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PLANT MEDICINE: THE USE OF MEDICINAL PLANTS AS GREEN MANURES IN THE
TREATMENT OF *STREPTOMYCES SCABIES* INFECTION AND POTATO SCAB DISEASE

By

Benjamin E. Bolcer

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Plant Medicine: The Use of Medicinal Plants as Green Manures in the Treatment of
Streptomyces scabies Infection and Potato Scab Disease

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ABSTRACT

PLANT MEDICINE: THE USE OF MEDICINAL PLANTS AS GREEN MANURES IN THE TREATMENT OF *STREPTOMYCES SCABIES* INFECTION AND POTATO SCAB DISEASE

By

Benjamin E. Bolcer

Potato scab disease is caused by the pathogen *Streptomyces scabies* and leads to significant crop losses annually worldwide. There is growing public and scientific concern regarding the human and environmental safety with the use of many pesticides and current disease control measures. In lieu of this, the use of medicinal plant species as organic green manure treatments against potato scab disease was evaluated in field studies. Green manure applications of calendula (*Calendula officinalis resina*) and comfrey (*Symphytum officinale*) simultaneous to potato (*Solanum tuberosum*) planting in field studies were evaluated for scab reduction qualities. Treatments were designed to mimic home garden/family farm type operations and were applied as a single layer of rough chopped plant material coverage directly along with potato tubers within the planted rows. These treatments consisted of calendula, comfrey, and a calendula/comfrey mixture. In pilot study, treatments of both calendula and comfrey showed potent potato scab disease reductions of approximately 20%. The use of comfrey as a green manure increased total potato production by 24%. Follow-up field study using comfrey as a green manure showed similar results with an overall decrease in scab disease of 18.8% and demonstrated increased harvest yields. Results of this experiment suggest the use of these green manures as a feasible, effective, organic means of potato scab control and warrant further research into the use of these and other medicinal plants in the treatment plant disease.

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This thesis follows the format prescribed by the APA Reference Style Guide of Northern Michigan University's Olson Library.

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INTRODUCTION

Expanding global populations place increased demand on crop production. Potatoes (*Solanum tuberosum*) are a staple crop among those cultivated to fill a now 7 billion hungry mouths. Estimations anticipate the demand for potatoes and potato products will only continue to grow and one day may exceed 600 million tons per year. To meet this demand farmers must overcome a host of obstacles. Of these, a major issue for potato cultivation is scab disease caused by the pathogen *Streptomyces scabies*, a pathogenic species of the often-beneficial *Streptomyces* genus. Scab disease causes worldwide crop losses exceeding 1 million tons per year (Potato Council, 2010). The economic impact of this disease is difficult to measure, but costs were estimated at \$15-17 million for Canada alone in 2002 (Hill & Lazarovits, 2005).

Adding to the farmer's challenges is the growing public and scientific concern over the human and environmental safety of the use of many pesticides and practices of current disease control measures. These concerns include the health impacts of chemical pesticide, herbicide, and fertilization processes to the human consumers as well as the soil and environment. Included in these concerns are the resultant low nutritional density of crops, shown in part to be due to inert soils and mineral loss caused by current disease control methods (Popp et al. 2013, Yousaf et al., 2013). This questions the long-term sustainability of such methods. Yet, new approaches to address the complicated issues of providing a healthier product while meeting productivity and profitability quotas are incentivized by increasing public pressure to pursue environmentally friendly practices coupled with a consumer willingness to spend more on organic, sustainable products.

The following research examines the use of certain plant species, specifically calendula (*Calendula officinalis*) and comfrey (*Symphytum officinale*), with known medicinal and antibacterial properties as “green manures” (a term used to describe the incorporation of fresh “green” plant material into the soil as amendment) to reduce potato scab disease. The following research hypothesized the antibacterial properties of the selected green manure plant species could be used as a superior treatment against the indwelling soil infection of *Streptomyces scabies*, the pathogen responsible for potato scab formation, and thereby reduce overall disease occurrence and increase marketable harvest. The addition of organic matter to the soil system could also improve soil fertility, further correlated with reduced scab occurrence, increase nutrient density, and further improve yields. This approach could enhance market value and aid in meeting consumer demand for an organic product.

