AN EVALUATION OF *STREPTOMYCES* PATHOGENICITY IN TURNIP

By

Kerry Ann VerMeulen

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Plant Pathology—Master of Science

2021

ABSTRACT

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Plant pathogenic *Streptomyces* spp. grow both inter- and intracellularly in host tissue and are good saprophytes. Thus, it is likely that these pathogens produce cell wall degrading enzymes in the process of infection and in saprotrophic growth. *Streptomyces* spp. isolated from scabby turnips were tested for the ability to grow and produce enzymes on media containing pectin, polygalacturonic acid, or cellulose as a sole carbon source. The production of cell wall degrading enzymes *in vitro* was tested. Disintegration of inoculated turnip root slices was used as an indicator of enzyme production. Most of the isolates with the highest tissue-degrading tendencies were among the highest enzyme producers; however, many isolates that did not elicit symptoms produced high levels of enzymes, so cell wall degrading enzyme production is not a characteristic unique to pathogenic species. Several *Streptomyces* isolates that elicited symptoms in turnip either lacked the ability, or caused significantly fewer, symptoms on potato. Seedling inoculations with most *Streptomyces* isolates resulted in hypocotyl stunting in most varieties. Reduced hypocotyl lengths were also correlated with symptoms on root structures. Inoculated roots were stunted, lacked lateral roots, and exhibited necrosis as compared to controls. Purple Prince turnip showed the most resistance to inoculation with isolates compared to other turnip varieties. Application of the phytotoxins thaxtomin and coronatine also caused stunted hypocotyls and symptoms on roots.

This thesis is dedicated to my children Katherine, John, Nathan, and Gordon, without whose loving support I would not have been able to discover the confidence, passion, and security I needed to embark on this journey. You have been my roots, anchoring me while I reached for the sun.

To my dear friend Laura, and the entire Moher family, for warmly and generously welcoming me to their home full of comfort, friendship, and love. You have sustained me.

“Love…
…is an ever-fixed mark
That looks on tempests and is never shaken;”
Sonnet 116, William Shakespeare

ACKNOWLEDGMENTS

I would like to thank Dr. Raymond Hammerschmidt for his leadership and guidance throughout this process. Dr. Hammerschmidt balances his searing intelligence with compassion and humor, which allows his students the confidence to try new things and the ability to explore. I will be forever grateful that he saw potential in an individual so long out of the classroom and gave me the opportunity to develop my skills and talents. New paths have opened to me which I could never have foreseen.

Thank you to my guidance committee for providing review and oversight. Thank you to Chris Behrens, Celeste Dmytryszyn, and Dr. Pratima Devkota, for making every day in lab a joy. I benefitted so much from the help and expertise they provided in their own unique ways. I appreciate all the people in the basement labs of Plant Biology and owe a special debt to Dr. Carmen Medina-Mora and Doug Minier, for sharing equipment, time, and expertise.

Thank you to Kyungmin Kim for the many hours spent on statistical analysis. Your assistance was invaluable, and I enjoyed our zoom sessions very much. Thank you for your patience answering my many questions. Thank you to the Michigan State University Histopathology Lab.

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CHAPTER 1. LITERATURE REVIEW

The Brassica Family

The Brassicaceae family (formerly Cruciferae) includes approximately 375 genera encompassing 3,200 species of plants and a large group of commercially and nutritionally valuable crops including arugula, bok choy, broccoli, Brussels sprout, cabbage, cauliflower, horseradish, kale, mustard, rapeseed, radish, rutabaga, turnip, and watercress (Ishida et al., 2014). Three important genera within the family are the *Armoracia*, *Brassica* and *Raphanus*. Horseradish, in the genus *Armoracia*, has been valued since ancient times as an herb for its pungent flavor and medicinal use in folk remedies (Agneta et al., 2013). Rich in bioactive glucosinolates and their derivatives, the crop has generated new interest as a preservative and medicinal additive (Agneta et al., 2013). The *Raphanus* genus includes radish and daikon which are cultivated as a vegetable crop around the world, but especially in China, Japan, and Korea (Kobayashi et al., 2020). This genus has been cultivated for more than 2,000 years and the evolution of wild radish into cultivated varieties is unclear (Wang et al., 2015).

The largest, and most commercially important genus in the family, *Brassica*, includes broccoli, Brussels sprouts, cabbage, rapeseed, rutabaga, turnip and more. Global *Brassica* oilseed production lags behind only palm and soybean, and it provides the second leading source of vegetable oilseed worldwide (Sparks, 2012). Turnip, or *Brassica rapa* var. *rapa*, is one of the oldest known cultivated vegetables and has been used “for human consumption since prehistoric times” (Fernandes et al., 2007). A white-fleshed, large global root “develops at the base of the leaf petioles” and its edible greens and stems provide a food source in addition to the root (Fernandes et al., 2007). Turnips are also a valuable forage crop for livestock and

present a rich source of energy and highly digestible nutrients (Benedict, 2012). Turnips are particularly popular throughout Europe and Asia. This is demonstrated by the three leading producers, which are China, Uzbekistan and Russia (Burton, 2016), whose climates are ideal for production of this cold-weather vegetable.

Turnip as A Host

Turnip is a high-yielding crop that is well suited for Michigan's climate. Its most vigorous root growth can occur at temperatures as low as 40–60 °F, which makes it a useful second-harvest crop. The fall crop provides the largest root and seeds planted in July can be used for animal grazing from September through November (Undersander et al., 2012). In 2017, the USDA National Agriculture Statistics Service Census stated that 3,916 acres of turnip were harvested nationwide, with an estimated 3,000 acres for the fresh market (NASS, 2017). Michigan ranks in the top four states for turnip production and cultivates about 770 acres per year: 20% of the nationwide total according to the Michigan Vegetable Council ("Vegetable Production in Michigan").

Home gardeners can easily grow these minimal-maintenance, fast-growing vegetables. Although Purple Top White Globe is the most common variety found in the U.S., there are many varieties and hybrids with assorted flavors, root textures, and color combinations. The Market Express turnip root matures in just 38 days, and the Scarlet Queen matures in 45 days and yields a bright red root with white flesh. The White Lady produces succulent green tops, and roots with a sweet flavor, tender texture, and white skin (University of Illinois Extension, 2020).

Turnip Diseases

The following diseases and pests affect *Brassicas* and may require crop rotation and fungicide or insecticide applications (Table 1.1). Some fields may not be suitable for turnip production for more than two years in a row, especially when clubroot or another soil borne disease has occurred. Insect pests include the flea, cabbage flea and striped flea beetle, as well as the turnip aphid/louse (https://hort.purdue.edu/newcrop/afcm/turnip.html). The turnip aphid/louse is especially problematic to *Brassicas* since it also vectors diseases like cabbage black ring spot, cauliflower mosaic, radish mosaic, and turnip mosaic virus (https://link.springer.com/referenceworkentry/10.1007%2F978-1-4020-6359-6_2594).

Table 1.1 Diseases and Pests of Brassica

Bacterial, Fungal and Viral Pathogens		
Common Name	**Pathogen**	**Symptom**
White Rust	*Albugo candida*	white blister-like pustules, white spots on leaf undersides
Leaf Spot	*Alternaria brassicae*	Small dark brown or black spots with a yellow halo. Target-like rings and necrotic leaves appear
Anthracnose	*Colletotrichum higginsianum*	small dry, circular, pale gray to straw-colored lesions lead to yellowing, leaf death
Clubroot	*Plasmodiophora brassicae*	Galls form on roots, results in yellowing, stunting, and wilting on leaves
Root Rot	*Rhizoctonia Solani*	Damping off, wirestem, and rot of bottom, root, and head
Scab	*Streptomyces spp.*	lesions, scabs, and pitting on roots that may include raised lesions

Table 1.1 (cont'd.)

Turnip Mosaic Virus	TuMV; family *Potyviridae*	Dark and light mosaic pattern on leaves, especially around main veins. Stunting and yellowing of plant. Aphid vectored
Insect and Nematode Pathogens		
Beetles; cabbage flea and striped	Chrysomelidae family	Shallow pits and rounded holes in leaves, stems
Turnip Louse/Aphid	*Lipaphis erysimi*	Stem and leaf damage, stunting, vectoring of TuMV
Root-knot nematode	*Meloidogyne hapla*	stunted growth, leaf yellowing and wilting

Scab Disease

Streptomyces scabies is the primary organism that causes the disease commonly referred to as scab. The disease is usually characterized by superficial or raised lesions (scabs), pitting, or russeting on the surface of the root or tuber (Loria et al., 1997). On turnips the disease usually presents as "crackling" (personal communication, Ben Werling, MSU Extension, Oceana County). Crackling is a description given to the uneven mottling or cracks that form in the underground, white area of the vegetable, often near the main root emergence (Figure 1.1). The disease cycle in Figure 1.2 depicts the formation of scab in potato, and the reproductive process of the pathogen in the soil matrix.

Figure 1.1 *Streptomyces* Symptoms on Turnips from Michigan Fields
Note. White arrow indicates crackling symptom.

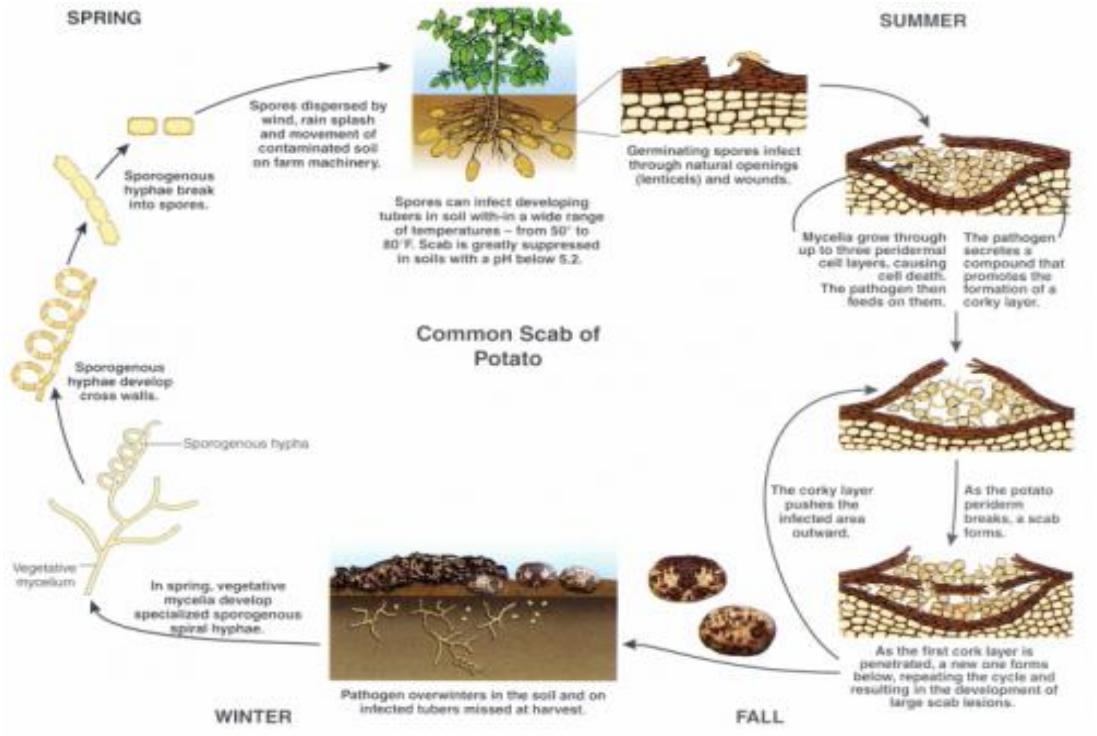

Figure 1.2 *Streptomyces* Scabies Disease Cycle in Potato
Note. Courtesy Michigan State Extension Bulletin E-2990 (Wharton et al., 2007).

Although *Streptomyces* can attack a wide range of host crops, the symptoms are a problem primarily on tuber-forming vegetables such as potato and taproots including beet, carrot, radish, and turnip (Hooker, 1949; Joshi et al., 2007; Loria et al., 1997).

Scab symptoms and disease development have been well studied in potato. Only limited studies exist for turnip, beet, radish, parsnip, and other crops, but the relationship between the scab caused by "actinomycetes species" on potatoes and that of scab appearing on other hosts grown in soils infested with potato scab has long been known (KenKnight, 1939). The type of lesion formed is thought to be determined by the combined effects of pathogen strain, environmental conditions, timing of infection, and host (Wharton et al., 2007). For example, russeting or crackling in turnip is a more prevalent symptom than scabbing, particularly on the white area of flesh that remains underground.

A study from 1937 examined the host range of *S. scabies* by growing a wide range of vegetables in fields known to be heavily infested by the pathogen. The 1937 study included cabbage, carrots, eggplant, onion, peanut, red pepper, spinach, and tomato (KenKnight, 1939). Only eggplant and peanut scab were noted, with lesions on eggplant similar to those found on the roots of turnip and rutabaga and small, raised lesions on peanut (KenKnight, 1939). Although the commercial losses due to disease in these other hosts may not be significant, their role in maintaining pathogenic *Streptomyces* spp. as inoculum in the soil is important (KenKnight, 1939).

Another study to determine crop rotation as an approach to control potato scab examined the host range of pathogenic *Streptomyces* spp. in beet, corn, cucumber, pea, radish, soybean, and wheat (Hooker, 1949). In this study, the effect of pathogenic *Streptomyces* spp.

applied to the roots of seedlings grown in soil agar was examined. Severe root necrosis was noted in all the plants except cucumber. Considerable resistance was seen in 18–dpi cucumber seedlings (Hooker, 1949). In pea, radish, and soybean, the fresh root weights were half of those obtained in controls and the aerial portions of the plants were both stunted and hypertrophied, possibly as a result of the necrotic condition of the roots and systemic invasion by the bacteria (Hooker, 1949). Beet had a more severe response than radish with even greater necrosis and almost complete inhibition of lateral root formation. Necrosis of wheat tips and lateral root emergence failure was also evident (Hooker, 1949). Although browning and some necrosis appeared on corn roots, mature corn plants did not seem susceptible to the pathogenic *Streptomyces* spp. Of all the plants tested, cucumber demonstrated the most resistance with no necrosis in the roots and little difference in fresh root weight compared to controls (Hooker, 1949). These experiments were duplicated in other small-seeded vegetables and legumes such as carrot, eggplant, lettuce, onion, and tomato, most of which exhibited some susceptibility. Alfalfa is noted as having more resistance than clover (Hooker, 1949).

The vast majority of studies have been performed on potatoes since they are the most commercially valuable, and susceptible (Loria et al., 1997). Tuber or root symptoms such as raised or pitted lesions versus superficial ones may indicate a difference between the *Streptomyces* spp. causing disease (Loria et al., 1997). Infections on sweet potato caused by *S. ipomoeae* are markedly different from infections caused by *Streptomyces scabies*, since *S. ipomoeae* will cause root rot, and reduced plant growth and yields, but not pitted or scabbed lesions (Loria et al., 1997). Crops that form large, fleshy taproots like turnip, and tubers like potato, are most susceptible to *Streptomyces* when lenticels are immature, which coincides

with the rapid growth and expansion of the tissue (Loria et al., 1997). *S. ipomoeae* has been shown to directly penetrate the root tissue of sweet potato (Clark & Matthews, 1987) and potato. This can be seen in Figure 1.3 where a *Streptomyces* filament is shown directly penetrating a potato periderm (Hammerschmidt, unpublished).

Figure 1.3 *Streptomyces* Periderm Penetration
Note. Photo courtesy of Dr. Raymond Hammerschmidt.

Streptomyces can survive indefinitely as a saprophyte in the absence of a host, and therefore is nearly impossible to eradicate from soil. The target of the main toxin produced by pathogenic *Streptomyces*, thaxtomin, is the plant cell wall, which may give this pathogen an unlimited host and tissue range (Joshi et al., 2007).

The pathogen does not kill the plant, nor does it cause systemic infection, but the damage on potato tubers or fleshy roots causes economic loss due to decreased marketability from the deep pitting (Loria, 1993). Decreased crop marketability can occur across multiple distribution channels including the fresh market, processed, and seed production (Loria et al., 1997). In the case of *S. ipomoeae* infections of sweet potato, significant losses result from

decreased yield and decreased marketability of the storage roots (Loria et al., 1997). *Streptomyces* has been ranked as the 4th most important disease of potato in its economic impact. *S. ipomoeae* is the most significant disease of sweet potatoes, but some resistant cultivars have been identified (Clark & Moyer, 1988; Ristaino, 1993).

Streptomyces **Ecology**

Streptomyces is the largest genus in the in the Actinobacteria phylum and one of the most commercially important. Actinobacteria contain at least 500 species of Gram positive, high G+C content (>55%), and primarily saprophytic bacteria (Barka et al., 2016). The seasonal abundance of Actinobacteria relative to other bacteria in the soil fluctuates from 20% during spring to over 30% in the fall when "large amounts of crop residues" are available (Barka et al., 2016). The phylum is reduced to just 13% of all bacteria in winter due to frost (Barka et al., 2016).

An ability to scavenge nutrients from diverse macromolecules leads to carbon cycling of relatively insoluble organic debris and makes *Streptomyces* a key player in soil ecology (Barka et al., 2016). *Streptomyces* can use chitin as both a C and N source, unusual among bacteria (Chater et al., 2010). Hyphal filaments adhere to and penetrate the insoluble "remains of fungi, plants, and other soil organisms," breaking them down with hydrolytic, excreted enzymes (Chater et al., 2010). The production of endo- and exo-chitinases has been shown to destroy and deform fungal hyphal complexes in co-culture studies against *Sclerotinia sclerotinium* (Fróes et al., 2012) and *Aspergillus parasiticus* is susceptible to thermophilic endochitinases produced by *Streptomyces* varieties (Gomes et al, 2001).

Although *Streptomyces* are found in marine and other aquatic environments, they are most prevalent in soils and comprise over 95% of all Actinomycetales found in the soil column (Barka et al., 2016). They prefer organic material and neutral to alkaline soils at a pH between 6–9, but some species like the phytopathogenic *S. acidiscabies* thrive at pH values below 5.2 (Loria et al., 1997).

Streptomyces Life Cycle

Streptomyces are known for their prolific secretion of cell wall degrading enzymes, and varied secondary metabolite production, including many lifesaving and commercially valuable antibiotics. To understand why this remarkable organism performs such complex extracellular protein secretion and transportation it is useful to examine how its life cycle, marked by morphological differentiation, leads to the production of chemically distinct inhibitors including antibiotics, fungicides, cytostatics, and a vast array of other secondary metabolites (Chater et al., 2010).

Vegetative Mycelia

Streptomyces do not form colonial clusters protected by a thick biofilm like many bacteria but grow hyphal mycelia that network and differentiate similar to fungi. These hyphal filaments grow exponentially through tip extension into a branched, vegetative mycelium when nutrients are plentiful (Chater, 2001). Another rare characteristic among bacteria is that this branching does not lead to cell fission but to a multicellular bacterium with hyphal compartments that contain multiple chromosome copies (Barka et al., 2016). When nutrients become depleted and starvation occurs, the vegetative mycelium is eventually cannibalized in a nutrient-recycling process that transforms the mycelia into sporogenic structures called aerial

hyphae (Barka et al., 2016). Through the production of cell wall and other polymer-degrading enzymes, *Streptomyces* metabolizes external nutrients and its own hyphae using a programmed cell death (PCD)-like mechanism that is tightly linked to and controlled by metabolic regulators (Barka et al., 2016; Miguélez et al., 1999). The evidence indicates strict metabolic links between nutrient metabolism, aerial hyphae formation, and antibiotic production (Rigali et al., 2006). One metabolic link is the N-acetylglucosamine (GlcNAc) byproduct of chitin breakdown. GlcNAc can both suppress or stimulate reproductive development depending on the source from which the GlcNAc is derived. This has been elucidated by studies of *Streptomyces* grown in rich media. When nutrients are plentiful because GlcNAc is coming from externally derived chitin sources, the sugar transporters activated by the bacteria are different than if the GlcNAc comes as a byproduct of the breakdown of its own cell wall as a result of PCD (Barka et al., 2016; Rigali et al., 2006). *Streptomyces* differentiates between monomeric and dimeric GlcNAc and responds accordingly to signal the onset of aerial development in starvation and cause the global elicitation of antibiotic production (Barka et al., 2016; Nothaft et al., 2010).

The autolytic degradation of the vegetative mycelia through PCD results in a pool of nutrients, and evidence suggests antibiotic production occurs to protect these amino acids, sugars, lipids, and nucleotides from motile organisms attracted to them (Barka et al., 2016; Rigali et al., 2006). The varied transcription factors that signal *Streptomyces* to stimulate or suppress antibiotic production have not been completely determined but are of continued interest to researchers, especially in light of recent studies indicating that *Streptomyces* may contain even greater antibiotic production capabilities than are currently recognized (Barka et al., 2016).

The prolific ability of *Streptomyces* to produce antibiotics has long been known, but their capacities continue to surprise researchers, as new studies indicate some species may harbor more than "50 different secondary metabolite gene clusters" (Barka et al., 2016). These clusters were suspected when the genome sequence of *S. coelicolor*, already a well-known producer of antibiotics, was found to contain over 20 biosynthetic clusters that had been previously unknown (Barka et al., 2016). Referred to as cryptic or "sleeping" antibiotics, the discovery of these sequences has prompted applied genomics research into activating the biosynthesis of these silent metabolites (Barka et al., 2016). The importance of already discovered *Streptomyces* products to humans including antibiotic, anticancer, anthelmintic, antifungal, antiviral, and cytostatic compounds, cannot be overstated. They are the originators of more than two-thirds of all naturally derived antibiotics, including polyketides which are used as both antimicrobials and immunosuppressants, the aminoglycosides of which Streptomycin is a member, and glycopeptides like Vancomycin to treat C-diff, MRSA and pneumonia, among other diseases (Barka et al., 2016).

Aerial Mycelia

Aerial hyphae are less branched and more than twice as wide as their vegetative mycelial counterparts. A sheath that forms on the exterior of the aerial hyphae lends structure and eventually becomes part of the spore coat (Barka et al., 2016). An additional function of the sheath may be to facilitate nutrient transport along the outer hyphal wall by providing a way for metabolites to diffuse from the basal colony up to the growing tips of the aerial hyphae (Chater et al., 2010). This sheath is hydrophobic on the air-facing side, and with assistance from high turgor pressure generated inside the hyphae, breaks through the moist soil-air surface (Barka et

al., 2016). The high turgor pressure, combined with the release of a powerful surfactant called a chaplin, provides a reduction in surface tension from 72 to 24 mJ m^{-2}, and enables the sensitive, fragile tips to reach into the air (Barka et al., 2016). An additional hydrophobic surfactant protein, SapB, is also widespread in *Streptomyces* and plays a role in the developmental growth of aerial hyphae (Rigali et al., 2006).

The aerial hyphae that lend *Streptomyces* colonies their signature fluffy appearance develop as a result of signaling events controlled by Bld regulators. Named after the bald or "hairless" aerial hyphae that develop in *bld* mutants, Bld regulators are required for exospores to fully differentiate through a "synchronized septation event" (Schumacher et al., 2017). One regulator in particular, BldD, sits atop a signaling cascade, repressing the transcription of an estimated 170 sporulation genes with targets that encode "critical components of the cell division and chromosome segregation" that eventually result in spore formation (Schumacher et al., 2017). The activity of BldD is regulated by a second messenger called 3',5'-cyclic diguanylic acid (c-di-GMP), "one of the most widespread second messengers in bacteria" (Schumacher et al., 2017).

Desiccation-resistant spores form on the chains of aerial mycelia and can endure in the soil matrix until nutrient availability stimulates them to germinate and form new vegetative hyphae (Chater, 2001). The morphology of these chains is often spiral resembling corkscrews, or wavy, and this structural differentiation allows for taxonomic distinction (Loria et al., 1997). The spores may be smooth, hairy, spiky or warty (Barka et al., 2016). The spore length on the long axis has been measured in *S. coelicolor* and is about 1.1 µm (Bennett et al., 2018).

