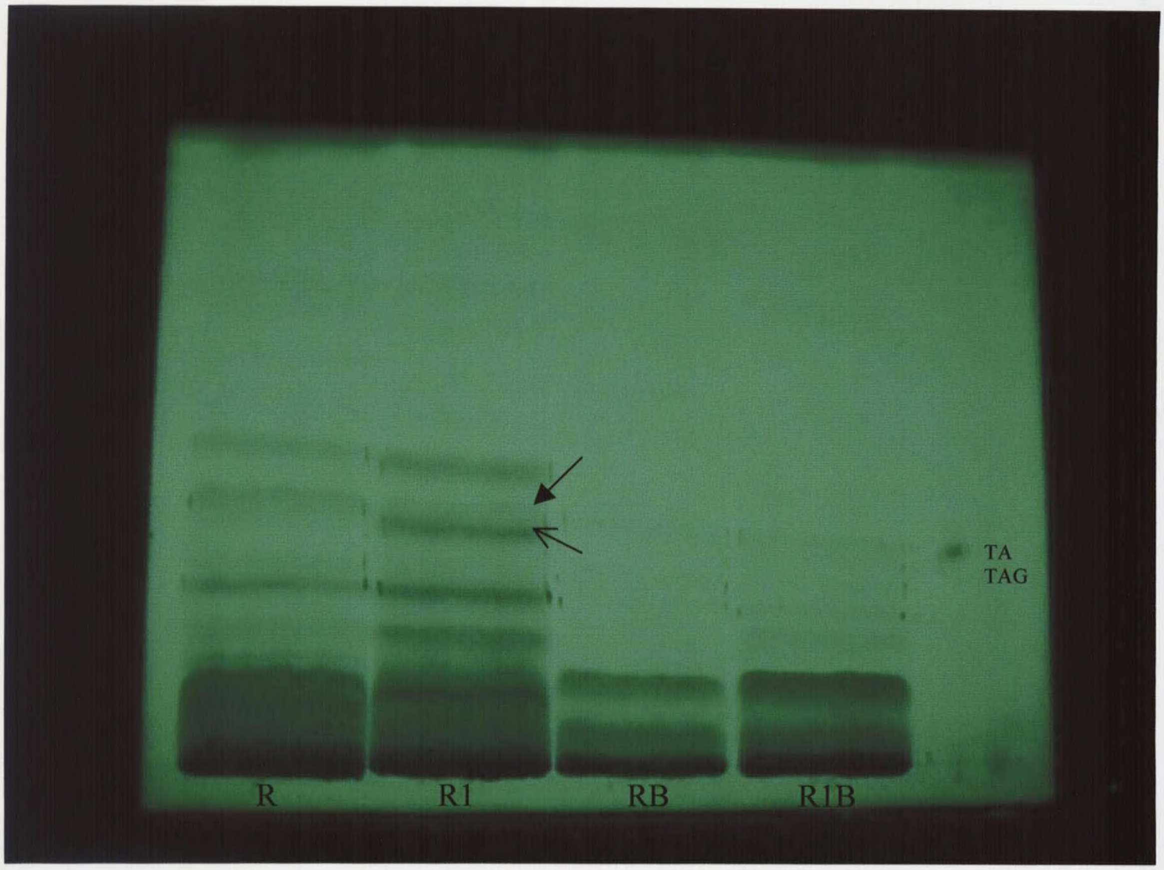


Figure 26. Thin Layer Chromatography of n-butanol Extracts from Potato Minitubers of Ranger (R) and R0.1-1 (R1); and Boiled Minituber Controls of Ranger (RB) and R0.1-1 (R1B) Observed Under UV.

→ =Thaxtomin A (TA), ⇾ = ThaxtominA-Glucoside (TAG)

Table 21. Thaxtomin A-glucoside Radioactivity in Potato Minituber Extracts from Ranger Cultivar and Thaxtomin A Selected Ranger Clones Injected with ^{14}C-Thaxtomin A.

Cultivar or Clone	Radioactivity (dpm/ Kg sample)	
	Treatments	Controls (2)
Ranger (S) (1)	1737	688
R0.1-1 (R)	4309	514
R0.1-2 (R)	3782	1648
R0.1-6 (R)	8484	1390

(1) S= Scab Susceptible, R=Scab Resistant
(2) Boiled Tubers

Glucosyl Transferase Activity in Scab Resistant and Susceptible Potatoes

The glucose conjugation mechanism is mediated by glucosyltransferase, an enzyme that transfers glucose from UDP-glucose to free –OH groups. This mechanism has been described in plants as a chemical compartmentation protection against toxins (Conn, 1985). I evaluated UDP glucose-thaxtomin A glucosyl transferase activity using Nooksack and Ranger minitubers (Table 22). Acetone powders of the two potato cultivars tested were extracted from these minitubers and mixtured in a reaction with thaxtomin A and UDP glucose-1-^{3}H. The enzyme was not purified in this experiment, therefore the specific enzyme that is transferring glucosyl residues to OH-groups in thaxtomin A is not known. However, according to Strobel and Hess (1997), the enzyme will be referred to UDP glucose thaxtomin A glucosyl transferase. Since pure enzyme was not used, some unspecific reaction would likely occur, explaining some radioactivity in the negative treatment controls (Table 22). Nonetheless, the relative enzyme activity of Nooksack was twice that of Ranger's, correlating with resistance to scab. Enzyme activity of Ranger was lower, statistically similar to that of many of the negative controls.

This suggests that glucosyl transferase activity in potato tubers may be related to resistance or susceptibility to scab. This test was done three times with similar results.