LITERATURE REVIEW

CHAPTER ONE: THE CURRENT PROBLEM

Cultivation of potatoes (*Solanum tuberosum*) began as early as 8,000 years ago in the Bolivian-Peru region of the Andes. Potatoes, domesticated from the nightshade family, were shaped from a potentially poisonous wild plant to a staple food crop comprised of over 5000 unique cultivars (Lutaladio & Castaldi, 2009). Cultivation began and thrived under Incan governments, who devoted vast resources to the sustainable production of all food crops, including potatoes (Kendall, 2005). The people of the region developed complex and creative agricultural techniques to supply the nutrition necessary to support the expanding growth of their civilization (Garcia et al., 1998). The tiered-structures the people built for food production still stand hundreds of years later and the soil within is remarkably still fertile today (Garcia et al., 1998).

The potato spread throughout Europe following the Spanish Inquisition and was grown for its starch-rich rhizome. It has now become an important staple in many world diets. This expansion was not without good reason. The potato is rich in minerals such as calcium, copper, manganese, phosphorus, and potassium. It is also high in B vitamins such as thiamine (B1), niacin (B3), pantothenic acid (B5), pyroxidine (B6), and folate (B9) (USDA, 2018). Furthermore, potatoes can provide 16% of the daily recommended amount of fiber and 35% of the daily recommended amount of vitamin C (USDA, 2018).

In the United States alone, 20 million tons of potatoes are grown and harvested annually, accounting for a \$3.5 billion industry (Canada, 2011). Potato production has also come to be a

vital food resource of both economical and nutritional value in developing countries, where 25% of the world's current potato production occurs and potato consumption has doubled since the 1960's (Lutaladio & Castaldi, 2009). Included in the list of growing markets is Bangladesh. In a country with a population exceeding 164 million people, potatoes now make up more than half of the total vegetables grown (Masum et al., 2011). Global potato production has continued to grow over the past decade with current world production of potatoes exceeding 376,000,000 tons annually (FAOSTAT, 2018).

Although production continues to rise, significant crop losses limit the ability of producers to meet the current demand. Like many other food crops, the potato is susceptible to a host of diseases that, along with storage failures, are currently estimated to diminish annual total production by upwards of 37% – 40% (Oerke, 2006). One of the diseases faced in potato cultivation is the potato scab disease. This disease is caused by the infectious pathogen *Streptomyces scabies*, a soil-dwelling saprophytic bacteria species, and presents with the development of lesions, or scabs, on the potato surface (Kinkel et al., 2012). While it is not a direct risk to human health, scab disease can degrade potato taste and quality leaving the tuber unpalatable and commercially unmarketable. The lesions also impair the integrity of the tuber skin-like covering, increasing the risk of rot during storage.

While chemical intervention does treat and prevent some of the pest and soil disease issues of potato production, growing public and scientific concern over the human and environmental safety impacts of these production measures suggests a need to search for alternative options (Dich et al., 1997; Hirsch-Ernst et al., 2009; Repetto & Baliga, 1997). Studies show the chemicals used to inhibit pathogen proliferation and eradicate pathogenic populations may damage the beneficial soil life important to the long-term sustainability of field soils and

crop production and further push the soil ecosystem towards a pathogenically selective environment (van Elisas et al. 2012, Yousaf et al., 2013). Research also demonstrates increased human consumption of these chemicals positively correlates with increased rates of certain diseases including cancer, immunosuppression, and alteration of the human microbiota (Dich et al., 1997; Hirsch-Ernst et al., 2009; Repetto & Baliga, 1997). With growing public awareness, recent consumer demand for organic and naturally grown products has prompted increased scientific and agricultural investigation into natural pest and disease management systems.

CHAPTER TWO: THE CULPRIT

Streptomyces bacteria reside in almost every soil system on the planet. These bacteria aid in numerous processes of the soil cycle through their saprophytic abilities. *Streptomyces* bacteria are also well known for their ability to produce a wide array of antibiotic secondary metabolites. This array includes streptomycin, the first antibiotic found to treat tuberculosis patients. These bacteria also produce many other secondary metabolites of both medical and agricultural importance including immunosuppressants, anti-tumor agents, and insecticides and pesticides (Bignell et al., 2014). Furthermore, these bacteria play a vital role in the soil microbial community. *Streptomyces* species constitute about 10% of the total soil flora and are so engrained in the soil community that geosmin, one of the secondary metabolites produced by this microorganism, gives the soil its “earthy” smell (Schrey & Tarkka, 2008, Seipke et al., 2012). A unique feature of these filamentous bacteria is their ability to break down complex polymers including chitin and lignin; a process normally reserved to the soil fungi (Schrey & Tarkka, 2008).