Protein Secretion

Streptomyces' ability to secrete proteins and proteases extracellularly may reside in their extraordinary and numerous signal transduction systems, including about 50 "extra-cytoplasmic sigma functions," which is unusual for bacteria and for comparison, E. coli has just one. These secretions enhance their ability to interact with and respond to the soil environment (Chater et al., 2010). The capability of microbes to produce secondary metabolites, which has evolved separately from primary, survival metabolism (O'Brien & Wright, 2011) is thought to provide physiological and defensive benefits, and may be involved in establishing mutualistic relationships. Secondary metabolites are intrinsically linked to the ecological niche of the microbe. In the case of *Streptomyces,* secondary metabolites such as antibiotics are likely microbial agents of warfare, the production of which is stimulated by complex inter- and intra-cellular communication and signaling (O'Brien & Wright, 2011). Secondary metabolites are the likely reason why *Streptomyces* have formed mutualistic relationships with eukaryotes like ants. Attine ant species harbor *Streptomyces* species because the antifungal compounds they produce provide protection for the ant's farms (O'Brien & Wright, 2011).

Melanoid pigments provide crucial protection from radiation and temperature extremes and are critical to survival and competitiveness of bacteria in the soil matrix (Srinivasan et al., 2016). Melanin compounds produced by *Streptomyces* have proven valuable for pharmaceutical and cosmetic use, and supply broad protection as antiviral, antibiotic, antitumor and immunosuppressive compounds and enzymes (Srinivasan et al., 2016). The pigment produced usually varies by species and may even differ within strains based on growth

medium, and age of colony. *Streptomyces* most commonly produce light to dark grey melanin, but a range of colors from pink, to red and orange have been characterized (Barka et al., 2016).

The *Streptomyces* genome encodes at least three different types of protein secretion systems—the Sec translocase pathway, the Esx secretion system, and the TAT pathway. The Sec translocase pathway is the main route by which proteins are secreted, as is common in most prokaryotes (Driessen & Nouwen, 2008). The Gram positive-specific Esx or Type VII secretion system is usually associated with virulence, but since this transport machinery is also found in non-virulent *Streptomyces* it likely serves many roles (Chater et al., 2010). Although the function of this transport machinery is unknown it likely plays a significant role based on analogy with similar systems in other organisms (Chater et al., 2010).

The TAT pathway differs from the other protein secretion systems because it "transports pre-folded proteins across the cytoplasmic membrane" and *Streptomyces* rely on this system for the excretion of the large amounts of proteins they produce (Chater et al., 2010). Knockout of the TAT pathway in *S. scabies* affects the production of melanin and these mutants show defects in sporulation (Chater et al., 2010; Schaerlaekens et al., 2004). Multiple proteomic studies indicate a lack of virulence in TAT mutants unable to secrete novel proteins, particularly enzymes like glycosyl hydrolases (Planckaert et al., 2018).

Streptomyces **as a Pathogen**

Streptomyces that are pathogenic to root vegetables include a growing number of species because of the transfer of a pathogenicity island or PAI—examples of which are rare in Gram-positive bacteria (Kers et al., 2005). These mobile gene clusters are commonly transferred across genera and are recognized by variant G+C content (Kers et al., 2005). The

pathogenicity island that transforms *Streptomyces turgidiscabies* is also an integrative and conjugative element, or ICE, which can mobilize and insert at site-specific locations during conjugation (Kers et al., 2005). In the study by Kers, this PAI was shown to be capable of integrating into the chromosome of nonpathogenic *Streptomyces* spp. and in one species, *S. diastatochromogenes*, confer pathogenicity (Kers et al., 2005). The PAI insertion occurs at a bacitracin resistance gene called *bacA,* and it encodes at least four virulence factors: thaxtomin, or *txtA,* phytotoxin proteins; a saponinase, *tomA*, that encodes for α-tomatinase (common in many fungal genomes); a necrogenic protein, *nec1*; and the Fas operon (Kers et al., 2005).

These modules possess a G+C content of 68% compared to the genome's usual 72% (Huguet-Tapia et al., 2011). The new protein expression capabilities conferred by a PAI often transform the host range or phenotypic interactions of the bacteria, which may cause a saprophytic species like *Streptomyces* to be transformed, under certain inducing conditions, and depending on the genetic background of the recipient strain, into a pathogen (Kers et al., 2005).

These inducing conditions likely involve the carbon catabolite repressor (CCR) regulatory operon CebR, which is constitutive in *Streptomyces*. CebR functions similar to the Lac operon and represses transcription of β-galactosidase and other glucosyl hydrolases to prohibit utilization of secondary carbon sources except in the absence of a preferred substrate like glucose or chitin (Marushima et al., 2009). CebR includes *Ceb* genes-E, F and G that are thought to encode ABC transporters, and *bglC*, a β-glucosidase that hydrolyzes cellobiose (CB) and cellotriose. Cellobiose is the most effective inhibitor of the DNA binding of CebR and likely serves as a pathogenicity signal in *S. scabies* to postpone starch and glucose utilization

(Planckaert et al., 2018). Cellobiose has been proposed as a signal that indicates expanding plant cell growth and expanding tissue where cellulose synthesis is taking place (Bignell et al., 2014). Research indicates cellobiose also represses the saprophytic breakdown of lignocellulose into glucose through a 1.91-fold decrease in glucose kinase enzymes, although this interaction occurs independent of carbon catabolite repression signals like CebR (Planckaert et al., 2018).

CB utilization becomes an important regulator in *Streptomyces* strains where the PAI has been acquired, because its metabolism leads to the loss of genetic cofactors that inhibit *txtR* (Planckaert et al., 2018). The repression of the CebR operon through the loss of cis-acting elements leads to the allosteric activation of the binding of *txtR* (Planckaert et al., 2018) and the production of thaxtomin. This pathway has been elucidated through various studies demonstrating an absence of thaxtomin production in common growth media, including luria and tryptic soy broths, and its production in living host tissue or plant-based media such as potato, oatmeal and oat bran broths (Kers et al., 2005). Suberin and cellobiose in combination will stimulate much higher production of thaxtomin than the addition of either carbon source (Bignell et al., 2014) on its own.

S. scabies is more closely related to several benign, saprophytic *Streptomyces* than to other pathogenic ones (Loria et al., 1997) and it has not been determined what characteristics make some *Streptomyces* more likely to become pathogenic. A few of the most studied, like *S. acidiscabies*, *S. ipomoeae, S. scabies*, and *S. turgidiscabies,* have a similar host range and produce similar symptoms, but are not closely related according to DNA-DNA homology, 16S rRNA sequences and ribosomal DNA sequence comparisons (Bukhalid et al., 1998). DNA-DNA hybridization data suggests that *S. ipomoeae* is not closely related to either *S. scabies* (39%

homology) or *S. acidiscabies* (17% homology) which supports the idea that plant pathogenicity in *Streptomyces* is the result of genes conferred through lateral transfer (Loria et al., 1997). In Table 1.2 the known strains of pathogenic *Streptomyces* spp. that have been characterized to date are listed.

Table 1.2 List of Pathogenic *Streptomyces* Species

Potato is Primary Host	
*Streptomyces acidiscabies***	*Streptomyces spp. GK18*
Streptomyces cheloniumii	*Streptomyces spp. IdahoX****
*Streptomyces europaeiscabiei****	*Streptomyces spp. MAFF225003*
Streptomyces luridiscabiei	*Streptomyces spp. MAFF225004*
Streptomyces niveiscabiei	*Streptomyces spp. MAFF225005*
Streptomyces puniciscabiei	*Streptomyces spp. MAFF225006*
Streptomyces reticuliscabiei	*Streptomyces stelliscabiei****
*Streptomyces scabies (scabiei)****	*Streptomyces turgidiscabies****
*Streptomyces spp. DS3024****	
Alternate Host or Disease	
*Streptomyces ipomoeae****	Soil Rot of Sweet Potato
*Streptomyces scabies (scabiei)****	Pod Wart of Peanut

Note. **Known to produce thaxtomins Bignell et al., 2014).

More recently characterized species include *Streptomyces europaeiscabiei*, commonly found in Europe, and *S. turgidiscabies*, first discovered in Japan but now found in China, North America, Korea and the UK (Dees et al., 2013). The genome of *S. turgidiscabies* has been

studied in detail. The study of this genome led to the discovery of the pathogenicity island that causes scab disease (Kers et al., 2005).

This species variety and lack of commonality can be seen in the range of morphological differences. For instance, the gray, flexuous spore chains produced by *S. scabies* are distinct from the pink to white spores produced by *S. acidiscabies*, which lacks melanin. As the name implies, *S. acidiscabies* can cause disease in soils with a pH below 5.0, which hampers the scab avoidance technique of lowering soil pH (Loria et al., 1997).

S. ipomoea differs the most because it attacks sweet potato and produces quite different symptoms from common potato scab. *S. ipomoeae* causes necrosis on fleshy sweet potato roots, and cankerous growths (Loria et al., 1997).

Conserved Target-The Plant Cell Wall

Saprophytic organisms like *Streptomyces* are well known for producing cell wall degrading enzymes at levels equal to those produced by phytopathogenic organisms, either fungal or bacterial (Dean & Timberlake, 1989). Plant pathogenic *Streptomyces* grow both inter- and intracellularly, and it is possible that the cell wall degrading enzymes made constitutively by *Streptomyces* in their role as non-pathogenic saprophytes are also used in pathogenic interactions. These interactions involve the plant cell wall (PCW), the cellulolytic and pectolytic enzymes capable of its degradation, and the chemical stimuli that govern bacterial signaling mechanisms. The primary PCW is composed of three major polysaccharide types: cellulose, pectin, and cross-linking glycans sometimes referred to as hemicelluloses. The amount and type of each polysaccharide changes over time as plants grow, develop, and respond to environmental and biological stimuli (Voiniciuc et al., 2018). Growing tissue requires not just

expansion but additional material deposition and recycling, coordinated to loosen and restructure the polysaccharides as dynamic changes occur in the structure and function of the PCW (Buchanan et al., 2011). The plant uses modifying enzymes continuously to alter the composition of the cell wall, ripen reproductive structures, promote organ growth, and for defense (Buchanan et al., 2011).

Cellulose is a linear, simple glucose polysaccharide and is the first cell wall component to be synthesized in the primary cell wall (Buchanan et al., 2011). Cross-linking glycans that attach to these microfibrils are branched and highly substituted with post-translational modifications that incorporate methyl, acetyl, and phenol groups. Abundant cross-linking glycans include xyloglucan (XyG), xylans, mannans, with XyG by far the most abundant in eudicots. XyG is believed to be important in tip elongation because of its abundance at the interface between the plant and its environment (Voiniciuc et al., 2018).

Pectins are rich in α-1,4-linked galacturonic subunits like homogalacturonan (HG), Rhamnogalacturonan I (RGI), and Rhamnogalacturonan II (Voiniciuc et al., 2018). Pectins can incorporate 12 different sugars with as many as 25 various glycosidic linkages, but the most abundant pectin in primary walls is HG (Voiniciuc et al., 2018).

Figure 1.4 shows a broad overview of primary plant cell wall components, and the relative abundance of each type, when known.

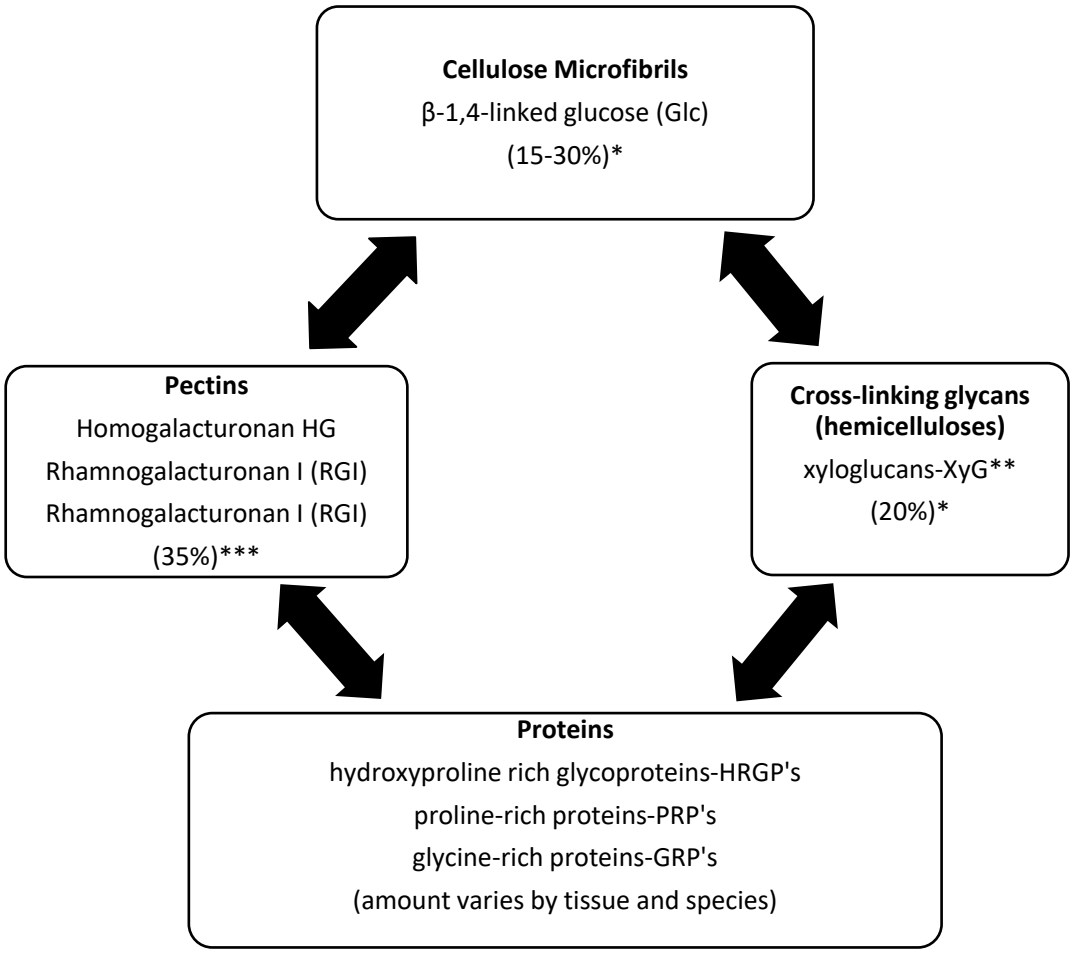

Figure 1.4 Primary Plant Cell Wall Components in Eudicots

Note: *Percent of dry mass weight in primary plant cell walls (Buchanan et al., 2011). **XyG is the major polysaccharide in eudicots. Other hemicellulosic polymers are xylan, glucuronoxylan, arabinoxylan, mannan, glucomannan and galactoglucomannan (Buchanan et al., 2011). ***Pectin content is highly variable by plant type. 35% is estimated for dicots, but some grasses could have as little as 2-10% (Kameshwar & Qin, 2018).

Disease Cycle

Common scab is most prevalent in dry soils with a pH between 5.2–7.0 (Loria et al., 1997). Strategies to reduce disease symptoms include raising and maintaining soil moisture when tubers or roots are in rapid growth stages and planting resistant cultivars when possible. Some known resistant cultivars of potato are Russet Burbank and Superior (Loria et al., 1997). Scab resistance in turnips has not yet been reported.

The optimum temperature for disease formation is 68–72 °F but the pathogen is active at range of temperatures from 50 to 88 °F (Wharton et al., 2007). The mode of entry of *Streptomyces* spp. is primarily through lenticels or wounds, (Wharton et al., 2007) although evidence exists for direct penetration (Hammerschmidt unpublished). The pathogen can spread easily from root to root, or through contaminated soil, but is prevalent in most potato growing fields throughout Europe, North America, South Africa, New Zealand, Australia, and Israel (Loria et al., 1997).

The early secretion of the necrosis-inducing peptide nec1 may be the first step in pathogenic *Streptomyces'* attack on plant host tissue. It is believed that *nec1* expression is not regulated by the carbon catabolite repression signal that governs thaxtomin and coronafacic acid production (Joshi et al., 2007). The small amount of glucose that is released around root tissue during cell wall expansion and modification may initiate colonization attempts by upregulating production of Nec1 (Joshi et al., 2007). *Streptomyces turgidiscabies* colonizes the exterior, intra- and inter-cellular portions of Arabidopsis root tips within seven days (Joshi et al., 2007). Studies show that Nec1 secretion is an important first step in colonization of host root tissue, since Δ*nec1* mutants exhibit only mild symptoms of infection with limited or no colonization of the epiphytic zone, and no intra- or inter-cellular growth observed compared to wild type (Joshi et al., 2007).

Thaxtomin causes a hypertrophic response in onion, radish, and tobacco seedling tissue (Scheible et al., 2003). The pathogen may also secrete a coronafacic acid derivative that induces tissue hypertrophy (Planckaert et al., 2018), and this phytotoxin, along with thaxtomin, is likely what leads to the comprised cell wall integrity seen in the disease (Loria et al., 1997). The roots

are especially vulnerable during rapid expansion, which takes place in turnips around 49–56 days (Gupta et al., 2001). The stimulation of thaxtomin synthesis may aid the penetration of the pathogen into the expanding tissue (Bignell et al., 2014). Hypertrophy, also induced by thaxtomin, likely causes the erumpent, or raised lesions, while pitted tissue is thought to be the result of toxin-induced cell death (Fry & Loria, 2002).

Role of Toxins in Pathogenesis

Thaxtomins

Thaxtomins are cyclic dipeptides derived from phenylalanine and L-4-nitrotryptophan entities of which at least eleven forms have been identified and which differ only in hydroxyl and N-methyl groups at particular sites (Bignell et al., 2014; King & Calhoun, 2009). One step of the biosynthesis of thaxtomin is catalyzed by nitric oxide synthase produced by nitric oxide (NO) derived from arginine (Kers et al., 2005; King & Calhoun, 2009). An intermediate called thaxtomin D is N-methylated on both the nitrotryptophyl and phenylalanine moieties (King et al., 2003). The last step in thaxtomin biosynthesis is the additional of hydroxyl groups to the phenylalanyl moiety. The thaxtomin phytotoxin appears to be unique to *Streptomyces* infections and its activity is disrupted by movement or replacement of any of the nitro groups, phenyl side chains or D,L configurations, indicating that these chemical features are required for phytotoxicity (King & Calhoun, 2009; Loria et al., 1997). Thaxtomin A is the most prevalently identified toxin and is produced by *S. scabies*, *S. acidiscabies, S. turgidiscabies* and many other species (Bignell et al., 2014). Thaxtomin C is the primary kind produced by *S. ipomoea*.

Thaxtomin inhibits crystalline cellulose biosynthesis in the cell wall (King et al., 2003; Loria et al., 1997) with a similar, but not identical, mode of action as the herbicide isoxaben and

appears to increase lignification and callose deposition (Scheible et al., 2003). This leads to a reduction in seedling shoot and root length, and radial swelling due to hypertrophy (Fry & Loria, 2002; Loria et al., 1997). This hypertrophy occurs even at thaxtomin concentrations below 1.0 µM, indicating that thaxtomin affects plant growth like a hormone (Loria et al., 1997).

Thaxtomin affects both monocots and dicots and so the target is thought to be conserved (Loria et al., 1997). The cell hypertrophy produced in seedlings mimics the erumpent lesions produced on the more mature tuber tissue, but there is evidence that both tubers and seedlings gain some resistance to thaxtomin A as they mature (Loria et al., 1997).

A non-hydroxylated member, thaxtomin C, is the primary form produced by the sweet potato pathogen *S. ipomoea,* although it is also made by *S. acidiscabies* and *S. scabies* (Loria et al., 1997). Mutants lacking thaxtomin C are unable to penetrate the adventitious root structure of sweet potatoes to cause infection (Guan et al., 2012). Thaxtomin C production is not stimulated by the cello-oligosaccharides that induce production of thaxtomin A, and the exact interaction of TxtR to induce thaxtomin production in *S. ipomoeae* remains to be discovered (King et al., 2003 and Guan, 2012).

Nec1

Nec1 is a necrosis-causing, water soluble phytotoxin with no known homologs that possesses a low G+C content (54%) indicative of lateral genus transfer (Bukhalid et al., 1998). Nec1 has a strong influence on root infection, is highly conserved among plant pathogenic *Streptomyces*, and also has a conserved plant cell target (Joshi et al., 2007). Unlike thaxtomin, which is “catabolically repressed,” Nec1 is produced in a rich growth medium early in the plant-pathogen interaction (Joshi et al., 2007). Studies indicate the presence of Nec1 just 20 hours

post infection (hpi) whereas thaxtomin is not apparent until 48 hpi (Joshi et al., 2007). Studies on Arabidopsis to isolate the effect of the Nec1 protein showed that the peptide causes "gall-like structures at the tips of lateral roots," with eventual necrosis of the roots (Joshi et al., 2007).

Coronatine Homologs

Another biosynthetic gene cluster discovered in *S. scabies* is highly similar to the coronafacic-acid (CFA) biosynthetic gene cluster (Bignell et al., 2014). CFA is the polyketide component of the nonhost-specific toxin coronatine (COR) that is produced by many pathovars of *Pseudomonas Syringae* and *Pectobacterium atrosepticum* (Bignell et al., 2014). The COR toxin in *Ps.* syringae causes hypertrophy and may function as a jasmonic acid (JA) mimic to suppress plant defense responses. Although *S. scabies* lacks biosynthetic genes that would enable the production of COR, it is likely to be producing similar COR-like toxins possible of enhancing virulence (Bignell et al., 2014). The CFA biosynthetic cluster has been identified in *S. scabies* but not in *S. turgidiscabies* or *S. acidiscabies* (Bignell et al., 2014).

Additional Toxins

Additional phytotoxins have been elucidated from select pathogenic *Streptomyces* species. In addition to thaxtomin, *S. scabies* has been reported to produce at least two types of concanamycin secondary metabolites (Bignell et al., 2014). First isolated from *S. diastatochromogenes,* the concanamycin family are polyketide macrolides characterized to exhibit antifungal, anti-neoplastic qualities, and to cause root growth inhibition in seedlings (Natsume et al., 1996).

Non-Toxin Virulence Factors

The third putative virulence factor identified as part of the Car8 PAI in *S.* turgidis*cabies* is encoded by genes with homology to a saponinase-encoding gene called *tomA* (Huguet-Tapia et al., 2011). Although deletion studies did not show a direct effect on virulence, its conservation in the PAI indicates a role that has not been determined. *TomA* has known homologs in *Clavibacter michiganensis* subsp *michiganensis* (CMM) and the coding regions flanking *tomA* "are predicted to encode 2 β-glycosidases, an ABC sugar transporter and TetR regulator". The *tomA* gene is syntenous in CMM and plant pathogenic *Streptomyces* may to share a virulence mechanism with CMM that involves carbohydrate metabolism (Huguet-Tapia et al., 2011). The research carried out in the present study seeks to yield additional information about the process of *Streptomyces* pathogenicity in turnip, the importance of cell wall degrading enzymes, the disease symptoms caused by isolated toxins compared to virulent *Streptomyces* strains, and whether there is resistance within turnip cultivars to the pathogen.

CHAPTER 2. CELL WALL DEGRADING ENZYMES IN THE TURNIP–*STREPTOMYCES* HOST PATHOGEN INTERACTION

Plant-pathogenic *Streptomyces* spp. grow inter- and intracellularly (Joshi et al., 2007) suggesting an important role for cell wall degrading enzymes in pathogenesis. The plant cell wall (PCW) is the most abundant source of organic carbon on the planet, a primary defensive barrier against many bacterial and fungal pathogens (Rai et al., 2015), and a primary phytopathogenic target. The ability to degrade plant cell walls has repeatedly and independently evolved in countless bacterial and fungal pathogens (Kubicek et al., 2014). Higher order animals like nematodes are believed to have acquired PCW degrading abilities from fungal and bacterial pathogens (Kikuchi et al., 2017). The production of cell wall degrading enzymes capable of penetrating roots, stems, leaves, or reproductive structures is well studied in various fungi (Mendgen & Deising, 1993) and is a hallmark of soft-rot bacteria. Soft rot diseases caused by *Dickeya* spp. and various *Pectobacterium* spp. occur primarily through the production of extracellular pectolytic enzymes and a wide range of cell wall degrading enzymes (Pérombelon, 2002). Pectin-degrading enzymes, in particular, are the first to be produced by many microorganisms when attacking plants or growing on purified cell walls. Depolymerization of pectin may be a prerequisite for further cell wall breakdown and pathogen colonization (Dean & Timberlake, 1989).