Table 22. UDP Glucose-Thaxtomin A Glucosyl Transferase Activity in the Acetone Powders of the Potato Cultivar Ranger and Nooksack

Treatments	Relative Enzyme Activity (dpm/mg protein)
1. Ranger enzyme plus thaxtomin A plus UDP glucose ^{3}H	706.4 b*
2. Ranger enzyme plus UDP glucose ^{3}H	379.3 bc
3. Ranger boiled enzyme plus thaxtomin A plusUDP glucose ^{3}H	180.9 bc
4. Ranger enzyme plus thaxtomin A	39.7 c
5. Nooksack enzyme plus thaxtomin A plus UDP glucose ^{3}H	1230.0 a
6. Nooksack enzyme plus UDP glucose ^{3}H	376.0 bc
7. Nooksack boiled enzyme plus thaxtomin A plusUDP glucose ^{3}H	92.5 c
8. Ranger enzyme plus thaxtomin A	19.9 c

(*) Numbers in column followed by the same letter are not significantly different at 5% LSD.

Discussion

Thaxtomin A is produced in potato broth and oatmeal broth (OMB) (Loria et al, 1995) and has been associated with pathogenic *S. scabies* in potato tubers (Lawrence et al, 1990). Production of thaxtomin A in vitro in OMB showed maximum production mainly in the lag phase of the bacterial growth curve (Figure 20). These results are consistent with Babcock et al (1993) who also described production of thaxtomins as secondary metabolites in late exponential and stationary phase, he commented that this is common for many toxic compounds produced by other Streptomycetes. Also, the addition of amino acids whose residues are an integral part of thaxtomin A inhibited the production of thaxtomin A in vitro (Table 17). This was previously described by Babcock et al (1993) for tyrosine and tryptophan in concentrations of 2.5mM. Our results showed that the production of thaxtomin A was inhibited by tyrosine and tryptophan at these concentrations but not by phenylalanine, however lower concentrations of the three amino acids, inhibited toxin production. Babcock and his coworkers explained this by a negative effect of nitrogen on the production of secondary metabolites or a direct effect of the amino acids in the toxin biosynthetic pathway. Furthermore, labeled phenylalanine amended in the media, showed that the bacteria are able to utilize this compound for many metabolites other than thaxtomin A (Figure 22). In addition, it was observed that the production curve of labeled thaxtomin A is similar to the standard thaxtomin A production, with a maximum around 10 days, with similar yields of about 4 mg /L.

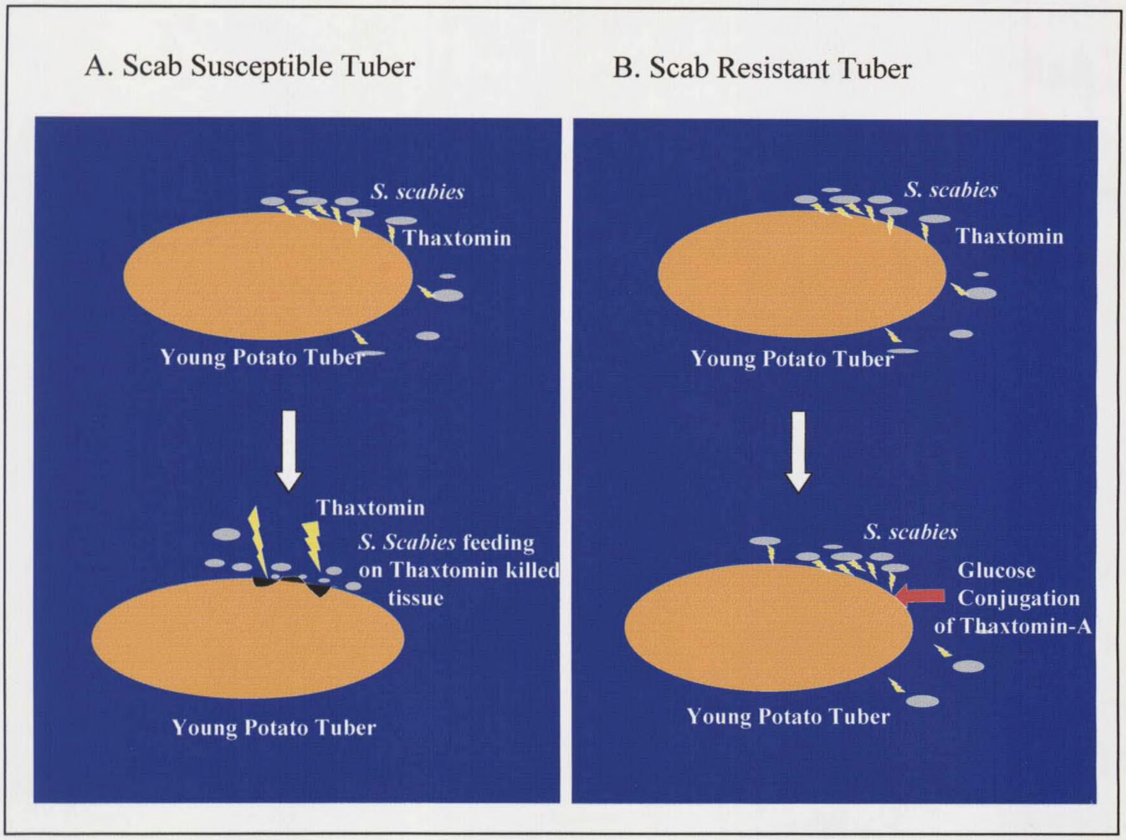
Glucosylation of toxins as a detoxification metabolism have been described for many microorganisms (Strobel and Hess, 1997; Park et al, 1994) and plants (Engelhardt et al, 1999). I hypothesized that the glucosylation of thaxtomin A by *S. scabies* in vitro suggests a similar mechanism of detoxification in scab resistant potato tubers.