Beyond interacting within their own populations, *Streptomyces* interact with soil bacteria, fungi, and plants. *Streptomyces* species have been found to colonize fungal hyphae of saprophytic, mycorrhizal, and pathogenic soil fungi and have been found to be both growth-promoting and/or growth-inhibiting depending on the situation or interacting species (Schrey & Tarkka, 2008). Most researched of these interactions is the *Streptomyces* and plant root interactions. These bacteria, well known to colonize the roots of various plant species, induce numerous actions of benefit or, though far less common, detriment to the plant. Many *Streptomyces* species aid in root colonization selection by releasing secondary metabolites that either aid or inhibit the certain bacterial species and mycorrhizal fungi trying to inhabit the root system of the plant (Schrey & Tarkka, 2008). Species of *Streptomyces* bacteria have also been found to promote root colonization, nodulation, and subsequent nitrogen-fixation by *Bradyrhizobium* and *Rhizobium* bacteria (Schrey & Tarkka, 2008). These bacteria may also directly promote plant growth through the synthesis of auxin, a potent growth stimulating hormone that aids in root growth and development (Seipke et al., 2012). In addition to secreting growth-promoting hormones, the microbes can aid in nutrient acquisition such as increasing iron and molybdenum assimilation along with other necessary growth factors (Seipke et al., 2012). Along with growth hormones, *Streptomyces* are also capable of secreting immune-modulating chemicals, which signal the plant to induce an immune response preventing infection by other pathogenic organisms. Higher proportions of antagonistic or nonpathogenic *Streptomyces* within a soil system is correlated with less disease within that system (Wanner, 2007).

Plant self-defense and disease resistance occurs with both local and systemic responses. Recent evidence suggests that *Streptomyces* interaction with the plant roots inhibits disease progression through multiple mechanisms beyond antibiotic inhibition of other soil-dwelling

pathogenic microbes (Olanrewaju & Babalola, 2019). Certain species are reported to induce a priming response in plants protecting the plants from above-ground pathogen attack as well as from root infection. This process, known as induced systemic resistance, is thought to be the result of plants viewing the non-harmful *Streptomyces* root-colonization as a “micro-infection” (Schrey & Tarkka, 2008). This is thought to lead to an up-regulation of jasmonic acid or ethylene signaling pathways that allows for a much quicker and stronger response of the plant to actual pathogen encounter, but it is not associated with detectable changes in gene expression in distant tissues (Schrey & Tarkka, 2008). However, not all *Streptomyces* species bring such benefits to their host plant species. Conversely to these symbiotic species, a certain subset of *Streptomyces* bacteria is pathogenic and detrimental to the production of a number of economically important root crop species. The most studied of these pathogenic species is *Streptomyces scabies*, the causative agent of common scab disease.

Streptomyces scabies invades the growing root tissues of the potato plant *Solanum tuberosum* resulting in the formation of raised or pitted necrotic lesions on the root tuber surface (Bignell et al., 2014). These bacteria are also capable of infecting a number of other root crop species including carrot, beet, radish, turnip, and parsnip under field conditions, which leads to the formation of similar lesions (Bignell et al., 2014). Research suggests the pathogen prefers to invade the plant roots or tuber during the early development of these structures, likely through the lenticel pore openings on the surface of these tissues (Dees & Wanner, 2012). These necrotic lesions greatly reduce the marketability of the crop and can lead to significant economic loss. Pathogenic success of these bacteria involves the production of a variety of secondary metabolites including phytotoxins, phytohormones, and proteins to manipulate host physiology and evade host response (Seipke et al., 2012). Some of these mechanisms include thaxtomin

phytotoxins, *nec1* necrogenic proteins, plant-immunity modulators, and melanin-like pigments, all of which *Streptomyces scabies* uses to its advantage when invading host tissues (Bignell et al., 2014). The majority of these phytotoxins and protein enzymes are secreted via a twin arginine protein transport (*tat*) pathway, which transports fully folded proteins across the cell membrane (Joshi et al., 2010). *Streptomyces scabies* utilizes the *tat* pathway in the secretion of 126 different proteins. Inactivation of this transport pathway results in the complete inhibition of virulence by *S. scabies* (Joshi et al., 2010).