Primary Plant Cell Walls

Primary plant cell walls are mainly composed of cellulose (15-30%), cross-linking glycans (20%), and pectin biopolymers (35%). The specific amount and composition of each varies among groups of plants and cell function (Buchanan et al., 2011). Primary cell walls are important because they allow for cell expansion during growth phases and provide the shape of the cell and tissues. When cell division occurs and more cells are formed, the intermediate space, or middle lamella, is filled with a pectin-rich substance that aids in cell to cell adhesion, nutrient flow, and defense. Lignin and other proteins are formed when the secondary cell wall, a more rigid and defensible structure, is formed after cell differentiation (Buchanan et al., 2011). The composition of the wall changes throughout the life of the plant in response to growth, reproductive development, defense, and abiotic stimuli (Kubicek et al., 2014). Differences exist between monocot and dicot plants. Dicots such as turnip are rich in xyloglucan and pectin (Kubicek et al., 2014).

Although the relative amount of each compound varies, the cell wall is comprised mostly of cellulose, a substantially crystalline, β-1,4-linked glucose polymer. Attached to this fibril platform, and connecting them, are cross-linking glycans that order the direction of growth and expansion of the cell while maintaining the inherent strength of the linear cellulose chains (Buchanan et al., 2011). This aggregate of cellulose and cross-linking glycans is embedded in a porous network of pectin (Buchanan et al., 2011). Hydroxyproline-rich glycoproteins (HGRPs), proline-rich proteins (PRPs) and glycine-rich proteins (GRPs) are added to the structure over time. Extensin, an HGRP, is believed to be part of the mechanism that

locks the cell wall into place after the process of elongation is completed (Buchanan et al., 2011).

Cellulose utilization has long been believed to require the synergy of multiple cell wall degrading enzymes. The crystalline microfibrils of cellulose prevent access and degradation by cellulases, requiring the synergy of both endo- and exo-acting enzymes, and the action of loosening compounds to increase the reactivity of the tightly-packed microfibrils before degradation can occur (Arantes & Saddler, 2010; de Souza Moreira et al., 2016). Working in coordination on the hydrolysis of the 1,4-β-glycosidic bonds that link cellulose, endo-acting cellulase enzymes create new ends for exo-acting cellobiohydrolases to attack. In addition, the presence of compounds that perform what is called “amorphogenesis” may be required (Arantes & Saddler, 2010). Amorphogenesis enhances the reactivity of cellulose fibers by reducing the degree of crystallinity and increasing the surface area of cellulose available to the enzymes (Arantes & Saddler, 2010). Biochemical and structural data suggest cellobiohydrolases may not be exclusively exo-acting. A single endo-processive cellulase in the GH9 family of enzymes is essential in the successful degradation of *Clostridium phytofermentans* (Gilbert, 2010).

Although there are three major forms of pectin, homogalacturonan (HG) is most commonly found in nature (about 65%;) (Keggi & Doran-Peterson, 2020). The degree of methylation or acetylation of HG can require organisms to remove these side groups before breaking down the pectin (Keggi & Doran-Peterson, 2020) complicating the elucidation of various pathways for HG deconstruction by microorganisms.

Table 2.1 Pectins of Eudicot Primary Cell Walls

Pectin Polysaccharide	**Backbone Chains**
Homogalacturonan (HG)	Polygalacturonic acid backbone
Rhamnogalacturonan I-alternating disaccharides	α-1,4-D-GalA and α-1,2-L-Rha
Rhamnogalacturonan II	13 different sugars with over 20 different linkages

Cellobiose-Mediated Signaling

Streptomyces are known saprophytes and prolific cell wall degrading enzyme producers. Their ability to switch from a saprophyte to a pathogen may result from their ability to colonize a living host versus dead organic matter (Planckaert et al., 2018). This ability to be a pathogen is likely to reside in the combined effect from CWD enzymes and toxins working synergistically. This ability to colonize enables pathogenic *Streptomyces* to occupy a niche for survival and reproduction when a saprophytic lifestyle is energetically unsuitable (Planckaert et al., 2018). The production of cellulose-degradation byproducts is a key step since cellobiose disables the repression of the thaxtomin biosynthesis activator gene *txtR* that is present in the pathogenicity island (Planckaert et al., 2018). The utilization of cellobiose as a virulence determinant is not well understood since cellobiose is also the most abundant carbon source in the pathways that provide glucose in saprophytic interactions. In the pathogenic interaction, cellobiose and cellotriose transported into the cell lead to signaling effects that stimulate thaxtomin production. In pathogenic interactions the presence of cellobiose downregulates the glucose kinase pathways, and instead of using glucose as a carbon source, alternative and more complex carbon sources are prioritized (Planckaert et al., 2018). Other plant materials that seem to trigger thaxtomin A biosynthesis come from xylan and suberin degradation products.

The role of these materials has not been as well characterized as the cellobiose interaction with the cellulose utilization regulator CebR (Planckaert et al., 2018). Proteomics studies show a link between the presence of suberin and the production of gylcosyl hydrolases and other extracellular enzymes that are likely needed in the degradation of cellulose and suberin (Planckaert et al., 2018). The importance of enzyme production is also indicated by the impaired virulence in *ΔtatC* mutants unable to produce glycosyl hydrolases (Planckaert et al., 2018).

Virulence Mechanisms

The variety of symptoms observed with *Streptomyces* spp. suggests a range of virulence mechanisms, which may differ by host and be relevant to the process of pathogenesis (Loria et al., 1997). For example, the superficial lesions with a netted pattern that have been called netted scab produce necrotic lesions not just on expanding plant tissue but on all underground parts (Loria et al., 1997). Netted scab is also more severe in high soil moisture and low temperature conditions, the opposite of conditions that suppress common scab (Loria et al., 1997). Some potato cultivars such as 'Green Mountain' are highly susceptible to common scab but resistant to netted scab, suggesting differences in virulence pathways (Loria et al., 1997). *Streptomyces ipomoeae*, the cause of soil rot of sweet potato, infects both the fleshy and fibrous root parts. The infected tissues become necrotic with evidence that *S. ipomoeae* causes rot by directly penetrating fibrous roots (Loria et al., 1997). The necrosis and rot caused by netted scab and *S. ipomoeae* indicate a soft-rot type of disease with a potential virulence role played by cell wall degrading enzymes.

Objectives

Pathogenic *Streptomyces* may penetrate potato tubers via immature lenticels (Loria et al., 1997) or via direct cell wall penetration (Figure 1.3; Hammerschmidt) indicating cell wall degrading enzymes. The role that cell wall degrading enzymes play in the virulence of pathogenic *Streptomyces* spp. isolates on turnip are of interest. *Streptomyces* produce cell wall degrading enzymes *in vitro,* but the role these enzymes play in pathogenesis is unknown. The first objective of this research was to determine whether there is a correlation between the pathogenic tendency of *Streptomyces* spp. isolated from diseased turnips and the ability of these isolates to degrade polysaccharide components found in plant cell walls such as pectin and cellulose. Secondly, the ability of isolates to grow on media containing pectin, carboxymethyl cellulose, or glucose as the sole carbon source was determined. Also, the ability of *Streptomyces* spp. isolates to produce cell wall degrading enzymes in these media and the type of enzyme produced was evaluated. Finally, the ability of *Streptomyces* spp. isolates to cause disease in turnip tissue discs was used to determine whether pathogenesis is correlated with pectolytic and cellulolytic enzyme production.

MATERIALS AND METHODS

The reference isolates used in this study were provided by the lab of Dr. Raymond Hammerschmidt from Michigan-grown turnips (except for 1-1 which was isolated from potato) exhibiting symptoms of scab. The isolate colonies were grown for 7-10 days and were maintained at 29 °C on oat bran media (Schaad et al., 2001). A 5-mm plug from a culture was used in the growth measurements on media containing pectin, cellulose, or glucose as the sole carbon source, as described below.

Petri Dish Assays for Enzyme Activity

A total of 47 isolates were placed on each of four different media: carboxymethylcellulose (CMC), and pectin at three pH levels (5, 7, or 8). A total of 33 isolates were grown on media containing glucose as the sole carbon source. All media contained 500 mL of a mineral salts solution (MSS) consisting of the following (per liter): 2 g $(NH_4)2S0_4$; 4 g KH_2PO_4; 6 g Na_2HPO_4; 0.2 mg $FeSO_4*7H_20$; 0.2 g $MgSO_4$; 1 mg $CaCl_2$; 10 µg HBO_3; 10 µg $MnSO_4$; 70 µg $ZnSO_4$; 50 µg $CuS0_4$; pH 7.4 (Hankin et al., 1971). In addition to MSS, the pectin media contained, per liter: 15 g agar, 1 g yeast extract, and 5 g citrus pectin (Hankin et al., 1971). Additional tests using select isolates 1-1, B98, NV1, NV4, PBd1, TB-CD, V4 and V5 were run in pectin media without yeast extract. The media were homogenized in a blender to mix the pectin thoroughly and the pH was adjusted to either 5, 7, or 8. The media containing CMC was made as described for the pectin mixture with the substitution of 40 g CMC, just 5 g agar and no yeast extract. The same ingredients and protocols were also followed to make the glucose media, substituting 5 g glucose for pectin or CMC, 15 g agar, and no yeast extract. Isolates were also grown in oat bran media for growth comparisons.

All media were autoclaved and cooled before being dispensed. Using a sterile serological pipet, five mL of test media were added to each 60 x 15 mm Petri dish. One plug containing bacterial growth was applied (bacteria side down) to the center of each Petri dish. Three replicates of each isolate were made. Plates were incubated at 29 °C for 3 to 5 days, or until growth reached 5–10 mm. Some plates exhibited condensation after a few days of growth and the moisture that collected on the lids was removed periodically.

Pectolytic Activity Determination

To detect pectolytic activity, the inoculated plates containing pectin were flooded with a 1% hexadecyltrimethylammonium bromide (CTAB) solution dissolved in 15% ethanol in water (EtOH) (Hankin et al., 1971). This solution "precipitates polysaccharides" that have not been degraded. Thus, pectolytic enzyme activity is seen as a clear zone around the colony (Hankin et al., 1971). The clear zone, including the area containing the plug of bacteria, was measured to estimate the level of enzyme activity. For photographic recording of pectolytic activity, selected plates were stained with a 0.1% (w/v) ruthenium red solution (Spooner, 1994). Areas of undegraded pectin become pink, resulting in clear "halos" where enzymatic activity has occurred (Spooner, 1994). Growth in glucose media was measured, but enzymatic activity was not.

The plates containing CMC were flooded with a 0.25% (w/v) solution of Congo Red (Carder, 1986). The area degraded by cellulase does not stain as red as the areas where cellulose is intact (Carder, 1986). The area indicated as degraded by cellulase was measured and select isolates photographed.

Isolates in Shake Culture

Select isolates were chosen for further study in shake cultures after the plate assays were completed. The isolates were selected for their relative ability to produce low, mid, or high levels of enzymatic activity according to the ring size produced in the various pectin and CMC media. The isolates selected were 1-1 (*S. scabies*) from potato, and from turnip; 3-2, 4TA, 5TB, 7T, 7 Pit, 11 Pit, B9.8, Hud1, NV1, and V5. The isolates were placed in several different types of media to see if growth or enzymatic activity was enhanced by media type. The media contained, per liter: (1) Simple pectin broth contained 5g pectin, 500 mL MSS. (2) Polygalacturonic acid (PGA) media contained 3g yeast, 5g PGA, 2g K_2HPO_4, 1g $MgSO_4$*7H20, and 2g $(NH_4)_2SO_4$ at pH 5.0 (Jacob et al., 2008). (3) Pectin media containing 3g yeast, 5g pectin, 2g K_2HPO_4, 1g $MgSO_4$*7H20, and 2g $(NH_4)_2SO_4$ at pH 7.0 (Jacob et al., 2008). (4)High pectin media contained 0.5g yeast, 10g pectin, 1g K_2HPO_4, 0.88 g $MgSO_4$*7H20, and 1g $NaNo_3$, and 1g KCl at pH 7.0 (Mohandas). (5)High CMC media contained 0.5g yeast, 5g CMC, 1g K_2HPO_4, 0.88 g $MgSO_4$*7H20, and 1g $NaNo_3$, and 1g KCl at pH 7.0 (Mohandas). (6) Oat bran broth containing 20g oat bran, 0.1g $FeSO_4$*$7H_20$, 0.1g $MnCl_2$*$4H_2O$, and 0.1g $ZnSO_4$ *7 H_2O (Schaad et al., 2001).

Each media type was mixed and sterilized in the same manner as the agar Petri dish assays, and 50 mL aliquots were measured into sterile 250 ml Erlenmeyer flasks. A loopful of selected bacterial spores was added to the media and placed on a shaker at room temperature and 103 rpm. After 6 days, another loopful was added to some flasks from cultures grown in oat bran broth on shaker if significant bacterial growth was not visible.

Cultures were placed on the shaker at 103 rpm and maintained at room temperature for approximately 28 days before being filtered through two layers of cheesecloth, and then

centrifuged at 6000 rpm for 10–15 min at 4°C. The supernatant was decanted into large falcon tubes, frozen at -20°C and then lyophilized. Samples were lyophilized in an attempt to concentrate proteins at close to 1mg/mL for enzyme assays. Two isolates (B9.8 and 5TB) were grown as described above in media number 3, 4, and 5 and then placed in incubated shakers at 29 °C to see if elevated temperature enhance enzymatic activity. From selected isolates, 1 mL samples were removed for the agarose well assays described below. These samples are described as culture filtrates (CF) in the biochemical analyses.

Assays of Culture Filtrates

Enzyme activity produced in shake culture filtrates was detected and quantified using the Bradford assay for total protein, (Bradford, 1976), the DNS reducing sugar (Miller) assay for enzyme activity, and agarose well enzyme assays.

Protein Determination

Using the Bio-Rad kit based on the Bradford protein assay, select culture filtrates (CF) were analyzed following lyophilization. Sample CF were reconstituted in 1 mL of a 60 mM tris buffer at pH 6.6. This buffer has negligible interference. A standard curve was prepared using bovine serum albumin (BSA) at concentrations from 0.11 to 0.90 mg/mL. For comparison, a 10% pectinase standard (PS) in tris buffer was also measured and a standard curve prepared. The absorption of samples was compared to the standard curve to estimate protein levels.

Agarose Well Assays

CF fractions were applied to the following buffered, pectin-agarose media wells containing: 1) 1.5 g agarose, 100 mL tris buffer at pH 6.6, and 0.5 g pectin, 2) 2.0g agarose, 50 mL acetate buffer (pH 3.9), 50 mL dH20, and 0.2g pectin, 3) 0.5% (w/v) ammonium oxalate, 0.2% sodium azide, 1.0g agarose, in 100 mL 0.2 phosphate buffer adjusted to pH 5.3 with sodium salt of PGA (0.1%), 4) 1g pectin, 2g agarose, 0.05g $MgSO_4$, and 5) 1g pectin, 2g agarose, 0.08g $MgSO_4$. Each medium was boiled, cooled, and poured to completely cover a glass Petri dish with a thin layer. Following media solidification, 5-mm wells were created with a cork borer. Into these wells 50 µL of CF from select isolates was applied and the plate incubated at 29°C for 24 h. Some CF samples were applied to the wells without centrifugation. Following the addition of 1% CTAB solution (Hankin et al., 1971) a 0.1% ruthenium red solution was added before the Petri dish was placed back in the incubator for 1 day. Results were photographed.

The procedure steps were carried out in several different media. Applying pectinase standards (PS) in concentrations from 10–90% achieved a standard of ring degradation in media #1. The PS concentrations showed ring degradation areas ranging from 1.9 to 2.5 cm and were used as a comparison for CF samples applied to the media.

DNS Reducing Sugar Assay to Detect Pectolytic Enzyme Activity

Following the DNS reducing sugar method modified by Miller (1959), CF samples from all isolates grown in media #3 were analyzed for their ability to release reducing sugars from pectin. Following lyophilization, CF samples were reconstituted in 2 mL of a 0.1M phosphate buffer at pH 7.3 (Arijit et al, 2013) and then centrifuged at 3000 rpm for 10 min. The supernatant was decanted, and the pellet discarded. For a zero-time measurement, each CF

sample was added to a 1.0 % citrus pectin solution in a 1:1 ratio, using 0.25 mL of each. The DNS reagent solution was added, and the mixture boiled for 5 min before absorption was measured at 540 nm for Value 1. To determine enzyme activity, the same procedure was repeated except the solution was incubated 30 min at room temperature before the DNS reagents were added. The mixture was boiled for 5 min. Some CF samples had precipitants following heating in water bath and were centrifuged and the supernatant decanted. Absorption was measured at 540 nm. Subtracting the zero-time value from the 30-minute value yields an estimate of the reducing sugars released as a result of pectolytic enzyme activity. A 0.1% (w/v) mono-D-galacturonic acid (mDGLA) solution was diluted to concentrations from 0.01 to 0.71 mg/mL for the standard curve.

Thaxtomin Analysis by Thin Layer Chromatography (TLC)

Streptomyces spp. isolates 1-1, 5TB, B9.8, NV1, PBd1, and V4 were grown in oat bran broth (media #6) for 30 days. Broth cultures were filtered through two layers of cheesecloth and extracted in equal volumes ethyl acetate (Doumbou et al., 1998). The ethyl acetate extract was concentrated through rotary evaporation at 35°C under vacuum. The remaining residue was redissolved in ethanol in two 0.5 mL portions and these samples were applied to silica gel G plates with UV_{254} indicator and developed with chloroform-methanol (95:5, v:v). Some samples were separated in 85% chloroform, 15% methanol. Bands were visualized with UV light (254 nm) and Rf values were calculated from visible bands.

Turnip Tissue Disintegration Assays

Purple Top White Globe (PTWG) turnip tissue discs were prepared according to the method used by Hammerschmidt (1984) for potato tissue discs. Turnips were maintained at room temperature for 24 hours prior to use, washed with Alconox, rinsed in deionized water, and patted dry. The surface of each turnip was sterilized in 70% ethanol and flamed. This sterilization was repeated twice per turnip. Working with ethanol and flame sterilized instruments, the ends of the turnip were removed, and a cork borer was inserted through the center to remove a 2-cm core (Hammerschmidt, 1984). From this core, discs were made by slicing through the tissue at 5-mm intervals to create uniform, sterile pieces of tissue (Hammerschmidt, 1984). These pieces were washed in sterile water and rinsed three times to remove starch. Four pieces were added to pre-sterilized glass Petri dishes containing Whatman #1 filter paper. A few drops of sterile water were added to each Petri dish to moisten the filter paper before placing the tissue discs inside (Hammerschmidt, 1984). A 5-mm plug of 7 to 10-day-old culture from a *Streptomyces* spp. isolate was placed in the center of each tissue disk. A plug of sterile, non-inoculated oat bran media (Schaad et al., 2001) was added to the center of the control. Three replicates were made per isolate.

Turnip tissues were also tested for a reaction to enzymes and toxins. Filter paper discs of 5-mm diameter were dipped in solutions of 0.1% and 0.01% pectinase, and 0.1% cellulase and placed at the center of the tissue discs. A set of filter paper disks were made with 150 µg thaxtomin per disk, and each disk was placed on the center of a turnip tissue disk. A total of 46 sets of tissue discs were made. These were stored in the dark at room temperature for 7 days. Following rating according to

Table 2.2, the discs were weighed, select isolates were photographed, and tissue samples were frozen at -20°C. 34 *Streptomyces* spp. isolates were tested on turnip tissue discs.

Table 2.2 Rating Scale Applied to Tissue Discs

0	"No browning or disintegration compared to control"
1	"Tissue discoloration at disc edges with no disintegration"
2	"Uniform discoloration with some disintegration"
3	"Brown tissue with moderate, consistent disintegration"
4	"Brown or black tissue with significant disintegration"

Note. (Spooner, 1994)

Potato Tissue Disintegration Assays

A select group of isolates was tested on 'Manistee' potatoes using the same method. Seedling assays (unpublished data) indicate that some turnip varieties with resistance to the *Streptomyces* spp. strain isolated from potato were susceptible to turnip isolates. Additional tests were initiated on potato discs for evaluation. The isolates tested on potato were 1-1, B9.8, NV4, PBd1, TB-CD, and V5. Two isolates (1-1, B9.8) were applied to beet, radish, and rutabaga. Turnip was the host for the experiment unless otherwise stated.

Turnip and Other Root Tissue Discs, Further Analysis

Compounds produced in infected tissues were extracted in ethanol by macerating frozen tissue discs using a frozen mortar and pestle. The tissue samples were separated into two equal portions. One portion was lyophilized and stored at -20°C for future studies. The other portion was extracted overnight in 10 mL ethanol/g tissue. Samples were covered with parafilm and heated in a water bath for 1 hr., then centrifuged and the supernatant reserved as fraction 1. The process was repeated with the remaining pellet. The second supernatant fraction was added to fraction 1 and the ethanol was removed using a rotary evaporator at 80°C. Sticky residue remaining in vials was washed with dH_2O and then extracted in ethyl

acetate following the method of Doumbou (Doumbou et al., 1998). Extracts obtained from these discs were redissolved in ethanol at a rate of 0.5 mL/g tissue and these samples were applied to Silica Gel G 254 silica plates and developed with chloroform–methanol (95:5). Bands were detected with UV (254nm) light and Rf values were calculated for each band.

Statistical Analyses

Statistical model for the rating of turnip tissue disintegration included treatment (isolate) as a fixed factor and Petri dish nested within the treatment as a random factor. Since the effect of treatment was significant ($p < .05$), the mean separation was performed using Fisher's least significant difference.

RESULTS

Petri Dish Assays for Enzyme Activity

Although most isolates grew in pectin media at a pH of 5, 7, or 8, a few isolates showed little to no visible growth and no measurable pectolytic enzyme production. It was determined that 44 out of 47 isolates grew on media containing carboxymethylcellulose (CMC) as a sole carbon source suggesting that they could produce cellulases. All but four out of 33 isolates were able to grow on glucose as the sole carbon source. Several strains failed to survive in storage and could not be included.

On glucose as the sole carbon source, growth was not robust compared to oat bran (Figure 2.1). In glucose, *Streptomyces* spp. failed to form grey colonies with sporulating hyphae, although some pigment was produced by some isolates (Figure 2.1). The colonies grown in glucose also did not spread as they did in oat bran, pectin, or CMC media, where spores scattered new colonies away from the starting plug. Pectolytic and cellulolytic enzyme activity by isolates grown on glucose were not measured.