In culture *S. scabies* not only produces thaxtomin A, but glucosides of it (TAG-T1 and TAG-T2). Thaxtomin A glucosides are more than 6 times less phytotoxic than thaxtomin A and it seems that TAG-T2 was less bioactive than TAG-T1 (Table 16). Evidence of the glucose presence in these compounds was provided by acid hydrolysis followed by chromatography that detected a compound with the same mobility as glucose (Figure 19), and by carbohydrate analysis that showed glucose as a main sugar present in these compounds. Further description of TAG-T1 reveled configuration β of the glucosyl residue established by the chemical shift and the relatively large coupling constant for the anomeric proton. In addition, mass spectroscopy showed a M.W. similar to thaxtomin A plus two anhydro hexoses. Although further chemical descriptions are necessary to better characterize this compound, I described TAG-T1 as Thaxtomin A-β-di-O-gluoside. In vitro, thaxtomin A production is inhibited by the addition of glucose (King et al, 1992). It may be explained in part by the glucose conjugation of thaxtomin A in vitro by the bacteria. Glucoside production showed a similar pattern to thaxtomin A; therefore, probably at the same time the toxin is being produced glucose is being conjugated to it, if glucose is available in the media at adequate levels. Moreover, according to King et al (1992) and King and Lawrence (1995) the phenyl portion of the phenylalanine residue in the thaxtomin A structure is necessary in the structural requirements for phytotoxicity. It is likely that glucose conjugation occurs in the two free hydroxyl groups in thaxtomin A (Figure 16 B). Toxin activity is probably decreased by physical interference of the active site of the toxin by the glucosyl groups.

When potato tubers were inoculated with labeled thaxtomin A, radioactivity was detected mainly in the outer layer of the microtubers (Table 18). According to Jones (1931) when the bacteria invade the tuber cells in an infection process, cells collapse and cease to elongate and become suberized, forming a barrier of wound cork. Then further development is due to the ability of the bacteria to grow through this suberized layer. The exact role of thaxtomin A in the tuber-bacteria interaction is not clear, but it can be speculated that necrotic tissue is produced as a result of toxin secreted from *S. scabies.*

This dead tissue is used as a nutrient source for the bacteria. Then, with further thaxtomin A production further disease development occurs. Pathogenesis depends on the pathogenicity of the bacteria, in this case production of thaxtomin and other enzymes, and the ability of the host to detoxify thaxtomin or produce resistant cork cells. This process is visualized in Figure 27.

I suggest that scab resistant tubers are able to metabolize thaxtomin A to TAG, which is 6-10 times less phytotoxic, thus avoiding cell collapse and necrosis. Using minitubers of scab resistant and susceptible individuals inoculated with ^{14}C-thaxtomin A, I demonstrated that resistant plants are able to produce a higher amount of a radioactive metabolite with an R_f similar to thaxtomin A-β-di-O-glucoside than susceptible ones (Tables 20, 21). In addition, I evaluated the thaxtomin A glucosyl transferase activity in scab resistant and susceptible plants and found almost twice as much enzyme specific activity in resistant than in susceptible individuals (Table 22). I did not characterize this metabolite, but I speculate it is probably a thaxtomin A conjugate, since it was the only additional radioactive compound detected in crude extract. Glucose conjugation of toxins by plants has been previously described for zearalenone, deoxynivalenol, ochratoxin A (Engelhardt et al, 1999), maculosin (Park et al, 1994) and Leucinostatin A (Strobel, 1997). Furthermore, the early stages of tuber development are most susceptible to *S. scabies* infection. This stage also is associated with very low levels of glucose in the peel (<0.1%, Goto, 1981). As we know, glucose inhibited thaxtomin A production, thus the susceptible stage is coincident with low level of glucose in the peel, and probably low thaxtomin A glucose conjugation or low activity of glucose transferase potential. Then, when tuber development continues, more glucose is transported to the sink tuber and probably glucose transferase activity and many other glucose dependent enzymes increase, making tubers less susceptible to infection. Whether scab susceptibility in plants is because of lower levels of glucose or low glucose transferase activity in susceptible stages or a short susceptible stage is not clear. Our studies although not conclusive, suggest that glucose conjugation is one mechanism of thaxtomin A detoxification in potato plants and it is related to scab resistance and susceptibility in potato plants.



A. Scab Susceptible Tuber

B. Scab Resistant Tuber

Figure 27. Hypothesis of the Mechanism of Interaction Potato-*Streptomyces scabies*-Thaxtomin A in Resistant (A) and Susceptible (B) Potato Tubers.

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CHAPTER 4

SUMMARY

Common scab is one of the most important potato diseases worldwide and many approaches have been use for its control. Resistant varieties have been one of the best solutions to this disease. Traditional potato breeding programs do not have good screening techniques that allow for early generation selection for scab resistance and generally rely on inconsistent field evaluations. The discovery of thaxtomin A and its important role in bacterial pathogenicity suggested new approaches in developing the scab resistance screening techniques, and to the mechanism of resistance to thaxtomin A in potato tubers.

This research focused on the development of an optimized scab resistant screening assay using thaxtomin A; and on the interaction between thaxtomin A and scab resistant and susceptible potatoes. To pursue these objectives I investigated the correlation between an optimized TPS-Thaxtomin A resistance assay with common scab resistance of potato tubers as determined by tuber sensitivity to the toxin and scab symptoms in field tests in naturally infested soils; and tested the hypothesis that glucosylation of thaxtomin A is related to potato plant resistance to pathogenic *S. scabies*. ^{14}C-thaxtomin A was produced and used for inoculation of scab resistant and susceptible lines followed by analysis of produced metabolites.