The life cycle of *Streptomyces* bacteria aids in part to this virulence and is a reason why this bacterial genus is so successful in soil systems worldwide. *Streptomyces* are spore-forming bacteria and are able to spread quickly throughout and between soil systems. This is highly problematic in terms of pathogenic *Streptomyces* as these spores can be picked up on many inanimate objects including agricultural machinery and tires, shoes, or even the potato seed tuber itself. The spores are then transferred to new locations, spreading the pathogenic bacteria from one field to another. These spores are also resistant to desiccation and antibiotic treatment, similar to the endospores produced by other bacterial species, making them much more difficult to destroy. The endospores are able to survive for extended periods outside of the soil system, aiding in their ability to travel between field sites. Once the spore lands on a suitable substrate, it enters a life cycle more similar to a fungus than other bacteria. As the spore germinates, cell division ensues and substrate mycelium begin to form gathering food and resources for the growing *Streptomyces* (Mcgregor, 1954). At a certain point during the life cycle, thought to be density-dependent and quorum-sensing driven, these bacteria shift from substrate mycelium formation to the production of aerial hyphae. It is during this period that antibiotic production increases, which is also mediated by bacterial quorum-sensing (Becker et al., 1997). While it is

likely that this aids in the protection of the newly forming spores, which bud off from the aerial hyphae, many of these secondary metabolites are released in sub-inhibitory concentrations suggesting that these compounds are acting as signaling molecules (Seipke et al., 2012).

During their life cycle, *Streptomyces* produce a number of secondary metabolites to enhance survival in the soil system. In *Streptomyces scabies* many of these secondary metabolites are involved in aiding the bacteria to colonize the roots and rhizome of the growing potato plant. Survival of a bacterial species is also inherently entwined with the ability of that species to proliferate and pass its genes onto the next generation. In the bacteria world, this can occur in one of two ways. The first mechanism is through asexual cell division, or in *Streptomyces*' case spore formation. The second mechanism is via sexual reproduction through such processes as conjugation, in which genes are horizontally transferred from a donor to recipient. Lastly in transformation, bacteria can acquire pieces of DNA from a lysed cell in its environs. The genes coding for the regulation and production of the virulence factors of *Streptomyces scabies* are all found on a large pathogenicity island (PAI), similar to those seen in many another pathogenic bacteria (Kers et al., 2005). As shown in studies of other PAI containing bacteria, this does allow for the transfer of virulence genes between *Streptomyces* bacteria. The occurrence of this transfer must be limited given the overly small percentage of pathogenic *Streptomyces* within heavily populated *Streptomyces* communities (Kers et al., 2005). However, the low G + C content of many of the open reading frames (ORF) within this PAI, as compared to the relatively high G + C content (~71%) of the remaining *Streptomyces scabies* genome suggests that these virulence factors were acquired from an outside source (Kers et al., 2005). The pathogenicity island of *S. scabies* is split into two segments. The first segment, known as the toxicogenetic region, contains the genes encoding for thaxtomin production and

regulation, while the second segment, known as the colonization region, contains the genes *nec1* and *tomA* (Dees et al., 2014). These genes, though found in many *S. scabies* strains, are not conserved amongst these pathogens and are not required for virulence despite their aid in the infective process (Dees et al., 2014).

The best studied of these virulence factors, and conserved amongst *Streptomyces scabies* strains, are the thaxtomin phytotoxins. These phytotoxins are capable of inducing scab-like lesions on aseptically cultured tubers and are able to cause necrosis on potato tissue (Bignell et al., 2014). Thaxtomin A is the primary thaxtomin produced by *S. scabies*. In addition to causing necrosis of plant tissue thaxtomin A is capable of stunting monocot and dicot seedling growth (Bignell et al., 2014). Research suggests that the primary action of thaxtomin is the inhibition of cellulose biosynthesis (Bignell et al., 2014). By impeding synthesis of cellulose, thaxtomin weakens the cell wall of plant tissue allowing easier entry into the growing tuber for the *S. scabies* pathogen.