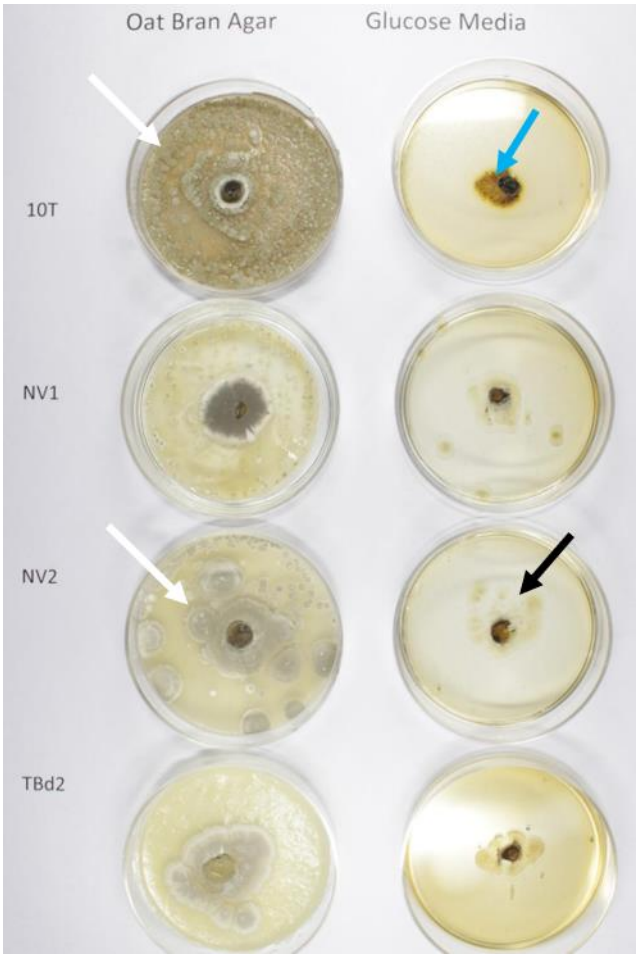

Figure 2.1 Comparison of *Streptomyces* Growth on Oat Bran Agar vs. Glucose Media

Note. White arrows indicate aerial hyphae formation by sporulating colonies. Blue arrow indicates melanin formation. Black arrow indicates sparse growth compared to oat bran media.

Zones of clearing, or activity, around the growing culture on media containing pectin or CMC were detected in most isolates indicating their ability to produce cell wall degrading enzymes. However, a few showed no measurable enzyme production in some media (Table 2.3).

Isolate 10T showed neither measurable pectinase production at all three pH levels nor cellulase production in CMC. Isolates 1T and 2T also produced no pectinase at pH 5 and little pectinase at 8; however, 1T produced some pectinase at pH 7, and 2T showed some cellulase production.

In addition to differences in the measurable size or diameter of the activity zone around the bacterial plug, there was also variation in the type of activity produced. For example, some isolates produced completely clear zones around the bacterial plug, even before the addition of CTAB, where it also appeared the agar had been degraded by the bacteria. These isolates were apparently capable of degrading both agar and pectin. Other isolates produced more hazy or indistinct zones around the plug where the top layer of agar appeared to have thinned but the enzymes had not diffused as readily. A few isolates produced areas where the media was dissolved in a uniform circle around the bacterial plug, and some isolates produced areas that spread out from the plug unevenly.

The production of crystal-clear areas of degradation with all media dissolved was rare and observed in isolates exhibiting both pathogenic and non-pathogenic characteristics. For example, the pathogenic isolate TB-CD in pectin media at pH 7 (with yeast extract) produced small zones where all media was disintegrated in the area of activity. In the same media (pectin at pH 7 with yeast extract) two isolates exhibiting saprophytic characteristics, Sup A3 and SupV3, also produced clear activity with disintegrated media. Isolate B9.8 by contrast, showed some of the largest zones of activity measured across all isolates and in all media, but with widespread and uneven areas without the complete dissolution of media seen in TB-CD, Sup A3

and SupV3. Isolate NV4 made smaller zones than B9.8, but they were more circular and distinct (Figure 2 a-b).

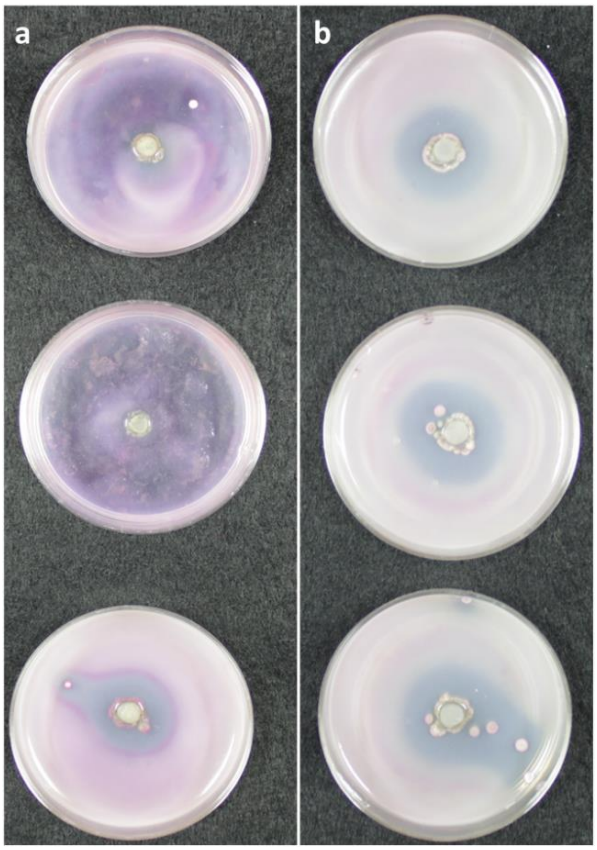

Figure 2.2 Enzyme Activity in Pectin Media at pH 7 (no yeast extract in media)
Note. a. B9.8 b. NV4

Some isolates produced “mottling” where the enzymatic activity was spread throughout the dish in an uneven manner and was difficult to measure visually. The cultures that contained mottling were measured as 0.0 cm due to an inability to visualize and measure a zone. These isolates often produced a large, visible area of activity in one replicate but mottling in one or two replicates that resulted in a high standard deviation for that isolate.

Table 2.3 lists the isolates tested and the average and standard deviation of their zone of activity.

Table 2.3 Means and Standard Deviations of Activity Zones as a Measure of Enzyme Activity

Isolate	pH 5		pH 7		pH 8		CMC	
	Mean (cm)	std dev	Mean (cm)	std dev	Mean (cm)	std dev	Avg (cm)	std dev
1-1	0.2	0.1	2.8	1.2	3.6	0.2	3.4	1.1
1 T	0.0	0.0	1.6	0.1	0.7	1.2	0.0	0.0
10 T	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
11 Pit	1.7	0.2	3.2	0.6	2.6	0.6	2.4	0.2
1-9	1.8	0.1	3.6	1.4	2.6	0.5	2.0	0.0
2T	0.0	0.0	0.0	0.0	0.1	0.0	1.2	0.2
3 surfB	1.8	0.2	1.9	0.4	0.2	0.2	2.7	0.3
3 T	3.1	0.7	0.2	0.3	1.8	1.5	1.7	0.3
3-2	1.2	0.3	1.4	0.4	1.4	0.3	2.1	0.4
4 T	0.0	0.0	1.0	1.0	0.0	0.0	2.3	0.2
4 TA	3.2	0.7	4.4	0.3	2.8	0.2	2.2	0.3
4 TB	3.4	0.4	2.7	0.6	0.2	0.3	2.6	1.6
5 surfA	2.3	0.2	0.0	0.0	0.0	0.0	3.4	0.2
5 surfB	0.0	0.0	2.7	2.4	0.0	0.0	3.5	0.5
5 surfC	0.0	0.0	3.7	0.7	0.5	0.9	2.8	0.3
5 surfD	0.0	0.0	1.8	1.6	0.0	0.0	2.3	1.3
5 T	0.0	0.0	2.4	0.5	2.1	0.4	2.1	0.4
5 TA	2.0	0.0	2.8	0.8	0.8	1.4	2.0	0.6
5 TB	2.2	0.4	2.8	1.0	2.9	0.3	2.2	0.9
6 surfA	1.7	0.3	2.3	1.0	0.4	0.2	3.3	0.8
6 surfB	0.7	1.2	1.9	0.4	0.4	0.2	2.3	0.3
6 surfC	4.0	0.0	0.0	0.0	0.4	0.5	2.1	0.5
6 T	0.5	0.5	0.0	0.0	0.9	0.6	0.0	0.0
7 T	4.0	0.0	0.0	0.0	0.2	0.1	2.7	0.3
7 Pit	4.2	0.3	1.6	1.4	0.2	0.1	1.6	0.8
8 T	0.0	0.0	2.5	0.5	0.0	0.0	1.0	0.8
B9.8	3.7	0.3	3.6	0.1	4.3	0.3	3.2	0.7
Hud1	4.0	0.0	2.6	1.2	1.1	0.9	1.7	0.2
NV1	2.3	0.6	2.7	0.9	3.3	0.4	1.8	1.8
NV2	3.0	1.0	1.2	1.0	3.0	0.6	2.8	1.0
NV3	1.0	1.2	2.8	1.3	3.8	0.3	0.2	0.1
NV4	3.8	0.3	1.4	0.1	3.8	0.3	0.3	0.0
PBd1	4.4	0.2	1.7	1.4	3.2	0.6	0.4	0.5
PitA1	2.8	1.6	3.3	0.6	1.6	0.2	0.4	0.4
PitV5	1.4	0.2	1.5	2.6	1.6	2.1	2.8	0.8
SupA1	0.1	0.2	1.5	0.5	3.2	0.7	0.3	0.1
SupA2	3.0	0.1	1.3	1.2	2.3	1.1	0.7	0.5
SupA3	0.0	0.0	2.8	0.3	2.6	2.3	0.4	0.4

Table 2.3 (cont'd.)

SupV3	1.8	0.0	2.6	0.5	0.9	0.4	0.6	0.4
SupV6	1.4	0.1	1.7	1.5	2.9	1.9	0.2	0.1
SupV7	0.0	0.0	0.0	0.0	1.5	2.0	0.8	0.7
TB-CD	0.0	0.0	0.5	0.5	3.5	1.7	0.2	0.3
TBd2	0.0	0.0	2.2	0.6	2.2	1.9	0.6	0.4
Tsup2	0.0	0.0	0.8	1.4	3.0	0.9	0.1	0.1
V2	0.0	0.0	1.7	0.1	2.5	1.3	3.1	1.8
V4	1.1	1.2	0.7	1.2	3.1	1.1	4.2	0.8
V5	1.0	1.7	1.3	1.3	2.3	2.1	3.3	1.6

Note. Zone of clearance (activity) was measured across the widest area of visible media degradation for each replicate.

Although fewer isolates produced zones of activity larger than 2 or 3 cm in pectin at pH 5 than at pH 7 or 8, or in CMC, this was the pH at which the highest percentage (11%) of zones larger than 4 cm were produced. The production of zones of activity larger than 4 cm was usually rare and was only seen in 2% of the isolates grown in CMC, or in pectin at pH 7 or 8 (Table 2.4).

Table 2.4 Percentage of Isolates Producing Zones of Clearance, by Media and Size

Isolate	**% > 2cm**	**% > 3cm**	**% > 4cm**
Pectin pH 5	36	26	11
Pectin pH 7	43	13	2
Pectin pH 8	47	21	2
CMC	51	17	2

Although 94% of all isolates tested produced at least a small zone of activity in CMC (the highest percentage for any media type) with an average of 1.75 cm, the top three disc-degrading isolates produced almost no enzymatic activity in CMC (Table 2.5).

Table 2.5 Cellulase Production by Top Three Disc Degrading Isolates

Isolate	Cellulase Activity	Turnip Disintegration Rating
TB-CD	0.17	4.0
PBd1	0.40	4.0
NV4	0.23	3.9
Mean*	1.75	1.9

Note. *Mean value of all isolates

Impact of Yeast Extract on Enzyme Activity for Select Isolates

Yeast extract also changed pectinase activity in many isolates. Although most *Streptomyces* spp. isolates were capable of growth on media limited to pectin, yeast extract may have provided an alternative source of carbon and so the tests were repeated on key isolates without yeast extract. The absence of yeast extract greatly diminished the ability of almost all the isolates to produce pectinase at pH 5. Although isolate 1-1 increased from 0.2 cm to 2.2 cm the high variability lessens confidence in this finding (Table 2.6). The ability of to produce pectinase at pH 5 was greatly diminished in PBd1 (from 4.4 cm to 0.5 cm) and NV4 (3.8 cm to 0.7; Table 2.6).

Isolates showed more ability to produce pectinase at pH 7 in media not supplemented with yeast extract. For example, five out of eight isolates showed increased enzymatic activity, with V4 showing an increase in average activity zone from 0.7 cm (yeast) to 4.2 cm (no yeast). V5 showed a similar result, going from a 1.3 cm activity zone with yeast extract to a 3.3 cm activity zone with no yeast extract (Table 2.6).

Although the activity zones were generally smaller at pH 8, most isolates were still capable of producing pectinase. The ability of isolate TB-CD to produce enzymes was the most reduced as shown by the decrease in activity zone from 3.5 cm to 0.2 cm (Table 2.6).

Table 2.6. Activity Zone Diameters for Key Isolates in Pectin Media Containing Yeast Extract vs. Pectin Media Without Yeast Extract

Isolate	Pectin Media											
	pH 5 Yeast		pH 5 NY		pH 7 Yeast		pH 7 NY		pH 8 Yeast		pH 8 NY	
	Avg	sd	Avg	sd	Avg	sd	Avg	sd	Avg	sd	Avg	sd
1-1*	0.2	0.1	2.2	2.0	2.8	1.2	1.8	0.2	3.6	0.2	2.4	0.7
B9.8	3.7	0.3	4.0	0.9	3.6	0.1	3.1	1.6	4.3	0.3	2.1	0.1
NV1	2.3	0.6	2.9	2.1	2.7	0.9	2.2	0.4	3.3	0.4	2.2	0.4
NV4	3.8	0.3	0.7	0.1	1.4	0.1	2.4	0.5	3.8	0.3	2.4	0.6
PBd1	4.4	0.2	0.5	0.6	1.7	1.4	2.7	1.6	3.2	0.6	3.2	0.6
TB-CD	0.0	0.0	0.0	0.0	0.5	0.5	1.7	2.4	3.5	1.7	0.2	0.3
V4	1.1	1.2	0.0	0.0	0.7	1.2	4.2	0.6	3.1	1.1	1.0	1.3

Note: No yeast extract present (NY). Average values are zones of clearance measured in cm. * Strain isolated from potato.

Isolate Growth in Liquid Culture

Cultures in simple pectin broth #1 were grown for 31 days with constant shaking. It was noted that NV1 appeared to have the most growth compared to NV4, PBd1 and V4. The isolates grown in media #1-3 appeared to grow well based on visual observations. In media #3 containing pectin, yeast, and various minerals, some isolates produced the grey and white growth indicative of sporulation that is seen when grown in oat bran agar. Some isolates formed globular balls (Figure 2.3). The media containing low quantities of yeast and high quantities of pectin (10 g pectin-media #4) produced bacterial colonies of yellow, ribbon-like filaments (Figure 2.3) with no globular balls visible. The isolates in media #5 containing CMC remained mostly clear with no visible balls, grey filaments, or other bacterial signs. These also did not yield bacterial pellets when centrifuged, unlike all the other media. It is possible the bacteria did not grow in the CMC broth at all.

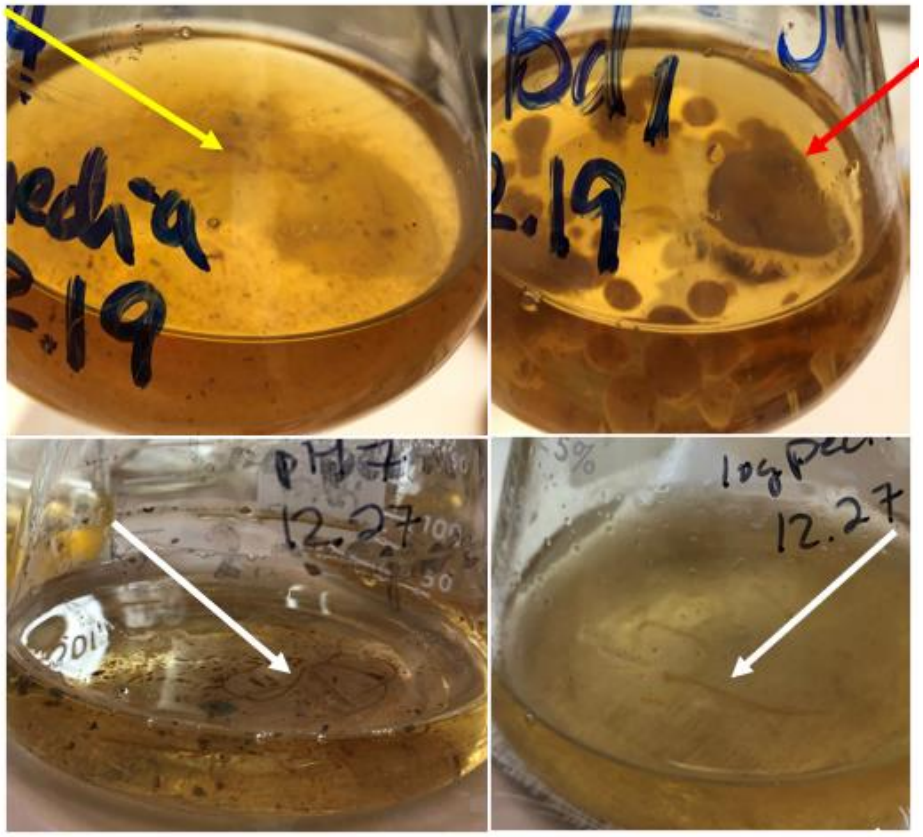

Figure 2.3 Growth Habits of *Streptomyces* Spp. Isolates in Pectin Media Shake Cultures

Note. Clockwise from top left: NV4 in Media #3, PBd1 in Media #3, PBd1 in Media #4, and NV4 in Media #4. Yellow arrow indicates regular, grey colonies typically seen in oat bran agar. Red arrow indicates globular growth in PBd1.White arrows indicate growth habit with yellow filaments.

Culture Filtrate Experiments

Total Protein Determination in Culture Filtrates

The Bradford protein assay was used to measure protein concentrations in CF samples prior to running agarose well and DNS reducing sugar assays. The sample CF used were from media #1 from isolates 1-1, 7T, 7 Pit, 11 Pit, B9.8, and a 10% pectinase standard (PS). The results indicated that the culture filtrates had low levels of protein, between 0.11 and 0.25 mg/mL (Table 2.7).

Table 2.7 Protein Concentration of CF Samples from Pectin Media #1 As Measured by Bradford Assay

Sample	10% PS	1-1	7T	7Pit	11Pit	B9.8
Protein mg/mL	>0.90	0.19	0.19	0.22	0.17	0.21

Agarose Well Assay for the Determination of Pectinase

A radial diffusion assay in agarose was attempted to quantify enzymatic activity in the culture filtrates. After many attempts in various media, CF samples in agarose #1 containing tris buffer at 6.6 with 0.5g pectin showed zones of dissolved media in from CFs of isolates 1-1 (turnip disc CF), and PBd1 and V4 (pectin shake CF). Although further attempts were made with culture filtrates from the same experiments, this result was not replicated (Figure 2.4). It is possible that the bacterial colonies had remained too long in the incubation period with the available nutrients and reached starvation. A shorter incubation time of 7–14 days is recommended for future attempts.

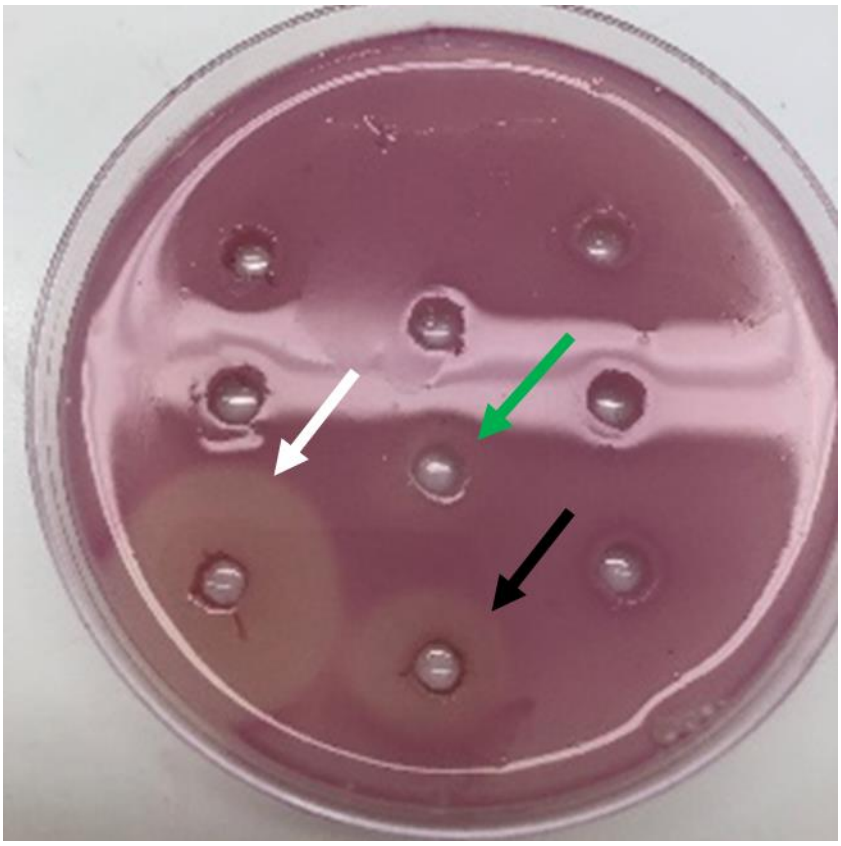

Figure 2.4 Agarose Well Assay Showing Pectolytic Degradation from Culture Filtrates

Note. Black arrow indicates 1.6 cm ring of pectolytic activity from CF of V4 isolate in pectin shake culture media #1 and green arrow indicates small ring from PBd1 CF from the same media. White arrow indicates 2.5 cm ring from CF of isolate 1-1 (turnip disc).

Measurement of Total Pectinase Activity Using DNS

Culture filtrate (CF) fractions B9.8 and 5TB from media #2 (PGA) and 5TB from media #1 (pectin) formed precipitates when heated while obtaining Value 2. An attempt was made to remove the precipitant through mechanical filtration (cheesecloth) and centrifugation, but the results may have been compromised by the formation of these precipitants. Enzymatic activity as measured by reducing sugars is listed below (Table 2.8).

Table 2.8 Reducing Sugar Released as a Measure of Pectinase Activity

Isolate	Media Type	Estimated mg/mL Sugar
5TB	1	0.0450
5TB	2	0.0525
5TB	4	0.0150
B9.8	2	0.0475
NV4	1	0.0200
PBd1	3	0.0400
V4	3	0.0200

Note. Media no. 1 contains 5 g pectin, 500 mL MSS. Media no. 2 contains 3 g yeast, 5 g PGA, 2 g K_2HPO_4, 1 g $MgSO_4$*7H20, and 2 g $(NH_4)_2SO_4$ at pH 5.0 (Jacob et al., 2008). Media no. 3 contains 3 g yeast, 5 g pectin, 2 g K_2HPO_4, 1 g $MgSO_4$*7H20, and 2 g $(NH_4)_2SO_4$ at pH 7.0 (Jacob et al., 2008). Media no. 4 contains 0.5 g yeast, 10 g pectin, 1 g K_2HPO_4, 0.88 g $MgSO_4$*7H20, and 1 g $NaNo_3$, and 1 g KCl at pH 7.0 (Mohandas)

Turnip Tissue Disintegration Assays

Table 2.9 Turnip Tissue Disintegration Rating 7 Days Post Inoculation

Isolate	Rating (0-4)*	Std. dev.
PBd1	4	0.0
TB-CD	4	0.0
NV4	4	0.2
V2	3	1.1
V5	3	1.0
1-1	3	1.3
NV2	3	1.1
6 surfC	3	0.8
B9.8	3	0.8
6 surfA	3	1.2
V4	3	0.9
7 T	2	0.8
1 T	2	1.2
Hud1	2	1.4
NV3	2	0.6
1-9	2	0.9
5 SD	2	0.9
PitA1	2	1.6
SupA1	2	0.5
TBd2	2	0.9
3-2	2	0.7
SupA3	2	0.5
5 SC	1	1.0

Table 2.9 (cont'd.)

5 SB	1	1.1
10 T	1	0.3
4TA	1	0.5
2 T	1	0.6
4 T	1	0.3
5TB	1	0.6
7 pit	1	0.6
4TB	1	0.4
5 TB	0	0.7
5 TA	0	0.3
NV1	0	0.3
Avg	2	
sd	1	
≥ 1 sd	3.0	

Note. *Rating scale as follows: 0 is no browning, 1 is browning with no disintegration, 2 is browning with some disintegration, 3 is browning with consistent disintegration, and 4 is necrotic tissue with significant disintegration.