Seedling screening using thaxtomin A permitted selection of scab resistant individuals. In these tests I evaluated the index of damage for individual seedling and used the advantage of potato clonal reproduction to multiply individuals identified as resistant. Seven days of TPS exposure to thaxtomin A at 1μM concentration allowed distinguishing between progenies that varied in resistance to scab. Thaxtomin A did not strictly select 100% resistant individuals, but it did allow a decrease in the range of population to be tested under field conditions by approximately 30%. Significant correlations were determined between seedling index of damage and tuber scab symptoms ($R=0.63$, $P=0.0001$; $R=0.49$, $P=0.0001$) and between tuber toxin sensitivity and tuber scab symptoms ($R=0.64$, $P=0.0001$; $R=0.75$, P0.01). It seems that thaxtomin A is an important factor in disease development, but not the only one. Therefore, future research should focus on combining other factors involved in pathogenicity such as esterase activity or glucose concentration with thaxtomin A as a tool for screening. However, using this technique I could select individual clones that were highly resistant to scab under very conductive field conditions for disease development. I could also identify individuals from Ranger self-cross progenies that were highly resistant to scab such as R0.1-1 and R0.1-6. In conclusion, the thaxtomin A seedling screening assay is a useful tool to identify scab resistant germplasm under homogeneous standard conditions, it allows a reduction in the population to be tested under field conditions and permits identification of highly scab resistant individuals for commercial purposes, as parents in breeding programs or as sources of resistant genes.

I described the glucosylation of thaxtomin A by *S. scabies* in vitro and suggest a similar mechanism of detoxification in potato tubers. I identified a glucoside compound from bacterial extracts than showed a molecular weight similar to thaxtomin A plus two anhydro hexoses. Evidence of the glucose presence in this compound was provided by acid hydrolysis followed by chromatography that detected a compound with the same mobility as that of glucose, and by carbohydrate analysis that showed glucose as a main sugar. Configuration β of the glucosyl residue was established by the chemical shift and relative large coupling constant for the anomeric proton. This compound was described as Thaxtomin A-β-di-O-gluoside. The results suggest that glucose conjugation occurs in the two free hydroxyl groups in thaxtomin A. Thaxtomin A-glucoside was 6 times less toxic than thaxtomin A in potato tubers. Probable toxin activity is decreased by physical interference by the glucose groups with the toxin active site in the thaxtomin A molecule. The exact role of thaxtomin A in the interaction of tuber and bacteria is not clear, but I speculate that necrotic tissue is produced as a result of toxin secreted from *S. scabies*. This dead tissue is used as a source of food for the bacteria. Then, further disease development and bacteria establishment will depend of the virulence of the bacteria strain, in this case the ability to produce of thaxtomin and other enzymes involved in pathogenisis, and the ability of the host to detoxify thaxtomin or produce resistant cork cells. I suggested that scab resistant cultivars are able to glucose conjugate thaxtomin A, decreasing its bioactivity, thus avoiding cell collapse and necrosis. Resistant plants are able to produce a higher amount of a radioactive metabolite with an R_f similar to thaxtomin A-glucoside than susceptible ones. In addition, almost twice as much glucose transferase specific activity was identified in resistant individuals compared to susceptible individuals. We did not characterize this metabolite, but it is probably a conjugate of thaxtomin A, since it was the only additional radioactive compound, besides the labeled thaxtomin A, detected in the crude extract. While these studies are not conclusive, they suggest that glucose conjugation is a mechanism of thaxtomin A detoxification in potato and it is related to scab resistance.

APPENDICES

APPENDIX A

THAXTOMIN A CHARACTERIZATION

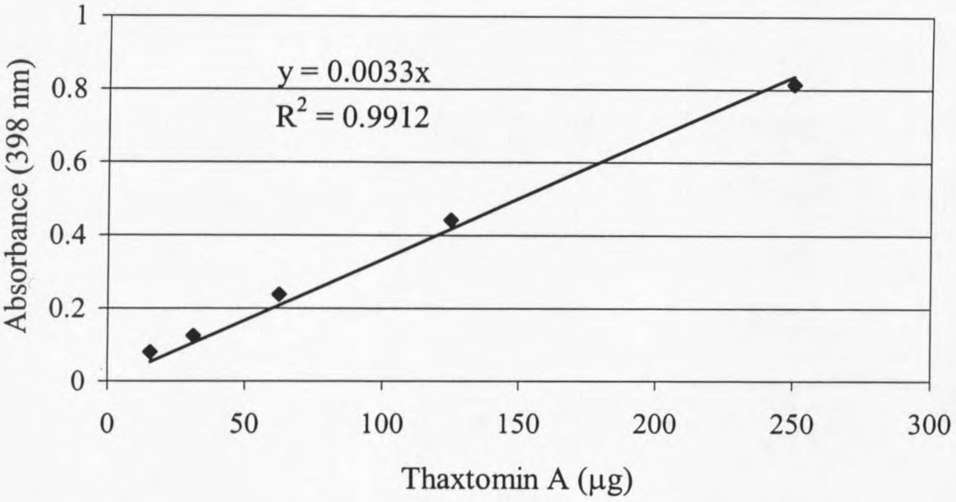

Figure 28. Thaxtomin A Standard Curve Determined by UV/vis Spectroscopy at A_{398}

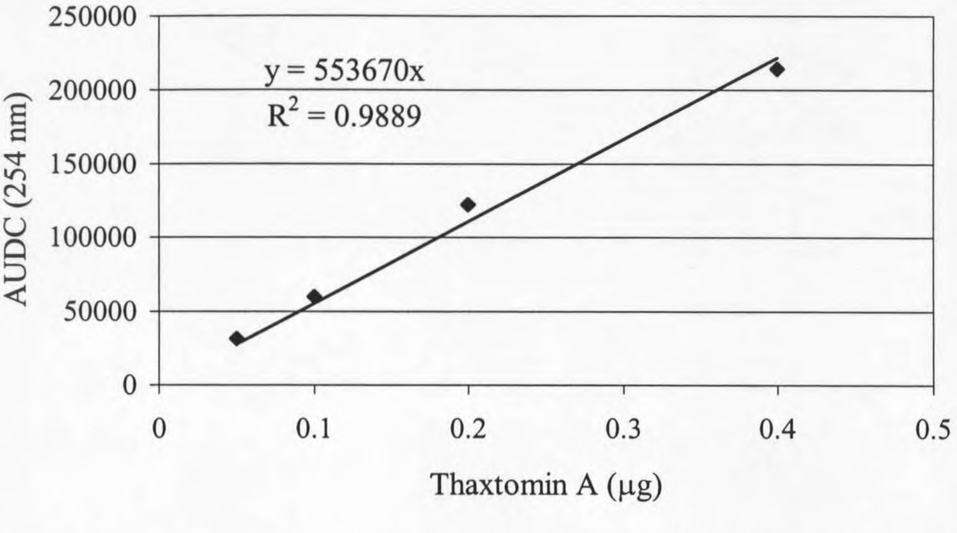

Figure 29. Thaxtomin A Standard Curve Determined by HPLC C8 Reversed Phase Column, in a Gradient 25-50% Acetonitrile at A_{254}. AUDC= area under de developing curve

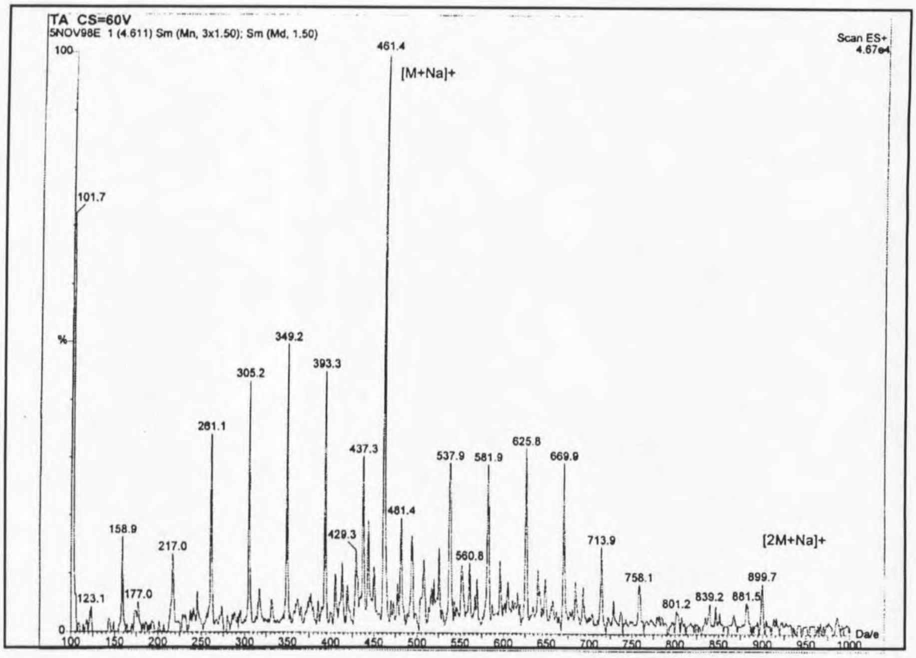

Figure 30. Thaxtomin A Electrospray Ionization Mass Spectroscopy. $[M+Na]^+$at 461.4, yielding a MW. of 438.4 .

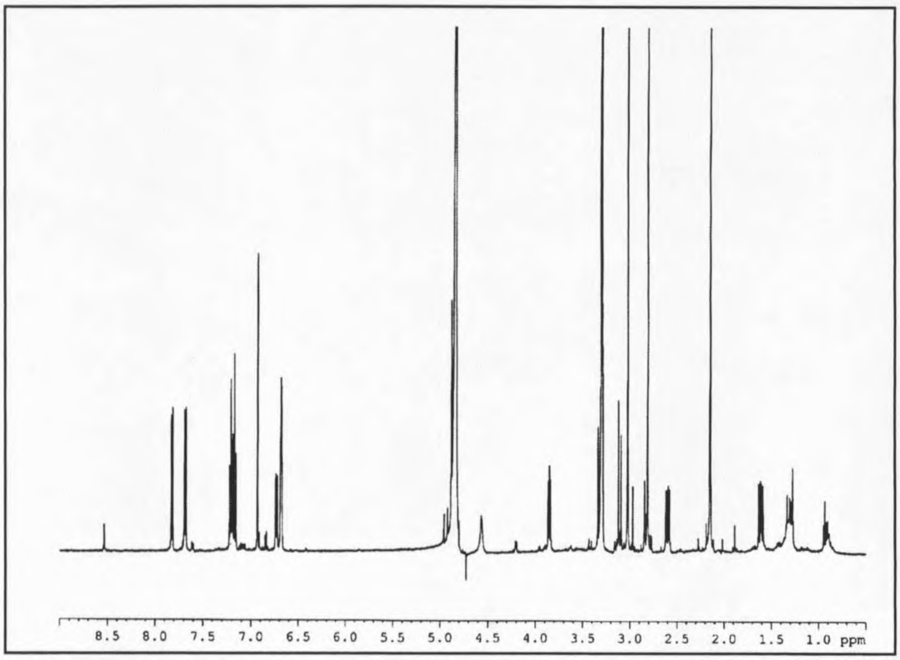

Figure 31. Thaxtomin A Spectrum from Nuclear Magnetic Resonance Spectroscopy (H-NMR).

APPENDIX B

THAXTOMIN A SELECTED CLONES

Table 26. Index of Damage of True Potato Seed Seedling Radical from Self-crosses of Ranger (R), Atlantic (A), Nooksack (N) and Norkotah (Nk) on Agar Amended with 0 to 50 µM of Thaxtomin A at 5, 7, 10 and 12 Days of Exposure, Analyzed by Toxin Level.