Table 2.10 Percent Isolates per Disease Rating Category

Rating category	**Descriptions**	**Percent**
0	No browning	9
1	Browning with no disintegration	26
2	Browning with some disintegration	32
3	Browning with consistent disintegration	24
4	Necrotic tissue with significant disintegration	9

Most of the 34 isolates applied to turnip tissue discs had the ability to cause browning with no disintegration with 65% of the Streptomyces spp. isolates showing a rating of 2 or higher, indicating at or above browning with some disintegration (Table 2.10). Those rated ≥ 3.0, or +1sd, showed browning with consistent disintegration, and three isolates with a rating of 4 showed necrotic tissue with significant disintegration.

A closeup of turnip tissue discs representing each rating category is seen in Figure 2.5. In Figure 2.6, the disintegration caused by three of the lowest overall enzyme producers compared to control is seen. Statistical analyses of the disintegration ratings of key isolates indicated significantly greater disintegration from the control in the ratings of isolates 1-1, B9.8, NV4, PBd1, TB-CD and V4 (Figure 2.7). The isolate NV1 was not different from control.

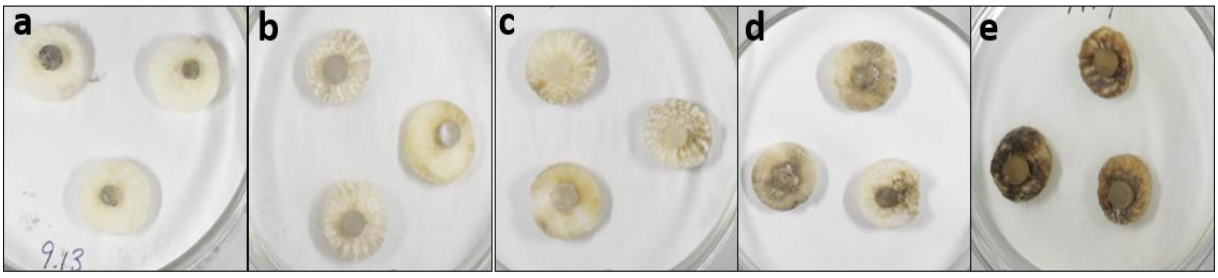

Figure 2.5 Turnip Disintegration Results by Rating Category

Note. A closeup of one of three replicates per isolate is shown with tissue disintegration rates in parentheses. a. NV1 (0) b. 4TB (1) c. 1-9 (2) d. B9.8 (3) e. NV4 (4)

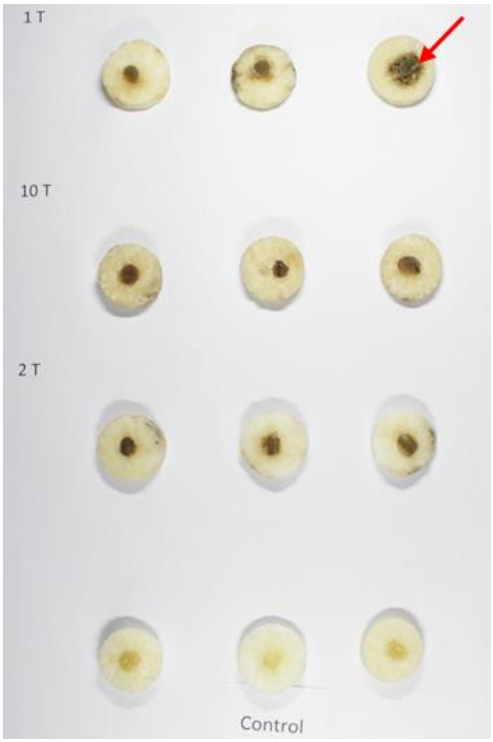

Figure 2.6 Turnip Tissue Discs–Low Relative Activity

Note. Red arrow indicates turnip disc disintegration by isolate I T.

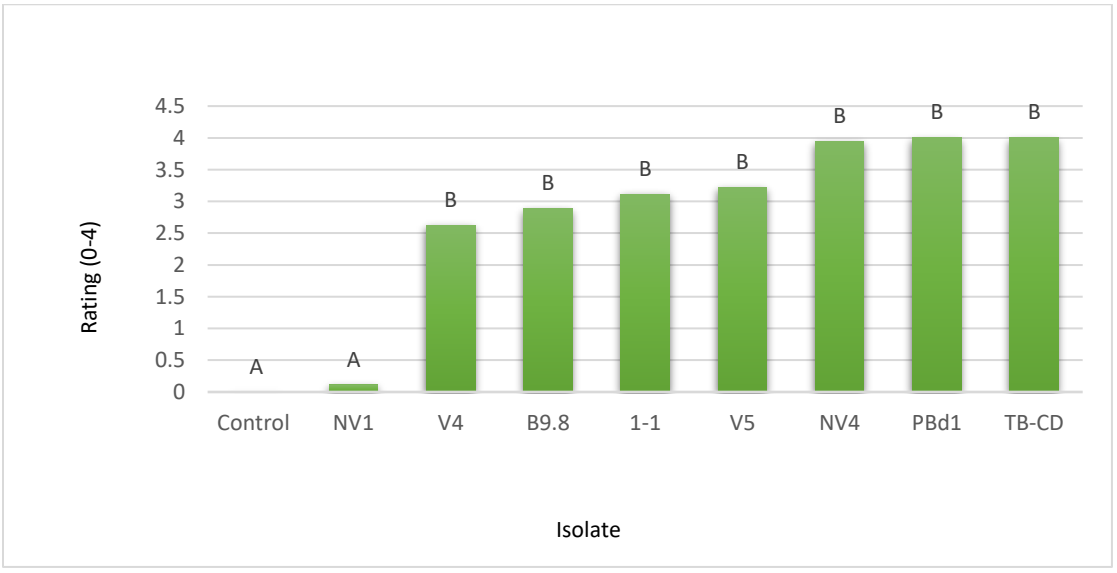

Figure 2.7 Turnip Disintegration Rating of Key Isolates

Note. Different letters indicate statistically significant differences among the isolates ($p < .05$).

Potato Tissue Disintegration

Isolates 1-1 (from potato), B9.8, NV4, PBd1, TB-CD, and V5 were selected for study on potato to see if differences in turnip variety susceptibility as indicated by seedlings assays in this study (Chapter 2) carried over into differences on tissue discs with potato as host. With the exception of isolate B9.8, which showed no difference in virulence between potato and turnip, there was a significant difference among all isolates tested. The most striking result is that PBd1, rated 4 on turnip tissue discs, was completely non-pathogenic on potato tissue discs (Figure 2.8).

Isolates NV4, TB-CD and V5 had some ability to degrade potato but they were not as virulent on this host as turnip. The strain isolated from potato, 1-1, was the only isolate that was more virulent on potato than turnip.

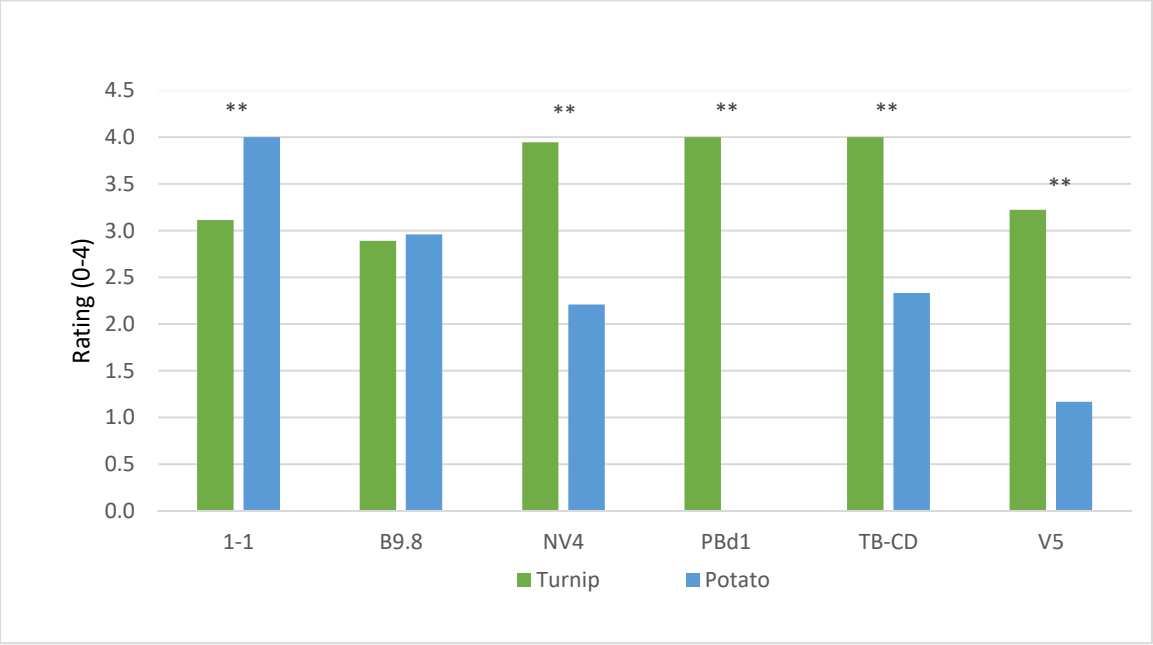

Figure 2.8 Disc Disintegration Ratings on Turnip vs. Potato

Note. A symbol ** indicates statistically significant differences between turnip and potato in the same isolate ($p < .05$).

Toxin Disintegration Assay

The discs treated with pectinase and cellulase standard solutions showed moderate tissue discoloration, but thaxtomin application did not result in tissue discoloration or disintegration (Table 2.11).

Table 2.11 Turnip Tissue Disintegration Rating 7 Days Post Treatment

Treatment	**Rating (0-4)***	**Std. dev.**
0.10 % PS	1	0.8
0.01% PS	2	0.6
0.10 % CMC	1	0.5
150 µg/mL thaxtomin	0	0.4

Note. *Rating scale as follows: 0-No browning or disintegration compared to control; 1, Tissue discoloration at edges with no disintegration; 2, Uniform discoloration with some disintegration; 3, Brown tissue with moderate, consistent disintegration; 4, Brown or black tissue with significant disintegration

Thin Layer Chromatography (TLC) of Culture Filtrates from Shake Culture and Tissue Disintegration Assays

Several bands were visualized for most CF when examined under visible and UV light (365 nm). The separated components of the CF were visible in the formation of bands, especially thaxtomin which appears yellow under visible light. The band colors ranged from blue, to white/blue, to purple, to yellow (Table 2.12).

Table 2.12 Fluorescing Bands Separated by TLC and Visible at 254 nm. Rf Values Were Calculated

Culture Filtrate	Rf Value							
	0.16*	0.39*	0.44	0.51	0.61	0.70*	0.86	0.97
1-1 (Oat Bran Shake)	X							
5TB (Oat Bran Shake)	X						X	
B9.8 (Oat Bran Shake)	X							
NV1 (Oat Bran Shake)	X	X		X		X	X	
NV1 (Turnip)					X	X	X	X
PBd1 (Turnip)	X	(0.32)	X		X	X	X	X
1-1 (Potato)	(0.19) X		X (Y)			X (0.75)	X	X
1-1 (Rutabaga)								X
1-1 (Beet)	X		X (Y)					X

Note. *Indicates some variation in exact Rf value among CF. Exact values, where different, in parenthesis. (Y) indicates yellow band. Yellow band is likely thaxtomin. Thaxtomin A and B, and their derivatives, have been previously identified by Rf values 0.27, 0.29, 0.35, 0.41, 0.45 (King et al., 1992).

DISCUSSION

This research to uncover the role of pectolytic and/or cellulolytic enzymes in *Streptomyces* pathogenesis concluded with several findings. Most *Streptomyces* spp. isolates are capable of making some pectolytic and/or cellulolytic enzymes and some isolates are far more robust producers of pectolytic and/or cellulolytic enzymes than others. There is a lot of variation, by *Streptomyces* spp. isolate, as to which pectolytic and/or cellulolytic enzymes is produced. Some non-pathogenic *Streptomyces* spp. isolates are highly robust producers of pectolytic and/or cellulolytic enzymes. There was no single kind of pectolytic and/or cellulolytic enzymes required for pathogenesis, but the most pathogenic strains are robust producers of at least one kind of pectolytic and/or cellulolytic enzymes. There is host specificity in some pathogenic *Streptomyces* spp. isolates.

In general, there was a high degree of variation in enzyme production by *Streptomyces* spp. isolate, and within each isolate by media type. For example, isolate V4 produced an enzyme ring of 4.2 cm in CMC but just 1.1 cm in pectin media at pH 5, and 0.7 cm in pectin media at pH 8 (both with yeast extract). These variations are likely due to the complex regulated carbon catabolism of *Streptomyces* that parse nutrient sources and initiate metabolic pathways not just on the type of nutrient but also the sugar transporter and molecular form in which it arrives through the cell membrane.

There were trends in which higher enzymatic activity correlated with necrosis in turnip tissue discs. Producers of pectinase at pH 8 showed a tendency to be correlated with virulence indicated by the result that five of the isolates that caused the most symptoms in turnip tissue discs produced their highest pectinase activity at pH 8. Three of the isolates that caused the

most symptoms in turnip tissue discs produced their highest pectinase activity at pH 5 and two in cellulase. None of the isolates that caused the most symptoms in turnip tissue discs produced their highest pectinase activity at pH 7. Seventy-one percent of the isolates that were rated 3.0 or higher (+1 standard deviation) in turnip tissue-degrading tendency produced zones of activity in pectin media at pH 8 that were ≥ 1 standard deviation. Only forty-three percent of the isolates that were rated 3.0 or higher (+1 standard deviation) in turnip tissue-degrading tendency produced activity zones ≥ 1 standard deviation at pH 5 or in cellulose. None of the isolates that were rated 3.0 or higher (+1 standard deviation) in turnip tissue-degrading tendency produced activity zones ≥ 1 standard deviation in pectin at pH 7.

Table 2.13 Summary of Top Ten Tissue Disc Macerating Isolates with Enzyme Zone of Clearance Values

Isolate	Zone of Clearance (cm)				Turnip Tissue Disc Rating 0-4 (4 most degraded)
	Pectin pH 5	Pectin pH 7	Pectin pH 8	Cellulose	
TB-CD	0.0	0.5	3.5 [a]	0.2	4.0
PBd1	4.4	1.7	3.2	0.4	4.0
NV4	3.8	1.4	3.8 [a]	0.3	3.9
V2	0.0	1.7	2.5	3.1	3.2
V5	1.0	1.3	2.3	3.3	3.2
1-1*	0.2	2.8	3.6 [a]	3.4	3.1
NV2	3.0	1.2	3.0 [a]	2.8	3.1
B9.8	3.7	3.6	4.3 [a]	3.2	2.9
6 surfC	4.0	0.0	0.4	2.1	2.9
6 surfA	1.7	2.3	0.4	3.3	2.6

Note. *Isolated from potato. All pectin media contained yeast extract. [a] Highest enzyme activity for this isolate was in pectin at pH 8. Turnip tissue disc rating score: 0 is no browning, 1 is browning with no disintegration, 2 is browning with some disintegration, 3 is browning with consistent disintegration, and 4 is necrotic tissue with significant disintegration.

The ability of an isolate to cause tissue disintegration on turnip discs is not solely due to the production of CWDE since several isolates that produced higher levels of CWDE *in vitro* were not able to cause disease in turnip. For example, NV1, a strong cellulase and pectinase producer, caused no disintegration on turnip tissue in comparison to isolate 1T that did not show evidence of much pectinase or cellulase production, yet still caused some disintegration on turnip tissue discs.

Toxins play an important role in pathogenicity and it would be interesting to repeat these enzyme assays only on those isolates known to possess the pathogenicity island and see if CWDE production is correlated with increased virulence, and if so, if virulence is correlated with a particular substrate.

Types of Enzymatic Activity Zones

It was observed that in addition to more typical zones of activity observed around bacterial growth on pectin agar, some other patterns of apparent degradation were observed. For example, some of the *Streptomyces* spp. isolates were capable of hydrolyzing agar, which is usually typically quite resistant to microbial degradation. While it is not particularly surprising for *Streptomyces* spp. to possess these enzymatic capabilities, it was interesting that this ability did not seem tied to higher overall pectinase or cellulase production. In fact, some zones of activity produced by the *Streptomyces* spp. that degraded agar as a nutritional source were smaller than average. It was a rare occurrence to see the media completely degraded to the bottom of the plastic Petri dish, but it did occur with some isolates. This tendency seemed to be associated neither with pathogenicity nor higher overall enzyme-producing capacity.

It may be important that the largest areas of degradation appeared far more frequently in pectin media at pH 5. Two possibilities are that either polygalacturonases were more diffusible in the pectin media or they were produced in greater quantities in some isolates. Diffusion of enzymes in plant tissue could be an important characteristic of plant cell wall penetration considering that pectin plays an important role in regulating the porosity of the plant cell wall, including the diffusion of enzymes that regulate growth and development. Enzymes that are more readily diffused may enable pathogens to inflict more damage on the plant cell wall than enzymes that are produced at a higher rate but are not able to spread to their target through the pectin layer, or middle lamella of more developed plant cell walls.

Effect of Yeast Extract on Enzyme Production

Yeast extract is often added as a nutrient for the degradation of pectin in commercial industries like fruit juice production, so it is not surprising that yeast extract would enhance pectin degradation. What is surprising is that enzyme production declined in the presence of yeast extract in isolates NV4, PBd1, TB-CD, V4, and V5 at pH 7. Yeast could be triggering a catabolite repressor in these isolates.

There was a high degree of variability in 1-1 with no yeast extract present that makes the results difficult to interpret. This isolate in pectin media at pH 5 with no yeast extract consistently demonstrated a wide range of variability in producing zones of clearing, which may indicate that yeast components are more susceptible to carbon catabolite regulation in this strain (known to be *Streptomyces scabies*) than to other strains.

Cellulase Production and Virulence

The production of cellulase by pathogenic isolates might also merit further study since cellulase was barely produced in the three most virulent isolates on turnip tissue discs. This was a surprising result when considering that overall, cellulase was the most consistently produced enzyme of the four types tested.

One of the low cellulase-producing isolates was PBd1, which was also unique as it was pathogenic on turnip but not potato. This might indicate that while cellulase production is not a strong predictor of pathogenicity in turnip, it may play a factor in pathogenicity against other hosts.

Growth in Various Media

Glucose

The lack of sporulation in glucose media is not surprising since it is well known that *Streptomyces* have repressors to block reproductive development such as aerial hyphae when glucose is available. This result demonstrates how effective and complete the carbon catabolite repression mechanisms that block development can be for this organism.

Disc Disintegration and Host Specificity

The three top-rated tissue disc degraders, PBd1, TB-CD, and NV4, were all originally isolated from turnip. Although the potato isolate, 1-1, was capable of degrading turnip tissue it was not as virulent as some of the turnip-isolated strains on turnip. Isolate 1-1 was significantly more virulent on potato discs than on turnip, indicating host specificity.

When these isolates were applied to potato discs, PBd1, the most virulent isolate on turnip tissue discs, had no ability to degrade potato. This may imply that some *Streptomyces* spp. isolates have developed adaptions uniquely suited to pathogenicity on turnip and may be worth further study.

One of these adaptations may be an ability to detoxify glucosinolates commonly produced by *Brassica*s like turnip. These compounds break down to isothiocyanates (Gouws-Meyer et al., 2018) which have been indicated to possess biostatic effects on *Streptomyces*. Isolates that have developed a capability to resist these compounds may be interesting for future study.

Although there was not a clear-cut and statistically significant indicator for CWDE production and virulence, a correlation seems likely based upon how many of the highest CWDE producers were also capable of causing disease on turnip. This result underscores the

importance to pathogenic *Streptomyces* spp. of possessing factors other than CWDE. Some of these factors, like toxins, have been explored further in Chapter 2 of this study.

The extent of host specificity by this pathogen has not been widely explored, and most literature points to a lack of host specificity by the toxin thaxtomin to conclude that host specificity is not likely. This conclusion may underestimate the importance of other factors in disease establishment by the pathogen. The results herein indicate that further study may be indicated.

CHAPTER 3. RESPONSE OF TURNIP SEEDLINGS TO INOCULATION WITH *STREPTOMYCES* AND TREATMENT WITH PHYTOTOXINS

Seedling pathogenicity assays are often performed as a quick and effective way to understand *in vivo* the dynamic interaction between plants and pathogens, and how these interactions affect long-term growth and survival (Maskell et al., 1999). Seedling inoculations have several advantages over soil-grown plant inoculation assays such as decreased growth and incubation periods, reduced greenhouse or growth chamber requirements, and ease of inoculation and handling (Ishiga et al., 2011). The pathosystem *Arabidopsis thaliana*-*Pseudomonas syringae* has been studied as a model of microbial pathogenesis and plant innate immunity, and the strain which causes bacterial speck of tomato *Pst* DC3000, has been used as a model for studying the molecular basis of plant-pathogen interactions. A more rapid and reliable assay for this model was delivered by switching from soil-grown plants to 2-week-old seedlings for easier inoculation with a bacterial suspension (Ishiga et al., 2011).

In a study by Maskell et al. (1999), the effects of turnip mosaic potyvirus (TuMV) and turnip yellow mosaic tymovirus (TYMV) in *Brassica oleracea*, wild cabbage, were investigated through the inoculation of seedlings germinated from five populations of wild cabbage. Seedlings at the three to five leaf stage were inoculated with TuMV, TYMV or water and the ability of the virus to cause reduced survival, reproduction and growth was measured (Maskell et al., 1999).

For *Streptomyces* bacteria, seedling inoculations provide a realistic simulation of pathogen entry through the immature tissues of host plants. In a 1949 study by Hooker, the growth responses of beet, corn, cucumber, pea, radish, and soybean seedlings grown in agar

inoculated with *Streptomyces* were evaluated. The root weights of soybean and pea seedlings inoculated with *S. scabies* were less than half of those grown in either water controls or with nonpathogenic *Streptomyces* cultures. Both Purple Top White Globe (PTWG) turnip and Early Scarlet Globe radish seedlings were susceptible to *S. scabies* (Hooker, 1949). This seedling study indicated that reduced root growth, severe impairment of lateral root development, and premature death could be predicted from the early effects of *Streptomyces* inoculation (Hooker, 1949).

Phytotoxins play a role in virulence and/or pathogenicity in several plant-pathogens systems. Thaxtomin has been used to determine relative resistance or susceptibility of potato to scab. However, the results of these studies suggest that screening with thaxtomin may not correlated 100% with resistance to the pathogen (Kaiser et al., 2020; Tegg et al., 2010). Part of this may be that pathogenic *Streptomyces* produces other toxins, including a coronafacic acid derivative that has properties similar to the well-studied toxin coronatine (Bignell, 2014). Thus, it is likely that any effective toxin screen for resistance may need to use more than one toxin.

Objectives

The purpose this study was to determine: 1) Disease reactions on the root, hypocotyl, and cotyledons of various turnip variety seedlings resulting from inoculations with selected *Streptomyces* isolates, and 2) The effect of the phytotoxins thaxtomin and coronatine on turnip and one radish cultivar. The overall objective was to determine if either pathogen inoculations and/or toxin application could be used to determine the relative resistance or susceptibility of turnip to pathogenic *Streptomyce*s.