Toxin Level (µM)	Index of Damage(1)															
	Exposure Time (days)															
	5				7				10				12			
	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk
50	2.9b*	3.0b	1.8a	2.7b	2.9a*	3.1a	2.8a	2.9a	5.0a*	5.0a	5.0a	5.0a	5.0d*	5.0d	5.0d	5.0d
25	2.9c*	3.0c	1.8a	2.6b	3.1a*	3.0a	3.0a	2.8a	5.0a*	5.0a	5.0a	5.0a	5.0d*	5.0d	5.0d	4.9d
15	2.9b*	2.9b	2.4a	2.7b	3.0a*	3.0a	2.9a	2.8a	5.0a*	5.0a	5.0a	5.0a	5.0d*	5.0d	5.0d	5.0d
10	2.8b*	2.8b	2.3a	2.6b	3.0a*	2.9a	2.9a	2.8a	5.0a*	4.9a	5.0a	4.9a	5.0d*	4.9d	5.0d	4.9d
5	2.7bc*	2.9c	1.6a	2.5b	3.5b*	2.8a	2.8a	2.9a	4.9a*	4.8a	4.8a	4.9a	4.9d*	4.8d	4.9d	4.9d
1	1.6c*	1.1ab	1.0a	1.3bc	2.3c*	1.6a	1.5a	2.1 b	3.3bc*	2.4a	3.1bc	3.7c	3.3c*	2.3c	2.9c	3.6c
0.5	1.2a*	1.0a	1.0a	1.0a	1.3a*	1.1a	1.3a	1.4a	1.9a*	1.7a	2.8 b	2.8 b	2.0b*	1.9b	2.7c	2.7b
0.1	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.1a	1.9 b	1.2 b	1.0a*	1.1a	1.6b	1.1a
0(2)	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.0a	1.0a	1.0a
0	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.0a	1.0a	1.0a	1.0a*	1.0a	1.0a	1.0a

(1) 1= Radical healthy, 2= Radical with necrotic flecks, 3= Radical with large necrotic areas, 4= Radical with large necrotic areas and deformed, 5= radical dead.

(2) Control with no toxin solvent (ethanol) in the media.

(*) Numbers in a row in each exposed time followed by the same letter are not significant different at 5% LSD.

Table 25. Index of Damage of True Potato Seed Seedling Radical from Self-crosses of Ranger (R), Atlantic (A), Nooksack (N) and Norkotah (Nk) on Agar Amended with 0 to 50 µM of Thaxtomin A at 5, 7, 10 and 12 Days of Exposure, Analyzed by Progeny.

Toxin Level (µM)	Index of Damage[1]															
	Exposure Time (days)															
	5				7				10				12			
	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk
50	2.9c*	3.0b*	1.8b*	2.7c*	2.9d*	3.1d*	2.8c*	2.9d*	5.0d*	5.0d*	5.0d*	5.0d*	5.0d*	5.0d*	5.0d*	5.0d*
25	2.9c	3.0b	1.8b	2.6c	3.1d	3.0cd	3.0c	2.8d	5.0d	5.0d	5.0d	5.0d	5.0d	5.0d	5.0d	5.0d
15	2.9c	2.9b	2.4c	2.7c	3.0d	3.0cd	2.9c	2.8d	5.0d	5.0d	5.0d	5.0d	5.0d	5.0d	5.0d	5.0d
10	2.8c	2.8b	2.3c	2.6c	3.0d	2.9cd	2.9c	2.8d	5.0d	4.9d	5.0d	5.0d	5.0d	5.0d	5.0d	4.9d
5	2.7c	2.9b	1.6b	2.5c	3.5e	2.8c	2.8c	2.9d	4.9d	4.9d	4.9d	4.9d	4.9d	4.9d	4.9d	4.9d
1	1.6b	1.1a	1.0a	1.3c	2.3c	1.6b	1.5b	2.1c	3.4c	2.4c	3.1c	3.1c	3.2c	3.2c	2.9c	3.9c
0.5	1.2a	1.0a	1.0a	1.0b	1.3b	1.1a	1.3b	1.4b	1.9b	1.7b	2.9c	2.9c	2.0b	2.0b	2.7c	2.6b
0.1	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.2a	1.9b	1.9b	1.0a	1.0a	1.6b	1.1a
0(2)	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a
0	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a	1.0a

(1) 1= Radical healthy, 2= Radical with necrotic flecks, 3= Radical with large necrotic areas, 4= Radical deformed with large necrotic areas, 5= radical dead.

(2) Control with no toxin solvent (ethanol) in the media

(*) Numbers in a column followed by the same letter are not significant different at 5% LSD.

Table 24. Percentage of Germination of True Potato Seed from Self-crosses of Ranger (R), Atlantic (A), Nooksack (N) and Norkotah (Nk) on Agar Amended with 0 to 50 µM of Thaxtomin A at 3, 5, 7 and 10 Days of Exposure, Analyzed by Toxin Level.

Toxin Level (µM)	Percentage of Germination (%)															
	Exposure Time (days)															
	3				5				7				10			
	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk
50	10.0b*	35.0a	2.5b	25.0ab	77.5a*	82.5a	32.5b	77.5a	85.0a*	82.5a	55.0b	80.0a	87.5a*	82.5a	60.0b	77.5a
25	15.0b*	52.5a	0.0b	20.0b	77.5ab*	75.0ab	62.5b	85.0a	85.0b*	80.0b	80.0b	100.0a	87.5ab*	80.0b	82.5b	100.0a
15	15.0bc*	75.0a	2.5c	32.5b	77.5a*	90.0a	77.5a	92.5a	87.5a*	97.5a	85.0a	100.0a	92.5a*	97.5a	92.5a	92.5a
10	30.0b*	82.5a	2.5c	32.5b	80.0ab*	97.5a	75.0b	85.0ab	87.5a*	97.5a	85.0a	92.5a	90.0a*	97.5a	85.0a	95.0a
5	13.3c*	72.5a	0.0c	50.0b	80.0ab*	87.5ab	70.0b	97.5a	83.3a*	92.5a	90.0a	100.0a	86.7a*	92.5a	95.0a	100.0a
1	37.5b*	87.5a	12.5c	27.5bc	72.5b*	100.0a	92.5a	85.0ab	82.5a*	100.0a	100.0a	92.5a	87.5a*	100.0a	100.0a	95.0a
0.5	40.0b*	98.0a	10.0c	52.5b	82.5a*	92.5a	80.0a	82.5a	87.5a*	95.0a	90.0a	90.0a	92.5a*	97.5a	90.0a	92.5a
0.1	42.5b*	80.0a	3.3c	30.0b	90.0a*	97.5a	85.0a	92.5a	95.0a*	100.0a	97.5a	97.5a	97.5a*	90.0a	100.0a	100.0a
0(1)	40.0c*	85.0a	10.0d	62.5b	95.0a*	95.0a	92.5a	92.5a	97.5a*	97.5a	97.5a	92.5a	100.0a*	90.0a	100.0a	92.5a
0	23.3c*	92.5a	0.0d	44.0b	90.0a *	97.5a	90.0a	93.3a	96.7a*	100.0a	100.0a	96.7a	100.0a*	100.0a	100.0a	100.0a

(1) Control with no toxin solvent (ethanol) in the media.

(*) Numbers in a row in each exposed time followed by the same letter are not significant different at 5% LSD.

Table 23. Percentage of Germination of True Potato Seed from Self-crosses of Ranger (R), Atlantic (A), Nooksack (N) and Norkotah (Nk) on Agar Amended with 0 to 50 µM of Thaxtomin A at 3, 5, 7 and 10 Days of Exposure, Analyzed by Progeny.

Toxin Level (µM)	Percentage of Germination (%)															
	ExposureTime (days)															
	3				5				7				10			
	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk	R	A	N	Nk
50	10.0c*	35.0c*	2.5a*	25.0e*	77.5a*	82.5a*	32.5f*	77.5a*	85.0a*	82.5a*	55.0c*	80.0a*	87.5a*	82.5a*	60.0b*	77.5b*
25	15.0cd	52.5bc	0a	20.0e	77.5a	75.0a	62.5e	85.0a	85.0a	80.0a	80.0b	100.0a	87.5a	80.0a	82.5a	100.0a
15	15.0cd	75.0ab	2.5a	32.5de	77.5a	90.0a	77.5bcd	92.5a	87.5a	97.5a	85.0ab	100.0a	92.5a	97.5a	92.5a	92.5ab
10	30.0abc	82.5a	2.5a	32.5cde	80.0a	97.5a	75.0cde	85.0a	87.5a	97.5a	85.0ab	92.5a	90.0a	97.5a	85.0a	95.0a
5	13.3c	72.5ab	0.0a	50.0abc	80.0a	87.5a	70.0de	97.5a	83.3a	92.5a	90.0ab	100.0a	86.7a	92.5a	95.0a	100.0a
1	37.5ab	87.5a	12.5a	27.5c	72.5a	100.0a	92.5a	85.0a	82.5a	100.0a	100.0a	92.5a	87.5a	100.0a	100.0a	95.0a
0.5	40.0a	98.0a	10.0a	52.5ab	82.5a	92.5a	80.0abcd	82.5a	87.5a	95.0a	90.0ab	90.0a	92.5a	97.5a	90.0a	92.5a
0.1	42.5a	80.0a	3.3a	30.0de	90.0a	97.5a	85.0abc	92.5a	95.0a	100.0a	97.5a	97.5a	97.5a	90.0a	100.0a	100.0a
0(1)	40.0ab	85.0a	10.0a	62.5a	95.0a	95.0a	92.5a	92.5a	97.5a	97.5a	97.5a	92.5a	100.0a	90.0a	100.0a	92.5ab
0	23.3bcd	92.5a	0a	44.0bcd	90.0a	97.5a	90.0ab	93.3a	96.7a	100.0a	100.0a	96.7a	100.0a	100.0a	100.0a	100.0a

(1) Control with no toxin solvent (ethanol) in the media.

(*) Numbers in a column followed by the same letter are not significant different at 5% LSD.

Table 27. Scab Index of Selected Individuals with (RR) and without (RRC) Thaxtomin A from Self-crosses of Ranger. 1998, Bozeman Miniplots.

Selection	Scab Index (1) (2)	
RR17	0.4	hijkl *
RR110	0.5	ghijkl
RR114	0.3	kl
RR119	0.0	l
RR132	0.0	l
RR133	1.7	abcde
RR135	1.3	abcdefgh
RR215	0.0	l
RR229	0.0	l
RR231	0.4	ijkl
RR238	1.7	abcde
RR36	2.2	a
RR322	0.8	efghijkl
RR335	0.7	fghijkl
RR339	0.1	kl
RR46	0.6	ghujkl
RR418	0.1	kl
RR431	2.2	a
RR436	0.8	efghijkl
RR442	2.1	ab
RR55	0.0	l
RRC21	1.6	abcdef
RRC22	ND	
RRC23	0.2	kl
RRC24	1.4	abcdefg
RRC25	1.8	abc
RRC26	0.2	kl
RRC27	ND	
RRC28	1.2	bcdefghi
RRC31	ND	
RRC32	0.9	defghijkl
RRC33	0.9	cdefghijkl
RRC35	1.8	abcd
RRC36	0.0	l
RRC37	1.0	cdefghijk
RRC38	0.7	fghijkl
RRC41	0.3	jkl
RRC42	1.4	abcdefg
RRC44	0.6	ghujkl
RRC45	1.3	abcdefghi
RRC52	0.3	jkl
Ranger	1.0	cdefghij
Nooksack	0.4	ijkl

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) ND=no data

(*) Numbers in a column followed by the same letter are not significant different at 5%LSD

Table 28. Scab Index of Damage of Selected Individuals with (NN) and without (NNC) Thaxtomin A from Self-crosses of Nooksack .1998,Bozeman Miniplots.