MATERIALS AND METHODS

Seedling Inoculation Studies

Purple Top White Globe (PTWG) turnip seeds were surface sterilized by washing for 5 minutes in a 70% ethanol solution, rinsed with sterile water, and then washed for 10 minutes in a 10% chlorine bleach solution (0.525% hypochlorite). Seeds were rinsed in sterile water and allowed to dry completely. Glass Petri dishes containing a disc of #1 Whatman filter paper were autoclave sterilized, moistened with sterile water, and surface-sterilized seeds were germinated in the dark. Nine Petri dishes with water agar had a line drawn across the middle of each plate. Five uniformly germinated seedling were place above that line and then *Streptomyces* isolates were streaked on the section of the agar media directly below the line. Each isolate was replicated two times. As a control, turnip seedlings were placed onto non-inoculated water agar media plates. The Petri dishes containing seedlings were placed upright to encourage roots to grow through the pathogen. The seedlings were grown under fluorescent lights with 12-hour photoperiod for 7 days. After 7 days, the hypocotyl length of each seedling from root juncture to just below the cotyledon juncture was measured. To compare the effects across different seedling varieties, each seedling hypocotyl length (HL) was divided by the average HL of the control for that variety. This normalized the observations since different varieties of seedlings naturally produce hypocotyls of different lengths. The *Streptomyces* isolates used in this study were 1-1, B9.8, NV1, NV4, PBd1, TB-CD, and V4. These were provided by the lab of Dr. Raymond Hammerschmidt from Michigan-grown turnips (except for 1-1 which was isolated from potato) showing symptoms of scab.

After HL measurement, qualitative ratings of necrosis, stunting and other visible signs of stress were made. The purpose of the qualitative ratings was to determine the symptoms correlated to a treatment effect on hypocotyl length. The qualitative observations were also used to evaluate the effect of the application of the toxins thaxtomin and coronatine. Qualitative observations included an evaluation of the following characteristics: necrosis (darkened or black tissue on any part of hypocotyl or root), stunting (how many hypocotyls were affected), hypertrophy (thickening), curling, and a root rating. The roots of each seedling were rated for the following symptoms:

- Thinning- decrease in root thickness, roots are weak and break
- Missing laterals-roots lack side branching and consist of one, singular root
- Stunting-roots were shorter than controls
- Necrotic or absent roots-some roots failed to grow, or a small root formed but was necrotic.

The root rating consisted of counting how many symptoms were visible and assigning a higher number with each additional symptom present. The higher the score from 0 (no effect) to 4 (the most effect), the more detrimental the pathogen/toxin was to the seedling (Table 3.1).

Table 3.1 Root Rating Applied to Seedlings

Symptom	**Symptoms Present**				
Thinning		x	x	x	x
Missing laterals			x	x	x
Stunted				x	x
Necrotic/absent					x
Root rating	**0**	**1**	**2**	**3**	**4**

Note. The presence of none of the symptoms resulted in a root rating of 0. One symptom resulted in a 1, and so forth. Roots showed fairly predictable progression of symptoms with thinning usually occurring first, followed by missing laterals, etc. so the table is designed to show the progression from 0, no symptoms, to 4, where necrosis or missing roots typically showed up in only the most affected seedlings.

After completing this experiment on PTWG, the other turnip and radish cultivars were tested using the same methods. The turnip cultivars used were Golden Globe (GGT), Japanese Baby (JBT), and Purple Prince (PPT) (Table 3.2). The radish cultivar used was Early Scarlet Globe (ESGR).

Table 3.2 Seedling Cultivars Used in the Study

Cultivar Name	Abbrev.	Attributes
Golden Globe	GGT	Golden skin, firm, crisp flesh, tasty tops, 55 days to harvest*
Japanese Baby	JBT	Pearly-white flesh, mild flavor, tasty tops, quick growing**
Purple Prince	PPT	Crisp, tasty white flesh, delicious tops, fast maturing***
Purple Top White Globe	PTWG	Pink to purple skin, white flesh, sweet, tender flavor *
Early Scarlet Globe (radish)	ESGR	Popular variety, bright red, crisp, tender, juicy flesh*

Note. *Burpee Seeds (www.burpee.com). **Renee's Garden Seeds (www.reneesgarden.com). ***West Coast Seeds (www.westcoastseeds.com).

This study was performed the same as the experiment on PTWG except the media was water agar instead of OBA. Seed of some cultivars required more than 1 day to germinate prior to placement in sterile Petri dish with inoculum, and some seed benefitted from 12 hours of light for germination.

The isolates used in the study (Table 3.3) were selected for their ability to cause disintegration on turnip discs (Chapter 1) and for their ability to produce cell wall degrading enzymes. The NV1 isolate was selected because it produces high levels of cell wall degrading enzyme activity but does not appear to be pathogenic to either turnip, beet, or radish seedlings. 1-1 was isolated from potato and has been confirmed as *Streptomyces scabies* by PCR amplification.

Table 3.3 *Streptomyces* Pathogenicity and Host Source

Isolate	Source	Pathogenic
1-1*	Potato	Yes
B9.8	Turnip	Yes
NV1	Turnip	No
NV4	Turnip	Yes
PBd1	Turnip	Yes
TB-CD	Turnip	Yes
V4	Turnip	Yes

Toxin Study

Seed was washed and germinated in the same manner and then placed in a row across the middle of the Petri dish. The phytotoxins thaxtomin A and coronatine were applied at the concentrations in Table 3.4 to pre-autoclaved, 4 x 0.5 cm strips of #1 Whatman filter paper. The toxin strips were air dried in a laminar flow hood before being placed into the Petri dish immediately below the seeds, covering the roots if present.

Table 3.4 Toxin Treatment Per Filter Paper Strip (nmoles)

Thaxtomin A	Coronatine	Toxins Combined	
		Thaxtomin A	Coronatine
0.18	0.25	1.83*	2.50*
1.83	2.50	3.65*	5.01*
9.13	12.52	9.13	12.52
18.26	25.05	18.26	25.05
91.32	125.23	91.32	125.23
182.65	250.47	**	**

Note. *Combined treatment amount differed from single treatment. ** Toxin level too high to permit growth

The Petri dishes containing seedlings were placed upright to encourage roots to grow through the toxin(s), under fluorescent lights with 12-hour photoperiod for 7 days. After 7 days, the effect of toxins applied to the cultivars was first determined by comparing hypocotyl lengths (HL) of treated seedlings. The hypocotyl length of each seedling from root juncture to just below the cotyledon juncture was measured, and examined for integrity, discoloration, necrosis, and other signs of stress. Following measurement of HL, qualitative observations of each seedling were made that included a rating of root health.

Turnip Seedling Histopathology

Tissue from the hypocotyl and root was cut from each of three PTWG seedlings grown in the presence of the following treatments: control, 50 ng thaxtomin, 50 ng coronatine and 50 ng of each toxin. Using a sterile razor, a 4-cm section was cut that included 2 cm of the hypocotyl and 2 cm of radical root with the hypocotyl/radical junction in the center. Tissue sections were immediately placed in formalin–acetic alcohol (FAA; formaldehyde: acetic acid: 70% ethanol, 5:5:9 v/v/v) fixative. After 48 hours, the FAA was decanted, and the samples were vacuum infiltrated with paraffin (melting point: 54-55°C) on the tissue processor (Sakura VIP 2000) and embedded in paraffin with an embedder (Thermo Scientific™ HistoCentre™ III). Paraffin-

embedded samples were cut into sections (6-µm thick) using a rotary microtome (model RM2245, Leica Biosystems) according to the methods of Devkota and Hammerschmidt (2019), with the embedding and sectioning done by the Michigan State University histology lab (Biomedical Physical Sciences, 567 Wilson Rd. Rm 2133).

Three sections were prepared from each tissue segment and were mounted on positively charged glass slides, processed through ethanol: xylene series, and stained with 0.1% T-blue by the histology lab. The toxin treatment effects and host responses in the sections were visualized using a light microscope at 10X magnification (DM2500, Leica).

Statistical Analyses

Hypocotyl length was normalized as a percent of the control to enable comparisons across a variety of seedling cultivars. Normality assumption was tested by using normal probability plots, and the dataset satisfied the assumption. Homogeneity of variance assumption was checked using manual Levene's test. Since the result indicated heterogeneous variance, possible models were compared based on the Akaike's Information Criteria and Bayesian Information Criteria to select the final model. Statistical model for hypocotyl length consisted of fixed effects of variety, treatment (isolate), and their interaction. Petri dish nested within the treatment and variety was included as a random factor. Heterogeneous variance by treatments was selected as a final model. Differences among isolates were presented separately by variety. When the effect of isolate was significant ($p < .05$), the mean separation was performed by using Fisher's Least Square Difference. The analysis was performed by using PROC MIXED procedure in SAS 9.4 (SAS Institute, USA).

RESULTS

Disease severity in all radish or turnip cultivars inoculated with *Streptomyces* spp. isolates was first determined by comparing hypocotyl lengths (HL) of inoculated seedlings. Following measurement of hypocotyl length, qualitative observations were made that included a rating of root health. Root health, based on Table 3.1, seemed to be the most important qualitative factor in predicting detrimental effect of pathogen inoculation.

Quantitative and Qualitative Results of Pathogen Inoculation of Purple Top White Globe (PTWG)

Quantitative Effects on PTWG

All of the isolates except NV1 showed a significant treatment effect in hypocotyl length from the control on PTWG (Figure 3.1). Pairwise comparisons indicated a significant degree of separation between the isolates PBd1, TB-CD, and V4 compared to the isolates B9.8 and NV4 (Figure 3.1).

As a percentage of the control, in seedlings inoculated with NV4, hypocotyl length was reduced to just 35% of the control and was statistically similar to isolates 1-1 (42%) and B9.8 (39%; Figure 3.1).

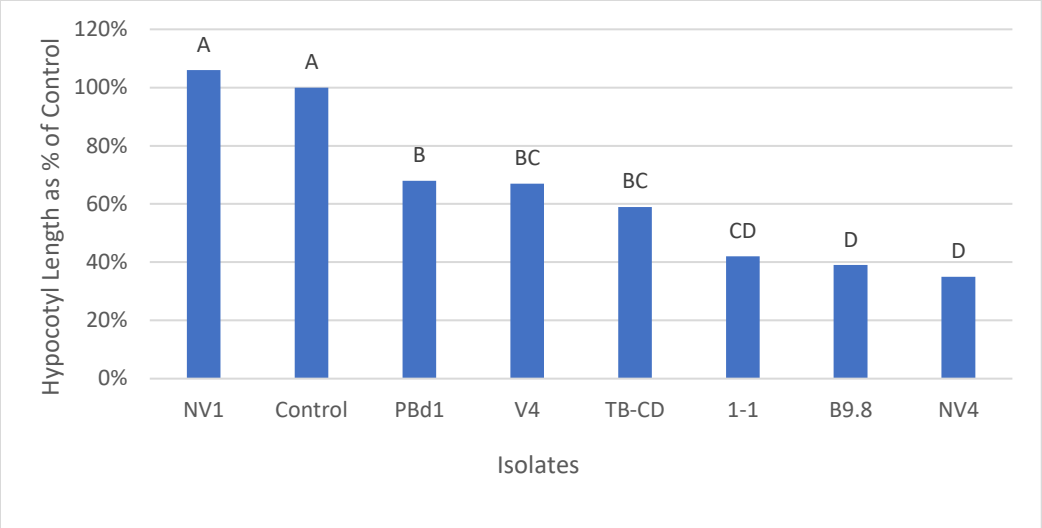

Figure 3.1 Purple Top White Globe Hypocotyl Length as % of Control for All Isolates
Note. Different letters indicate significant differences among isolates at .05 significance level.

Qualitative Effects PTWG

PTWG was susceptible to necrosis and stunting in 1-1 and some hypertrophy was seen (Table 3.5). Hypertrophy, or a thickening in the hypocotyl, was most often seen in the thaxtomin treatments. Hypertrophy was less common with the turnip pathogen inoculations.

In addition to stunted growth, the seedlings grown in B9.8 disintegrated readily, had stunted roots with no lateral roots, some black hypocotyls, small cotyledons, and browning (Table 3.5). B9.8 inoculation resulted in the shortest hypocotyls but did not present as many visible symptoms as 1-1. In Figure 3.2 the effect of pathogen inoculation between the control and NV4 (NV4, B9.8, and 1-1 were statistically similar) is shown. The healthy roots of the control contain many lateral roots with root hairs, and all are white and of normal size. NV4 stunted the hypocotyls and roots of the seedlings and caused some hypocotyls to curl. The roots were thinned, broken and disintegrating, and some roots have not formed.

The characteristics evaluated in the qualitative rating of inoculation with PBd1, the least detrimental pathogen to PTWG, can be seen in Figure 3.3. There is a difference in hypocotyl length, root thinning, lack of lateral roots, and root stunting. The control had hypocotyls exceeding 5 cm in length and the roots had lateral roots emerging with smaller root hairs. This contrasts with the poor growth of the seedlings in PBd1, where the tallest hypocotyl reaches about 3 cm and the roots extend through one, often truncated, main shoot. PBd1 was statistically similar to TB-CD and V4 (Figure 3.1).

Table 3.5 Qualitative Symptoms on PTWG Caused by Streptomyces Spp. Isolates

Symptom	Isolate				
	1-1	B9.8	NV4	PBd1	V4
necrosis	4	1	2	0	1
stunting	8	5	9	2	5
hypertrophy	4	2	0	0	2
curling	1	3	3	1	2
root rate*	3.0	3.0	2.7	2.0	1.6

Note. *Refer to root rating characteristics in Table 3.1

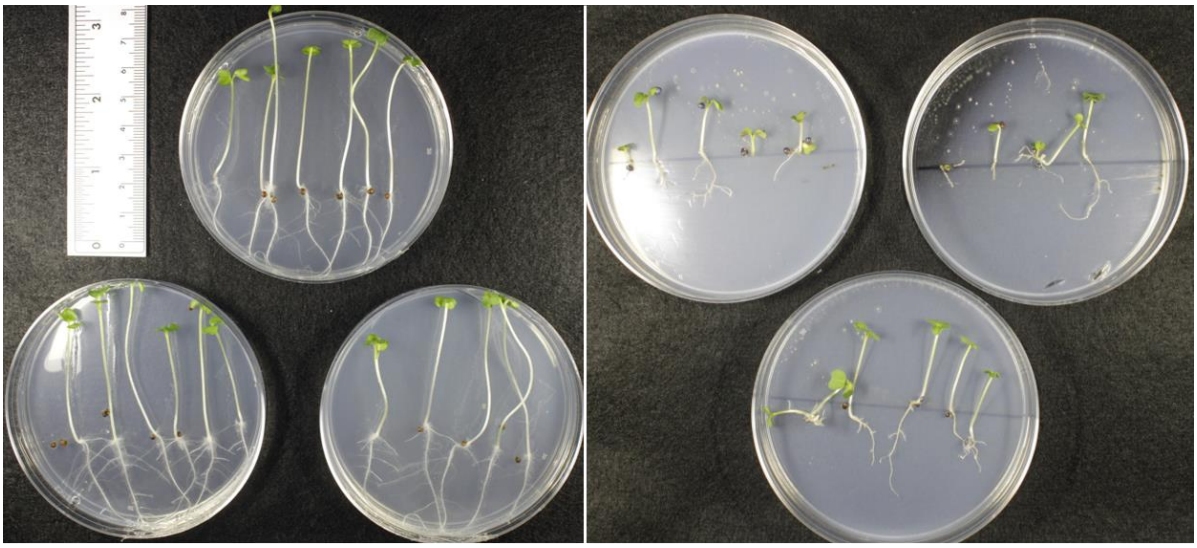

Figure 3.2 PTWG Control vs. NV4 Isolate
Note. Control on left and NV4 on right

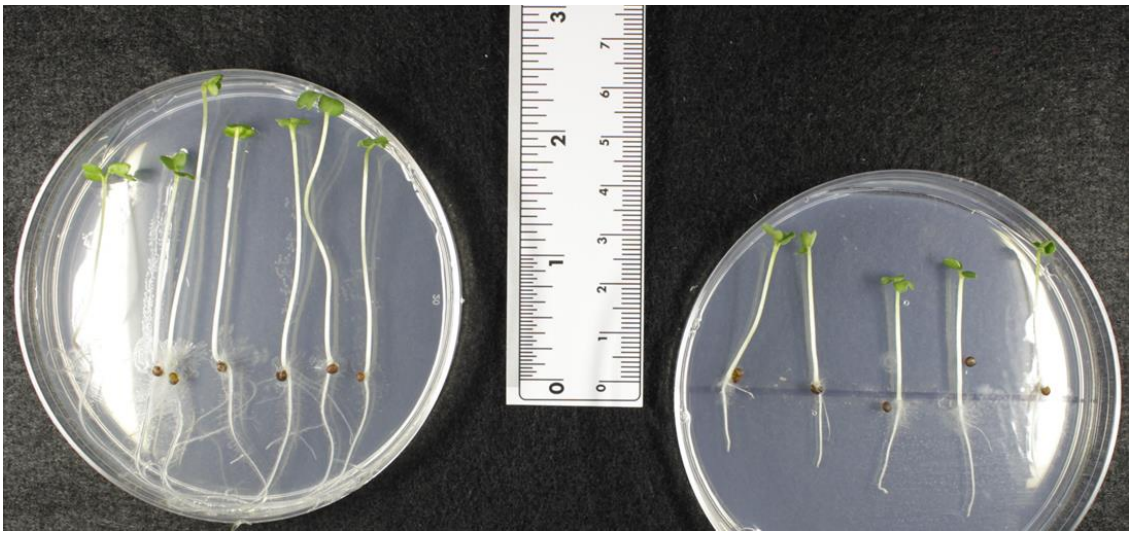

Figure 3.3 PTWG Control vs. PBd1 Isolate
Note. Control on left and PBd1 on right

Quantitative and Qualitative Results of Pathogen Inoculation of Purple Prince Turnip (PPT)

Quantitative effects on PPT

The application of isolates to PPT resulted in a growth stimulation effect from NV1. There was a significant difference in HL produced when inoculated with this isolate that exceeded the control HL by 24%. There was no treatment effect of TB-CD on PPT. There was a statistical similarity in the treatment effect of isolates NV4, PBd1, B9.8, and 1-1, and between PBd1, B9.8, 1-1, and V4 (Figure 3.4).

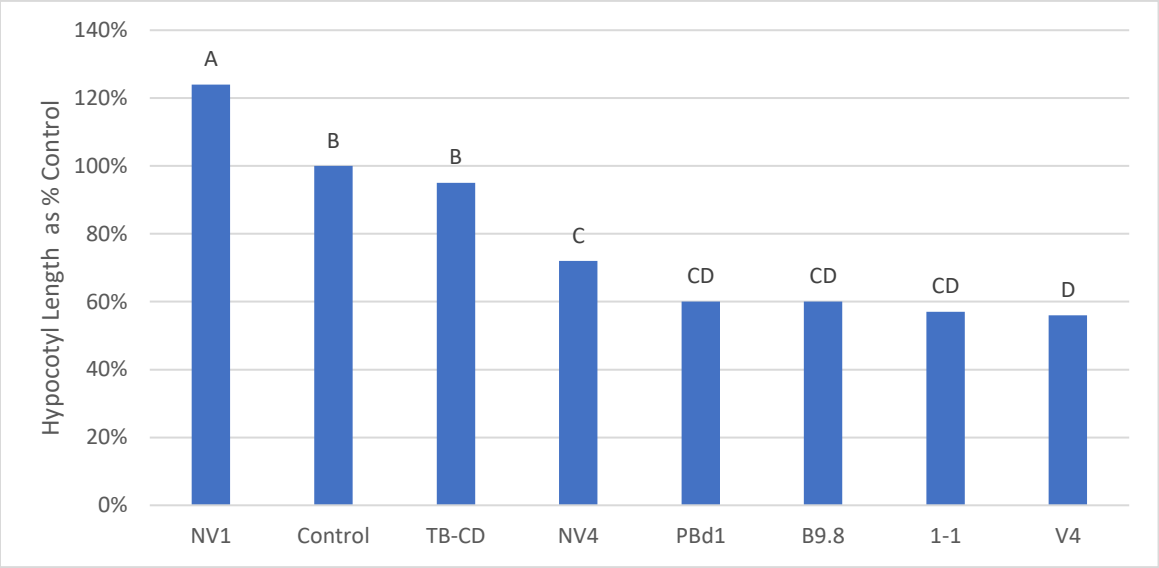

Figure 3.4 Purple Prince Turnip Hypocotyl Length as Percent of Control

Note. Different letters indicate significant differences among isolates at .05 significance level.

Qualitative effects on PPT

The most distinctive qualitative effect in PPT was the root growth (Table 3.6). Although the HL of PPT were not as markedly stunted as in the other isolates, the turnip showed significant symptoms of distress (Figure 3.5 and Table 3.6), severe root stunting and lack of lateral roots.

A side-by-side comparison of the treatment effect of NV4, B9.8 and 1-1 can be seen in Figure 3.5. The hypocotyls of the control were longer and healthier than in either B9.8 or 1-1. The hypocotyls in Petri dish B. inoculated with NV4 also appear more robust and healthier when compared to the other two pathogens, B9.8 and 1-1. The roots of the seedlings in Petri dish B. (root rating 2) show some lateral root development. The roots of the seedlings in both C. and D. show severely stunted, thin roots lacking lateral root formation (Figure 3.5).

Table 3.6 Qualitative Symptoms on Purple Prince Turnip Caused by *Streptomyces* Spp. Isolates

PPT	**Isolate**				
	1-1	**B9.8**	**NV4**	**PBd1**	**V4**
HL as % Control	57%	60%	72%	60%	56%
Stunting	2	1	1	4	3
Necrosis	3	1	0.5	0	1
Root rate	3.3	3.3	2.0	2.3	1.7

Note. *For root rating see Table 3.1

Figure 3.5 Treatment Effect on Hypocotyl Length of NV4, B9.8, and 1-1 on PPT
Note. A. control, B. NV4, C. B9.8, D. 1-1

Quantitative and Qualitative Results of Pathogen Inoculation of Golden Globe Turnip (GGT)

Quantitative effects on GGT

All of the isolate inoculations, even NV1, resulted in a significant difference in treatment effect to hypocotyl length from the control (Figure 3.6). Post-hoc tests indicated a significant degree of separation among the isolates NV1, TB-CD, NV4, and V4 and B9.8 and 1-1 (Figure 3.6), with similarities among TB-CD, NV4, V4, and PBd1, and similarities between PBd1 and 1-1. As a percentage of control, seedlings inoculated with B9.8 achieved a hypocotyl length of just 27%, with isolate 1-1 (36%) also having a profound effect. The hypocotyl stunting of B9.8 on GGT was the highest across all seedling varieties and isolates.

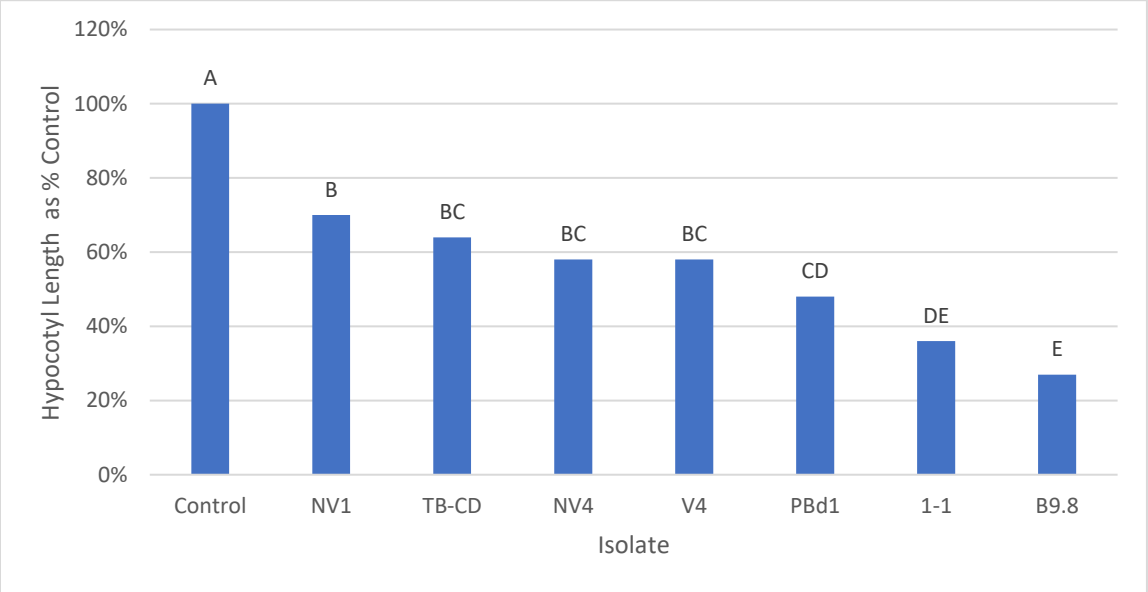

Figure 3.6 Golden Globe Turnip Hypocotyl Length as Percent of Control

Note. Different letters indicate significant differences among isolates at .05 significance level.