Selection	Scab Index (1) (2)
NN115	0.0 h *
NN140	1.2 cde
NN227	0.5 efgh
NN324	0.0 h
NN52	1.9 ab
NNC11	0.0 h
NNC12	1.4 bcd
NNC15	1.1 cde
NNC16	0.6 efgh
NNC21	0.0 h
NNC22	ND
NNC23	2.1 ab
NNC24	0.6 efgh
NNC25	0.4 fgh
NNC27	0.2 gh
NNC28	0.7 efgh
NNC31	1.1 cdef
NNC32	0.4 fgh
NNC33	1.8 bc
NNC34	ND
NNC36	0.6 efgh
NNC37	0.4 fgh
NNC38	0.3 gh
NNC39	0.9 defg
NN543	0.6 efgh
NNC42	0.7 efgh
NNC43	2.6 a
NNC44	0.4 fgh
NNC52	0.2 gh
Ranger	1.0 def
Nooksack	0.4 fgh

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) ND=no data

(*) Numbers in a column followed by the same letter are not significant different at 5%LSD

Table 29. Scab Index of Selected Individuals with (RN) and without (RNC) Thaxtomin A from Crosses of Nooksack x Ranger .1998, Bozeman Miniplots.

Selection	Scab Index (1)(2)		Selection	Scab Index (1)(2)	
RN13	0.0	o *	RNC21	0.9	ghijklmn*
RN111	0.1	no	RNC22	0.7	ujklmno
RN113	0.3	lmno	RNC23	1.2	fghijk
RN122	1.2	fghijk	RNC24	0.1	mno
RN124	2.7	a	RNC25	1.9	abcdef
RN126	1.5	defghi	RNC26	0.0	o
RN129	0.4	klmno	RNC27	2.3	abcd
RN130	1.2	fghijk	RNC28	0.1	mno
RN132	0.2	lmno	RNC29	1.3	efghi
RN144	0.0	o	RNC210	2.6	ab
RN21	1.5	cdefghi	RNC211	0.2	lmno
RN22	0.1	mno	RNC31	2.2	abcde
RN24	0.2	lmno	RNC32	0.4	jklmno
RN26	0.0	o	RNC33	0.0	o
RN27	1.9	fghijk	RNC34	0.2	lmno
RN29	1.3	fghijk	RNC35	ND	
RN216	1.3	fghi	RNC36	0.1	mno
RN222	0.7	ijklmno	RNC37	1.5	defghi
RN224	0.2	lmno	RNC38	1.4	efghi
RN229	0.0	o	RNC41	2.4	abc
RN237	1.4	efghi	RNC42	1.5	defghi
RN242	ND		RNC43	0.0	o
RN243	2.4	ab	RNC44	1.5	defghi
RN246	0.0	o	RNC45	0.7	ijklmno
RN248	0.3	lmno	RNC46	2.3	abcd
RN39	0.1	mno	RNC51	0.0	o
RN316	1.7	bcdefgh	RNC52	1.0	ghijklm
RN321	0.1	mno	RNC53	0.0	o
RN34	1.9	bcdefg	Ranger	1.0	fghijkl
RN413	ND		Nooksack	0.4	klmno
RN426	0.3	lmno			
RN449	1.0	ghijklm			
RN59	0.3	lmno			
RN13	0.8	hijklmno			

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(2) ND=no data

(*) Numbers in both column followed by the same letter are not significant different at 5%LSD

Table 30. Scab Index of Clones Selected either with and without (C) Thaxtomin A from Ranger (RR) and Nooksack (NN) Self-crosses and Nooksack x Ranger Cross. 1999, Ronan, Field Experiment.

Selection	Scab Index (1)		Selection	Scab Index (1)		Selection	Scab Index (1)	
RR17	3.2	abc*	NN115	2.1	abc*	RN13	0.7	c*
RR110	2.4	abc	NN140	2.5	abc	RN111	2.3	abc
RR114	2.2	abc	NN227	3.3	abc	RN113	1.9	abc
RR119	1.0	bc	NNC16	2.7	abc	RN129	1.6	bc
RR215	1.3	bc	NNC21	0.9	bc	RN132	3.0	abc
RR322	1.7	bc	NNC24	1.4	bc	RN144	1.1	bc
RR335	3.0	abc	NNC25	0.9	bc	RN22	3.2	abc
RR46	0.8	c	NNC27	1.0	bc	RN222	2.8	abc
RR418	2.5	abc	NNC37	2.3	abc	RN229	1.4	bc
RR436	2.8	abc	NNC38	2.3	abc	RN248	1.5	bc
RRC26	1.6	bc	NNC39	1.5	bc	RN39	0.8	c
RRC28	1.4	bc	NNC42	2.8	abc	RN321	2.6	abc
RRC52	0.8	c	NNC52	3.0	abc	RN59	2.9	abc
RANGER	4.5	a				RN525	3.2	abc
NOOKSACK	2.6	abc				RNC22	3.1	abc
						RNC26	1.5	bc
						RNC211	2.3	abc
						RNC34	0.9	bc
						RNC36	2.7	abc
						RNC43	2.8	abc
						RNC45	3.6	ab
						RNC51	1.6	bc
						RNC52	2.3	abc

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%.

(*) Numbers in columns followed by the same letter are not significant different at 5%LSD. Average of 3 replications.

Table 31. Scab Index of Clones Selected either with and without (C) Thaxtomin A from Ranger (RR) and Nooksack (NN) Self-crosses and Nooksack x Ranger Cross. 1999, Ronan, Field Experiment.

Selection	Scab Index (1) (2)
RR132	2.3
RR229	ND
RR339	ND
RR55	2.4
NN324	2.8
RN24	2.7
RN26	ND
RN246	0.9
RN449	ND
RRC23	2.4
RRC32	1.0
RRC33	1.1
RRC37	0.5
RRC44	2.7
NNC15	1.0
NNC22	5.0
NNC28	2.9
NNC32	3.2
NNC36	1.0
NNC44	1.4
RNC24	0.7
RNC28	2.7
RNC32	3.3
RNC33	2.9
RNC53	2.3

(1) 0=no scab, 1=1% 2=10%, 3=25%, 4=50%, 5=75% and 6> 95%. Data from 1 replication.

(2) ND= no data