Qualitative Effects on GGT

The seedlings in this treatment group had very thin roots that were not stunted but were necrotic, weak, and lacking lateral root formation (Figure 3.7). Hypertrophy was seen in the application of thaxtomin but was rare in the treatments containing pathogens. GGT had the highest occurrence of hypertrophy in response to the application of pathogens compared to all the other isolates, with nearly twice as much as PTWG. The high rate of hypocotyl stunting in GGT correlated with a root rating of 4 (Table 3.7).

Figure 3.7 Treatment Effect of B9.8 on Golden Globe Turnip

Note. Orange arrow indicates enlarged seedling with weak roots (root rate 4) and thickened hypocotyl referred to as hypertrophy.

Table 3.7 Qualitative Symptoms on Golden Globe Turnip Caused by *Streptomyces* Spp. Isolates

GGT	Isolate				
	1-1	**B9.8**	**NV4**	**PBd1**	**V4**
HL % of control	36	27	58	48	58
Stunting	11.0	12.0	7.0	9.0	6.0
Necrosis	3	5	0	0	0
Root Rate	4.0	4.0	1.3	3.0	2.3

Note. *For root rating see Table 3.1

Quantitative and Qualitative Results of Pathogen Inoculation of Japanese Baby Turnip (JBT)

Quantitative effects on JBT

JBT showed the most restriction in HL as a result of the application of NV1, which was either non-pathogenic or acted as a growth stimulator in PPT, PTWG and ESGR. Only GGT and JBT showed statistically significant shortened hypocotyls from NV1 (Figure 3.8). The most detrimental pathogens were NV4, B9.8 and 1-1. Figure 3.9 shows the devastating treatment effect caused by 1-1 on JBT.

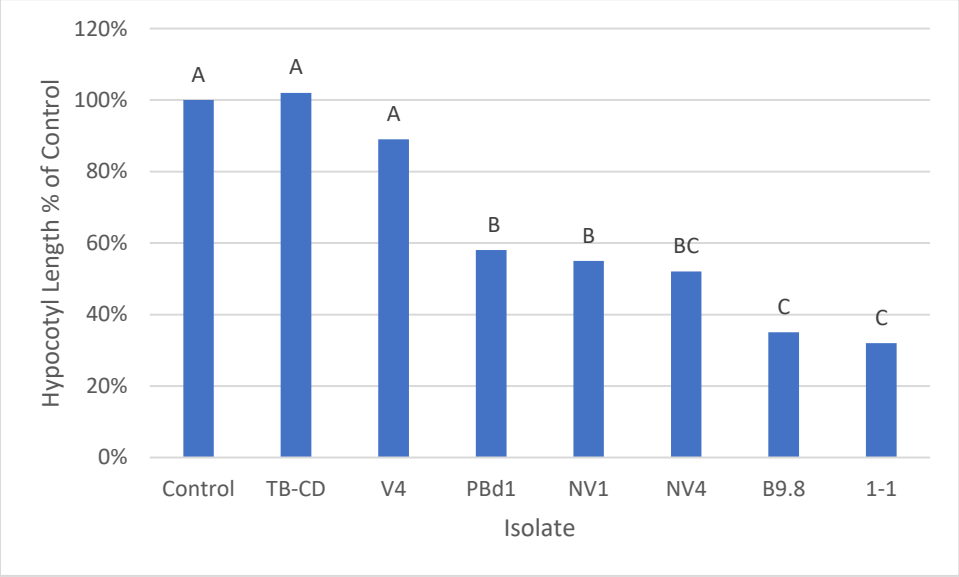

Figure 3.8 Japanese Baby Turnip Hypocotyl Length

Note. Different letters indicate significant differences among isolates at .05 significance level.

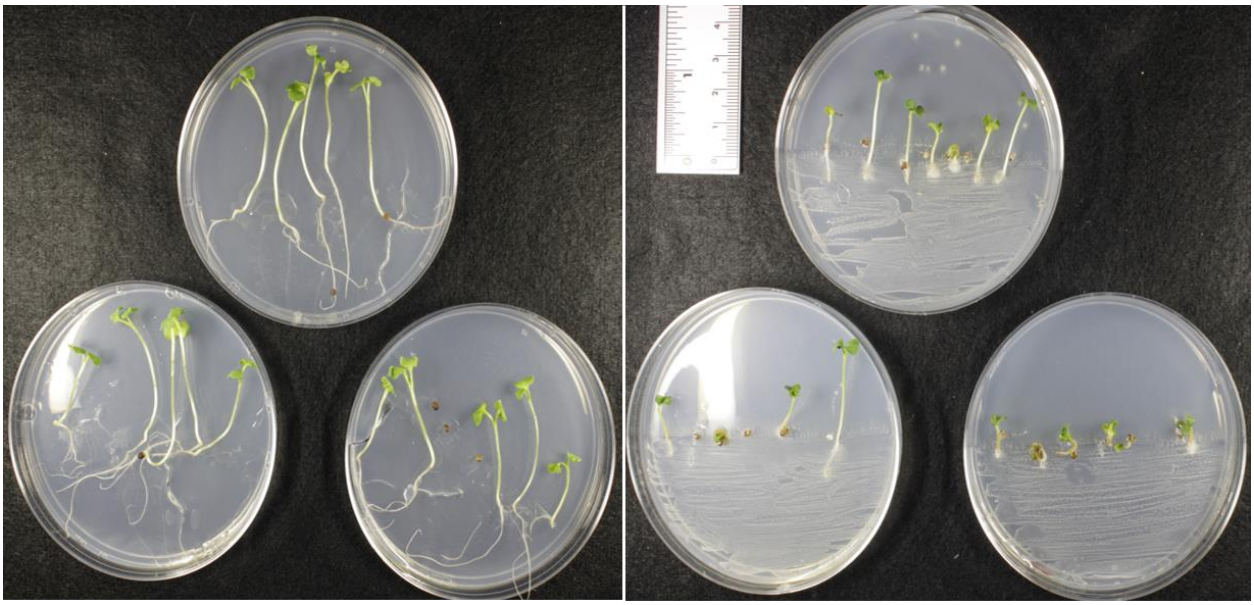

Figure 3.9 Effect of 1-1 on JBT Hypocotyl Length

Note. control (left), 1-1 (right)

Qualitative Effects on JBT

JBT seedlings also showed more signs of necrosis than other varieties, and two of the highest root ratings across all varieties (Table 3.8). In isolate PBd1 there was less stunting observed, although the roots had a higher rating.

Table 3.8. Qualitative Symptoms on JBT Caused by *Streptomyces* Spp. Isolates

	Isolate				
JBT	**1-1**	**B9.8**	**NV4**	**PBd1**	**V4**
HL as % control	32	35	52	58	89
Necrosis	7	10	0	1	0
Stunting	13	15	10	4	0
Root rate	4	4	1.7	2.5	1.5

Quantitative and Qualitative Results of Pathogen Inoculation of Early Scarlet Globe Radish (ESGR)

Quantitative Effects on ESGR

TB-CD had a definite growth-stimulating effect on radish (Figure 3.11). The other results were similar to the treatment effects seen in the turnip varieties, with 1-1 having the biggest effect on hypocotyl length. The pathogens V4 and NV1 were not statistically different from the control, and PBd1, NV4, B9.8, and 1-1 were statistically similar.

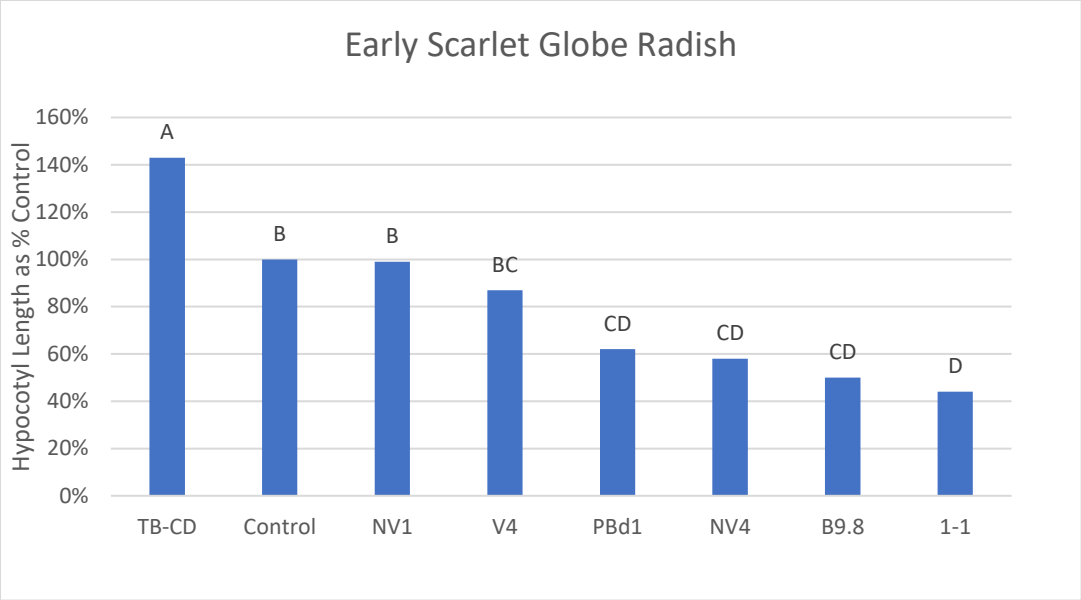

Figure 3.10 Effect of Isolates on ESGR Hypocotyl Length

Note. Different letters indicate significant differences at .05 significance level.

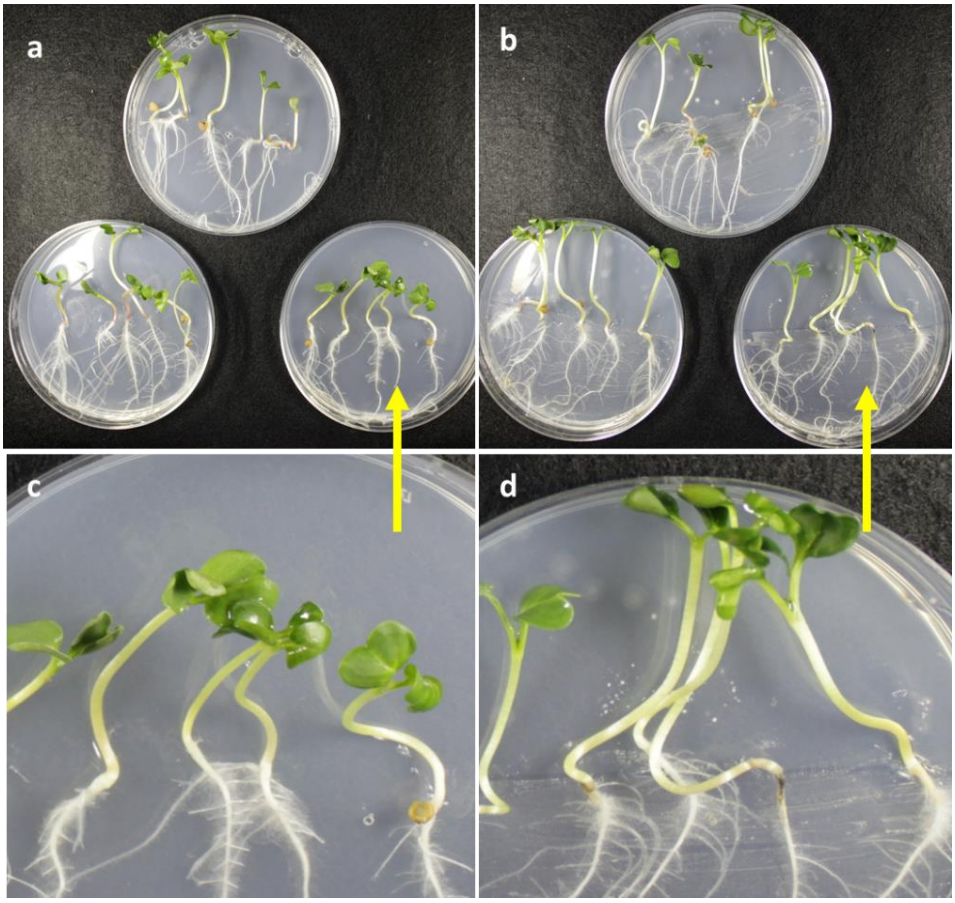

Figure 3.11 TB-CD Promotes Hypocotyl Growth in ESGR

Note. Yellow arrows indicate the Petri dish from which the enlarged section was obtained.

Qualitative Effects on ESGR

Table 3.9 Qualitative Symptoms on ESGR Caused by *Streptomyces* Spp. Isolates

ESGR	**Isolate**				
	1-1	**B9.8**	**NV4**	**PBd1**	**V4**
	55%	47%	54%	55%	78%
Necrosis	3	5	1	0	0
Stunting	n/a	15	9	3	1
Root rate	n/a	4	0.7	2.8	1

Overall Comparison of Host Cultivar Against *Streptomyces* Isolates

The shortest hypocotyls as a percentage of the control, across all seedling varieties including radish (ESGR), were measured in seedlings inoculated with 1-1 (32%) and B9.8 (27%). Depending on seedling cultivar, all isolates, including NV1, caused at least one significant difference in hypocotyl length compared to control (Table 3.10). In the case of isolates TB-CD and NV1 however, the statistical difference was often the result of hypergrowth, not stunting.

Table 3.10 Hypocotyl Lengths as a Percent of Control for Each Variety and Isolate

	Isolate						
Host Variety	**1-1**	**B9.8**	**NV1**	**NV4**	**PBd1**	**TB-CD**	**V4**
ESGR	44%	50%	99%	58%	62%	143%	87%
PTWG	42%	39%	106%	35%	68%	59%	67%
PPT	57%	60%	124%	72%	60%	95%	56%
GGT	36%	27%	70%	58%	48%	64%	58%
JBT	32%	35%	55%	52%	58%	102%	89%

PTWG Compared with PPT

There was a significant difference in HL between Purple Prince turnip (PPT) and the other host varieties for most pathogens. PPT seemed the most resistant to pathogens of the cultivars tested, including radish. This is seen in the consistently higher HL as % of control in PPT compared to other cultivars. PPT performed better than PTWG against each pathogen except PBd1 and V4, for which there was no significant difference (Table 3.11). In addition, the lowest HL of 56 % (V4) was significantly different from the lowest HL seen in PTWG of 35% (B9.8; Table 3.10).

Table 3.11 Hypocotyl Length as % Control for PTWG vs. PPT

	Isolate						
	1-1	**B9.8**	**NV1**	**NV4**	**PBd1**	**TB-CD**	**V4**
PTWG	42%	39%	106%	58%	68%	59%	67%
PPT	57%	60%	124%	72%	60%	95%	56%
LSM *p*-value	.0377	.0024	.0003	<.0001	ns	<.0001	ns

Note. ns indicates lack of significant difference at $p < .05$.

PTWG Compared with GGT

Although the data appear to show that GGT performed worse than PTWG against B9.8, there was not a significant difference in HL as % of control (Table 3.12). There was a significant difference in HL as % of control in isolates NV1, NV4, and PBd1. GGT did not respond to growth stimulation effects seen in ESGR or PPT, with no HL above 100% for either NV1 or TB-CD (Table 3.10).

Table 3.12 Hypocotyl Length as % Control for PTWG vs. GGT

	Isolate						
	1-1	**B9.8**	**NV1**	**NV4**	**PBd1**	**TB-CD**	**V4**
PTWG	42%	39%	106%	35%	68%	59%	67%
GGT	36%	27%	70%	58%	48%	64%	58%
***p*-value**	ns	ns	<.0001	0.0039	0.0138	ns	ns

Note. ns indicates lack of significant difference at $p < .05$.

PPT Compared with ESGR

PPT had significantly higher hypocotyl length when compared to ESGR, for all isolates except TB-CD and V4, for which there was no significant difference.

Table 3.13 Hypocotyl Length as % Control for PPT vs. ESGR

	Isolate						
	1-1	**B9.8**	**NV1**	**NV4**	**PBd1**	**TB-CD**	**V4**
ESGR	44%	50%	99%	58%	62%	143%	87%
PPT	57%	60%	124%	72%	60%	95%	56%
***p*-value**	0.002	0.0017	<.0001	<.0001	0.01	Ns	ns

Note. ns indicates lack of significant difference at $p < .05$.

PPT Compared with GGT

PPT performed better than GGT against all isolates except V4. PPT was susceptible to possible growth regulator effects of NV1 whereas GGT was not.

Table 3.14 Hypocotyl Length as % Control for PPT vs. GGT

	Isolate						
	1-1	**B9.8**	**NV1**	**NV4**	**PBd1**	**TB-CD**	**V4**
GGT	36%	27%	70%	58%	48%	64%	58%
PPT	57%	60%	124%	72%	60%	95%	56%
***p*-value**	0.0049	<.0001	<.0001	0.0102	0.0182	<.0001	ns

Note. ns indicates lack of significant difference at $p < .05$.

PPT Compared with JBT

PPT performed better than JBT with a significant difference against all isolates except TB-CD and V4, for which there was no difference in HL.

Table 3.15 Hypocotyl Length as % Control for PPT vs. JBT

	Isolate						
	1-1	**B9.8**	**NV1**	**NV4**	**PBd1**	**TB-CD**	**V4**
JBT	32%	35%	55%	52%	58%	102%	89%
PPT	57%	60%	124%	72%	60%	95%	56%
***p*-value**	0.0008	<.0001	<.0001	0.0002	0.0437	ns	ns

Note. ns indicates lack of significant difference at $p < .05$.

Effect of Phytotoxins

Thaxtomin Effects on Hypocotyl Length

The effect of thaxtomin application to seedlings was dependent upon thaxtomin concentration. At very low concentrations of 0.18-1.83 nmoles thaxtomin, two of the turnip cultivars, GGT and PTWG, and the radish, ESGR, grew hypocotyls of equal or greater length than controls. At moderate toxin concentrations of 9.1 nmoles (50 ng) thaxtomin ESGR, PPT, and PTWG hypocotyls were stunted to less than half of the control hypocotyls. A noted decrease in seedling health was seen at all concentrations greater than 50 ng (9 nmoles) of toxin. At 100 ng thaxtomin the seedlings lack substantial roots, thickened hypocotyls were observed, and some necrosis of root and hypocotyl was common. Above 100 ng, most seedlings lacked root structures and tissues were blackened, clubbed, and disintegrated. At higher toxin concentrations of 18.30–182.60 nmoles there was universal stunting of hypocotyls along with deformation, blackening, and disintegration of hypocotyls, roots, and cotyledons.

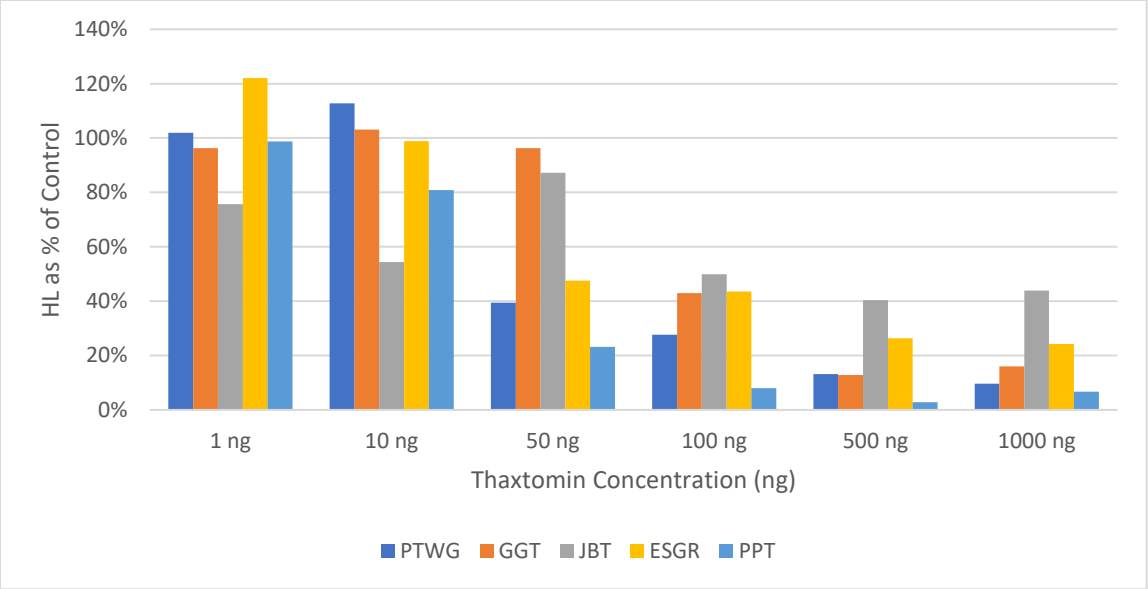

Figure 3.12 Effect of Different Concentrations of Thaxtomin on Hypocotyl Length in Seedlings of Five Varieties

PTWG

PTWG response to thaxtomin application was representative of the response seen in most other varieties. In addition to hypocotyl length, qualitative effects were rated and are listed in Table 3.16. Seen in Figure 3.13, thaxtomin has caused hypertrophy on PTWG seedlings, roots that have failed to grow past the toxin strip, chlorosis, and some browning of hypocotyls.

Table 3.16 Qualitative Effects of Thaxtomin Application to PTWG

PTWG				
ngrams	**50 ng**	**100 ng**	**500 ng**	**1000 ng**
nmoles	**9.13**	**18.26**	**91.32**	**182.65**
necrosis	0	0	10	10
stunting	13	15	15	15
hypertrophy	11	13	15	15
curling	1	4	6	3
chlorosis	1	4	1	4
root rate	3	3	4	4

Figure 3.13 PTWG in 100 ng (18.3 nmoles) Thaxtomin
Note. This is a close-up of one of three Petris dishes in the replication.

Most of the plant varieties were affected by thaxtomin at low concentrations, although the difference between control and low thaxtomin concentrations often resulted in longer hypocotyl lengths. Both ESGR and PTWG grew longer hypocotyls in 0.18 nmoles than in controls, and PTWG and GGT grew longer hypocotyls in 1.83 nmoles than in controls. Only JBT and GGT had HL that were statistically the same as the control at 9.10 nmoles (Table 3.17).

Table 3.17 Hypocotyl Length as Percent of Control in Thaxtomin Treatments for All Varieties

Thaxtomin Concentration						
ng	**1**	**10**	**50**	**100**	**500**	**1000**
nmoles	**0.18**	**1.83**	**9.10**	**18.30**	**91.30**	**182.60**
ESGR	122%	99%	48%	44%	26%	24%
PPT	99%	81%	23%	8%	3%	7%
PTWG	102%	113%	39%	28%	13%	10%
GGT	96%	103%	96%	43%	13%	16%
JBT	76%	54%	87%	50%	40%	44%

Note. Dark background indicates hypocotyl length statistically different from control at .05 significance

One isolate, GGT, showed some resistance to changes in hypocotyl length at thaxtomin concentrations up to 50 ng. This result was statistically significant compared to the hypocotyl lengths of all the other cultivars, except JBT, in 50 ng thaxtomin. This possible resistance to the toxins was broadly seen at thaxtomin concentrations from 0.18–9.10 nmoles (1 ng–50 ng; Table 3.18).

Table 3.18 Pairwise Comparison of Different Varieties to GGT

	Treatment Level					
ng	**1**	**10**	**50**	**100**	**500**	**1000**
nmoles	**0.18**	**1.83**	**9.10**	**18.30**	**91.30**	**182.60**
ESGR	ns	0.0034	0.0027	ns	ns	ns
PPT	0.0013	ns	0.0006	0.0006	ns	ns
PTWG	ns	ns	0.0003	ns	ns	ns
JBT	0.0007	<.0001	0.017	ns	ns	ns

Note. ns indicates lack of significant difference at p <.05.

There was also a visible difference in qualitative symptoms between GGT vs. PPT, ESGR, and PTWG at a thaxtomin concentration of 9.10 nmoles (50 ng; Figure 3.14).

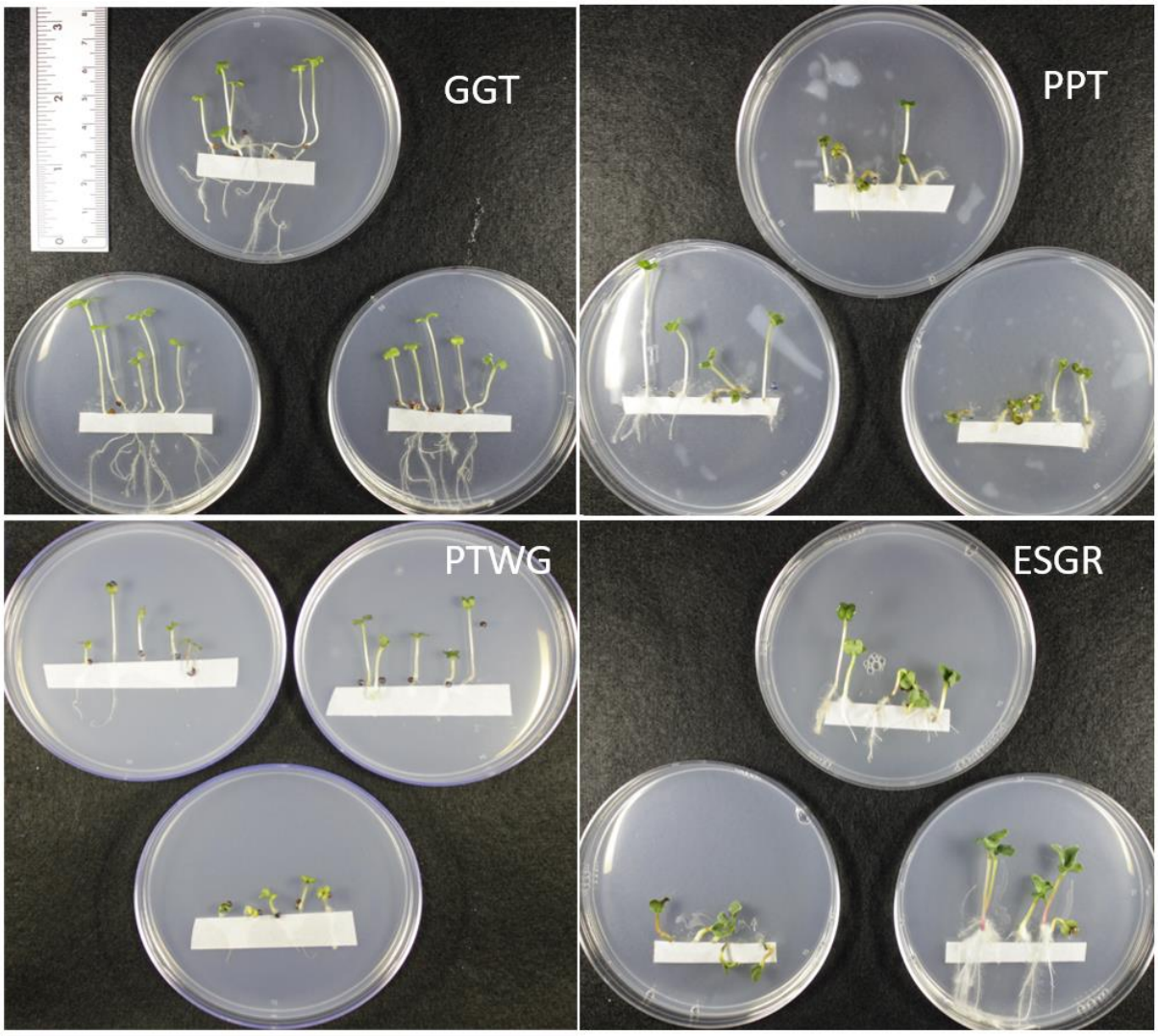

Figure 3.14 Comparison of GGT, PPT, PTWG, and ESGR in 9.10 nmoles Thaxtomin

The difference between GGT and the other seedling cultivars is striking. Whereas the roots in each replicate of GGT have grown far past the toxin strip, only ESGR has grown roots in one replicate that extend past the toxin strip. PPT has signs of necrosis that is lacking in GGT, and PPT, PTWG and ESGR all have severely stunted hypocotyls that GGT does not.

Coronatine

Coronatine treatments also shortened hypocotyls, but the effect was not as acute, and the seedlings were able to tolerate higher molar concentrations than thaxtomin. For example, 18.26 nmoles (100 ng) of thaxtomin reduced hypocotyl length of all tested varieties to 50% or less of control, whereas 25.05 nmoles of coronatine (100 ng) shortened just three of the varieties below 50%. At 91.32 nmoles (500 ng) of thaxtomin the seedlings were universally compromised with hypocotyls between 3-40% of control. At 125.23 nmoles (500 ng) coronatine, some HL were still between 29-48% control.

Seedlings treated with coronatine also had fewer visible signs of distress, with no incidence of necrosis or hypertrophy. The most common symptoms were stunted hypocotyls and roots, and chlorotic cotyledons. Figure 3.15 lists the results of hypocotyl length as % of control for all cultivars.

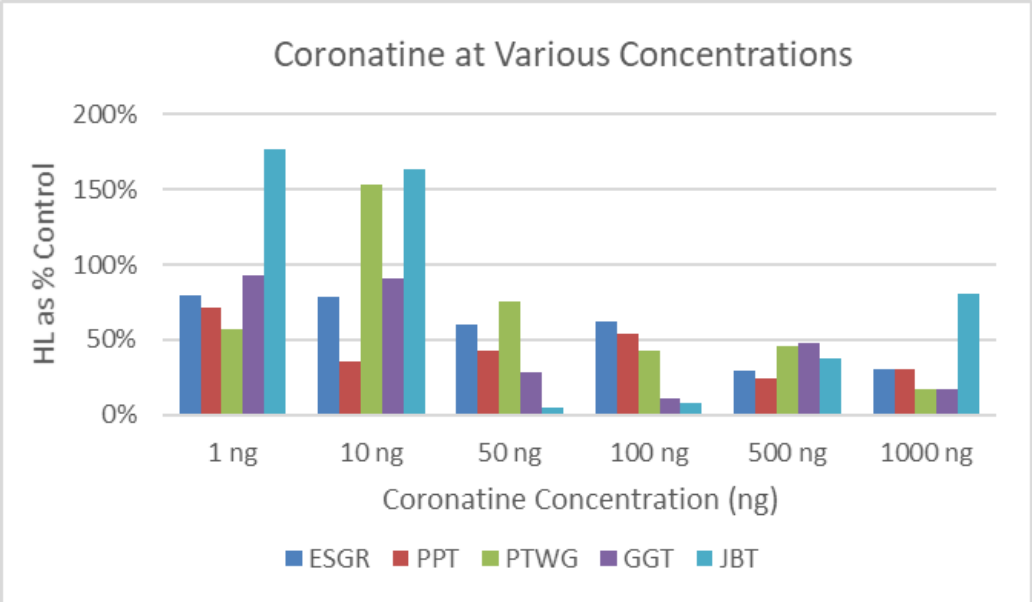

Figure 3.15 Hypocotyl Length as % of Control for all Varieties Treated with Coronatine

The hypocotyl lengths of PTWG were significantly different at all concentrations except 10 ng. Although there was a trend of coronatine causing hypergrowth in both JBT and PTWG at low concentrations, the results were not statistically significant. PPT showed the most susceptibility to coronatine, with stunted hypocotyl length values that were statistically significant at all concentrations.

Figure 3.16 shows a comparison of select PTWG seedlings following treatment with thaxtomin or coronatine. The two seedlings photographed were selected as representative of the most and least affected in each treatment replicate. There is some variation between the most and least affected seedlings at 50 ng concentrations for both thaxtomin and coronatine, but at 100 ng thaxtomin all seedlings were dramatically stunted, most were clubbed and exhibited a severe loss of root structure. The coronatine application of 100 ng did not cause such a consistent reduction in hypocotyl length as thaxtomin, but the seedlings were still affected, particularly in the lack of robust root formation. Although the roots of the seedlings treated with 100 ng coronatine are longer than in the 100 ng thaxtomin, they lacked the lateral roots seen in healthy seedlings.

Figure 3.16 PTWG in Various Toxin Treatments
Note. a. 50 ng thaxtomin b. 100 ng thaxtomin c. 50 ng coronatine d. 100 ng coronatine

Table 3.19 lists in more detail the effects of coronatine on PTWG at various concentrations. From 25.05 nmoles and higher concentrations the effects on root formation correlates with a loss of growth similar to inoculation with pathogenic *Streptomyces* spp. isolates and higher levels of thaxtomin.

In seedlings treated with coronatine, stunting and lack of lateral root formation correlates more with chlorotic tendencies than with the necrosis and hypertrophy seen in thaxtomin treatments (Table 3.19).

Table 3.19 Qualitative Effects of Coronatine Application to PTWG

Coronatine				
ngrams	**50 ng**	**100 ng**	**500 ng**	**1000 ng**
nmoles	**12.52**	**25.05**	**125.23**	**250.47**
necrosis	0.0	0.0	0.0	0.0
stunting	4.0	14.0	14.0	15.0
hypertrophy	0.0	0.0	0.0	1.0
curling	0.0	2.0	1.0	4.0
chlorosis	0.0	2.0	3.0	8.0
root rate	1.7	3.0	3.0	3.7

Effect of Both Toxins Combined

The application of both toxins to the seedlings resulted in similar effects to that seen in the separate applications of each toxin, but symptoms appeared at lower concentrations. The seedlings performed poorly at lower toxin concentrations than was seen with the application of one toxin at a time. The effect on PTWG from increasing concentrations of both thaxtomin and coronatine could be seen in the progression from a hypocotyl that reached about 4 cm with full, green cotyledons to a seedling with 0.5 cm hypocotyl length and chlorotic cotyledons. These seedlings' roots were missing. The experiment was not repeated with 1000 ng of each toxin since 500 ng of each was consistently toxic to the plants.

Figure 3.17 Effect of Both Toxins Combined on PTWG
Note. A. Control B. 50 ng each C. 100 ng each D. 500 ng each

Histological Examination of Toxin Effects

To examine the effects of toxin application on a cellular level, treated tissues were examined by light microscopy at 10X magnification. PTWG seedlings grown in 50 ng thaxtomin, 50 ng coronatine, and 50 ng of both toxins were sampled at 72 and 144 hours post inoculation (hpi).

Normal plant growth of rectangular, elongated cells is seen in Figure 3.18 A. The effects of thaxtomin in increasing the width of the cell are apparent in Figure 3.18 B., as well as green spots. In the control the cells are disk shaped and in the presence of thaxtomin they have become nearly square. In 50 ng CT, the normally rectangular epidermal cells have become practically round (Figure 3.18 C.), and this trend continues with 50 ng of each toxin but now the cells are showing a tendency to be more tall than wide (Figure 3.18 D.), a reversal of growth seen in the control. Rather than disk-like and more rectangular, the growth has become oval, almost square. This hypertrophy seen on a cellular level was observable in the stunted, hypertrophied appearance documented in both the pathogen inoculation and in the application of toxins.

Measurements of representative cell lengths indicate a trend towards shortened epidermal cells with the addition of toxins, with the most reduction of epidermal cell length seen in 50 ng of each toxin (Table 3.20). The ratio of length to width went from 4:1 in control at 72 hpi, to 1:1 in 50 ng of each toxin at 72 hpi.

Table 3.20 Measurement of Representative Cell Lengths at 10X Magnification

Average µm	Time (hpi)	Toxin ng			
		0	50 thaxtomin	50 coronatine	50 Each
Length	**72 hpi**	45	31	28	8
Length	**144 hpi**	58	64	33	22
Width	**72 hpi**	12	17	17	13
Width	**144 hpi**	13	25	20	13

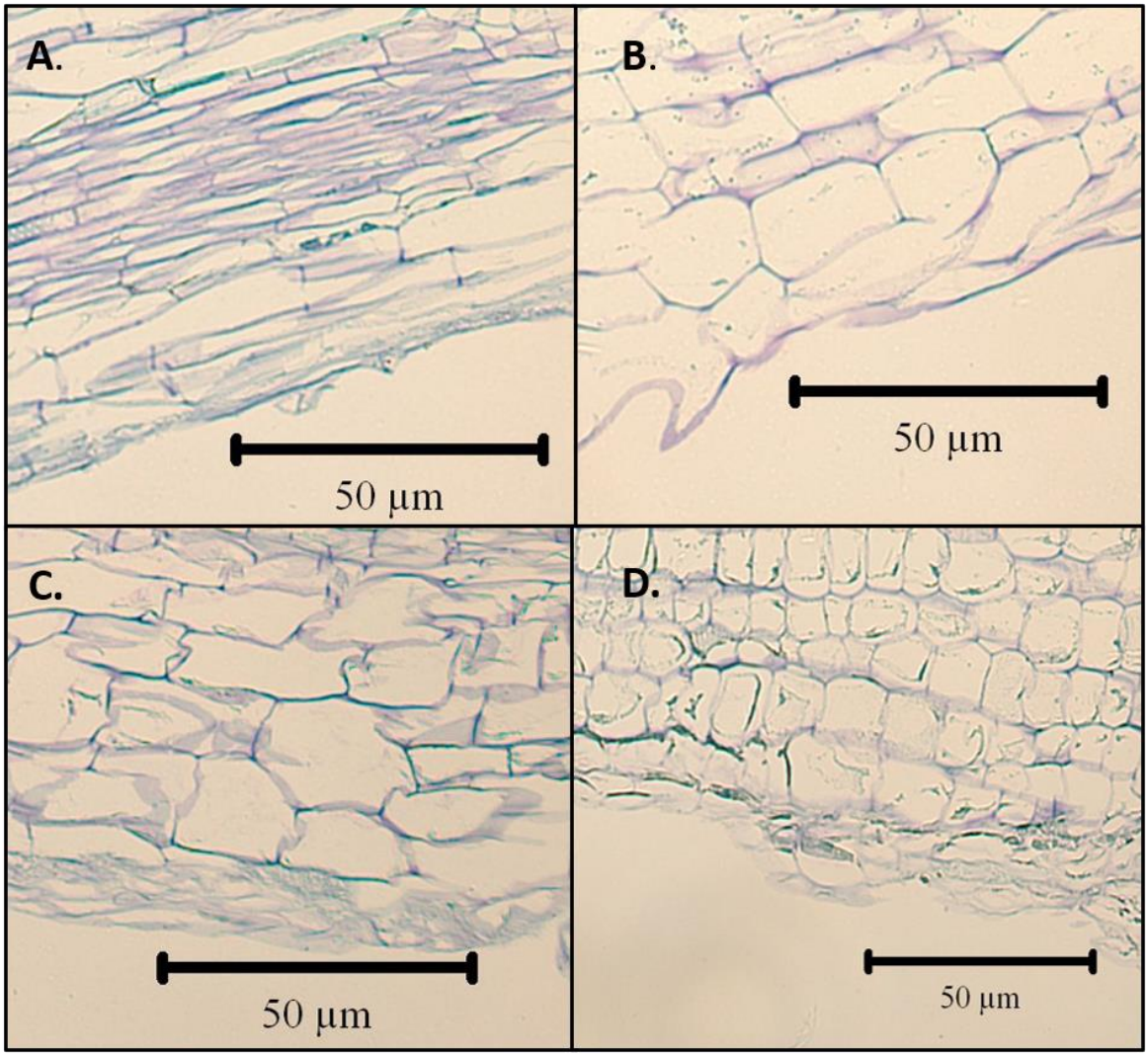

Figure 3.18 Comparison of Treated Tissue at 72-hpi in Thaxtomin, Coronatine, and Combined Toxins at 10X Magnification

Note. A. control, B. 50 ng thaxtomin, C. 50 ng coronatine, D. 50 ng both toxins combined

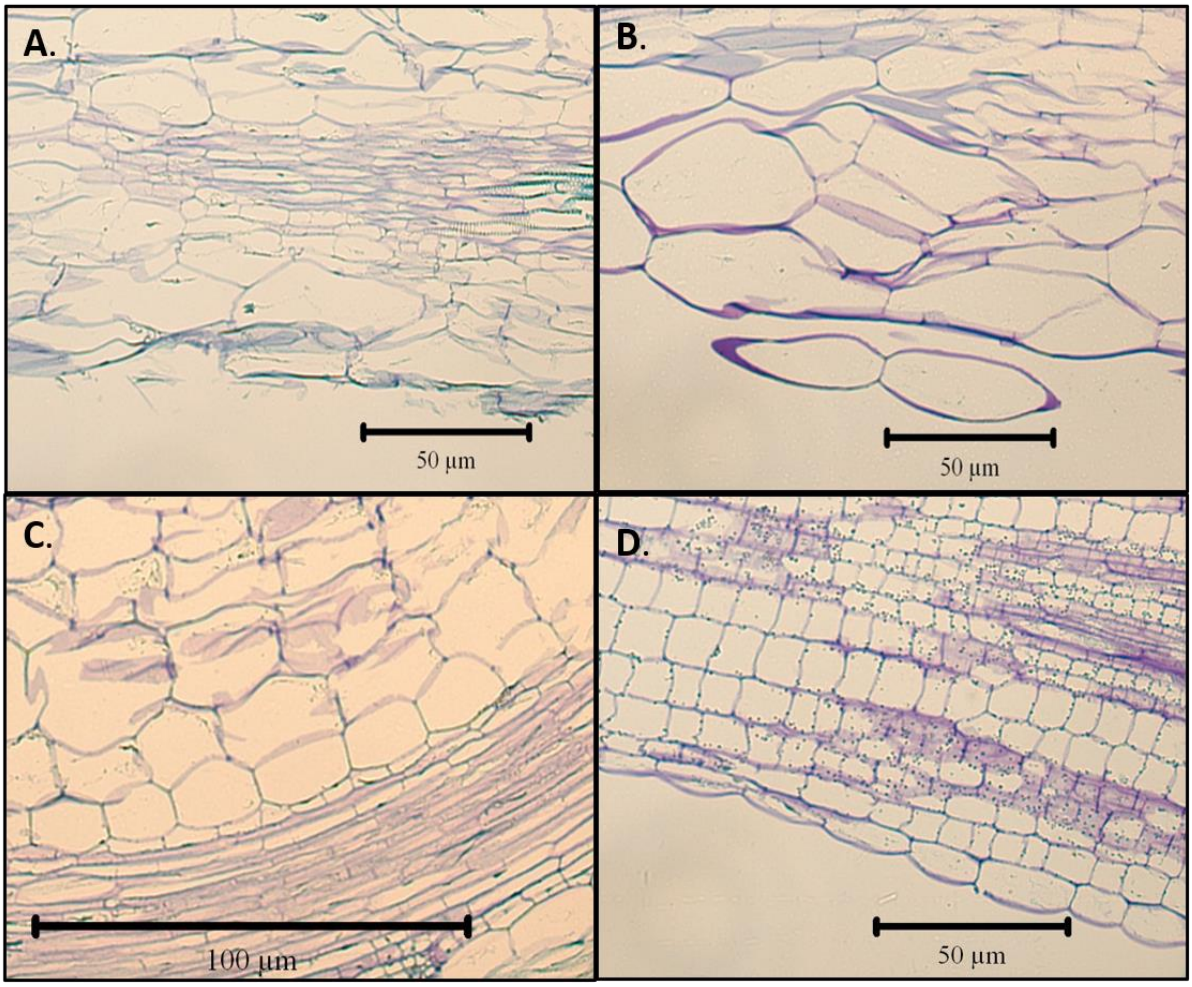

Figure 3.19 Comparison of Treated Tissue at 144-hpi in Thaxtomin, Coronatine, and Combined Toxins at 10X Magnification

Note. A. control, B. 50 ng thaxtomin, C. 50 ng coronatine, D. 50 ng both toxins combined

DISCUSSION

Symptoms Caused by Streptomyces Isolates

There was a high degree of variation in symptoms caused by different *Streptomyces* spp. isolates on the same host, and differences between hosts in response to the same *Streptomyces* spp. isolate. The same strain of *Streptomyces* could produce different symptoms on different hosts. The most consistently pathogenic isolates—B9.8, 1-1, NV4, and PBd1—caused a statistically significant hypocotyl length reduction in every host tested, although the degree of reduction varied.

Every isolate, including the "non-pathogenic" NV1 caused statistically significant hypocotyl length reduction in at least one host. Growth stimulation by NV1 and TB-CD was observed on some hosts. This could be due to the production of the plant hormone indole-3-acetic acid (IAA) by the bacteria. IAA has been shown to be produced in some *Streptomyces* that act as plant growth-promoting rhizobacteria (PGPR) (Salwan et al., 2020). It is possible that NV1 and TB-CD have characteristics of PGPR, and this trend may warrant further study, especially in alternate hosts such as potato, turnip, and radish.

There was a surprising degree of variation in the type of symptom produced by *Streptomyces* spp. in different hosts, although the *Streptomyces scabies* isolate 1-1 consistently caused disease that included necrosis.

It would be interesting to repeat the histological examination following inoculation with *Streptomyces* isolates to see how closely plant defense responses mimic what is seen with

phytotoxins. Additional replications that would allow for statistically accurate comparisons of epidermal cell's lengths and widths would also be informative.

Indications of Resistance in Turnip Varieties

Of all the hosts tested, PPT showed the most evidence of resistance to the pathogen and further study of this turnip for *Streptomyces* resistance may be indicated. It would be interesting to see how this variety performs in growth chamber or field tests when inoculated with various strains of *Streptomyces* spp. It was surprising that PPT was not resistant to the toxins after showing resistance to the isolates. It is possible that the plant is producing defense compounds to fight the bacteria rather than de-toxifying phytotoxins or does not induce the production of the toxins.

The isolates GGT and JBT showed a strong trend to be the most susceptible to the pathogens, although surprisingly, GGT may have some resistance to thaxtomin. GGT was also different from the other turnip varieties in showing susceptibility to inoculation with NV1.

JBT appeared to show resistance in all three replicates to thaxtomin at 50 ng (9.13 nmoles) but at no other concentration. This result probably needs to be retested since it seems likely to be an experimental error that caused the hypocotyls to evade the toxin. It is possible that they escaped encountering the toxin rather than resisting it.

Effect of Phytotoxins

Thaxtomin did not induce a significant growth difference to most hosts until the 50 ng (9.13 nmoles) concentration. At and above this level, the health of seedlings dropped off sharply. Higher concentrations of coronatine seemed to be tolerated and when qualitative ratings were considered, 100 ng coronatine affected roots more than hypocotyls and further

study may reveal that seedlings could be “rescued” if removed from the toxin. Rescue of seedlings inoculated with the same of amount of thaxtomin seems unlikely due to the severe tissue symptoms seen.

The application of both toxins caused tissue death more quickly than with either toxin alone. Since it is likely the pathogen produces more than one toxin in nature, it seems likely that less of each would be needed to cause disease.

It would be interesting to repeat the histological examination with additional replications to allow statistically accurate comparisons of epidermal cell’s lengths and widths.

Further Studies

Testing the effects of the necrogenic protein nec1 by applying the toxin to tissue seedlings inoculated with various strains of the pathogen would be interesting as this is the first toxin produced during infection. It would be informative to see if this necrosis-inducing protein would lead to the release of plant cell wall sugars like cellobiose, thereby causing thaxtomin production to increase.

The next step in analyzing host specificity may be to inoculate potato seedlings with *Streptomyces* strains that were not capable of causing symptoms of disease on tissue discs to see if the host specificity indicated by the assays is seen in seedlings.

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