Thaxtomin is a cyclic dipeptide composed of L-phenylalanine and L-4-nitrotryptophan subunits (Bignell et al., 2014). The 4-nitro group and L-L configuration are necessary to the phytotoxic activity of these compounds. A multitude of genes are involved in the regulation and production of the thaxtomin phytotoxins (txt) and they are clustered on two operons (Bignell et al., 2014). The first operon contains *txtA*, *txtB*, *txtH*, and likely *txtC* genes, while the other operon contains *txtD* and *txtE* genes. These genes are highly conserved between *S. scabies* and other similar pathogenic *Streptomyces* species. The bioproduction of these molecules by *S. scabies* begins with the production of nitric oxide (NO) from arginine catalyzed by nitric oxide synthase coded for by *txtD*. *TxtE* codes for cytochrome P450, which then catalyzes the nitration of L-tryptophan by NO. The newly produced L-4-nitrotryptophan acts as substrate to the *txtB* encoded

non-ribosomal peptide synthase (NRPS) and is combined with L-phenylalanine, a *txtA* NRPS substrate, to give the thaxtomin D intermediate. Thaxtomin D is then methylated creating the thaxtomin A final product. The production of this compound is regulated by the *txtR* encoded AraC-family transcription regulator, and is dependent on the presence of cellubiose, which serves as a ligand for the *txtR* protein (Bignell et al., 2014).

The *nec1* gene, found on the colonization segment of the PAI, further contributes to the virulence of these pathogens. The *nec1* gene encodes for a necrogenic protein capable of inducing necrotic lesions on excised tuber tissues, causing cell swelling, and the formation of gall-like necrotic structures on growing root tissue (Joshi et al., 2007). The low G + C content (~54%) of this gene suggests it was acquired via horizontal gene transfer from an unrelated taxon of bacteria (Bukhalid et al., 1998). However, the lack of homology to any other known bacterial gene suggests that this protein may be a novel virulence factor (Bukhalid et al., 1998). Evidence also suggests that gene transfer of this virulence factor occurs between the majority of pathogenic *Streptomyces scabies* strains, as well as *S. acidiscabies* and *S. turgidiscabies* (Bukhalid et al., 1998). The exact function of *nec1* is still uncertain. The necrotic nature of the protein may aid the bacteria in establishing an infection and gaining access to the underlying tissue of the growing root structure. However, this may not be its only function. *Nec1* proteins were found in media after 20 hours of growth, much earlier than the 48 hours of growth needed to detect thaxtomin production, which suggests that this virulence factor is not regulated through the same mechanisms as thaxtomin production and may aid in earlier infection of the root cells (Joshi et al., 2007). There is also evidence to suggest that this protein inhibits plant immune response to thaxtomin phytotoxins, enhancing the maximal infectivity of these pathogenic organisms (Joshi et al., 2007).

Certain *Streptomyces scabies* strains also possess a *tomA* gene homologous to many pathogenic fungal species (Seipke & Loria, 2008). This gene codes for a tomatinase (saponinase) enzyme that acts as glycosyl hydrolase degrading phytoanticipins of the plant immune response (Seipke & Loria, 2008). In fungal pathogens of the tomato plant this enzyme is used to detoxify α -tomatine, which would otherwise induce cell lysis of the fungal pathogen (Seipke & Loria, 2008). Exact function of this enzyme in the infection cycle of *S. scabies* is still unclear. While this enzyme is not necessary for virulence of these species, it may function to reduce immune response of the host plant (Seipke & Loria, 2008). This enzyme may also function to protect spore formation during the lifecycle of *Streptomyces* bacteria. Interestingly, when *Streptomyces* lacking the *tomA* gene were exposed to α -tomatine vegetative growth of the bacteria was not inhibited, but aerial hyphal growth and spore production were altered (Seipke & Loria, 2008). When the *tomA* gene was present, hyphal growth and spore formation were uninhibited (Seipke & Loria, 2008).

In addition, *S. scabies* also produces concanamycin and coronatine-like (COR) phytotoxins. Concanamycins are known to act as ATPase inhibitors and were shown in study to inhibit root growth, but their contribution to common scab disease is still uncertain (Bignell et al., 2014). The production of COR-like compounds is coded for on a biosynthetic gene cluster remarkably similar to the coronafacic acid gene cluster of *Pseudomonas syringae* (Bignell et al., 2014). While *S. scabies* lack the coronamic acid (CMA) genes necessary to produce COR, the novel genes in *S. scabies* suggest it likely produces metabolites similar to the minor coronafacoyl metabolites of *P. syringae* (Bignell et al., 2010). Though shown to add to the development of root disease of tobacco seedlings, the COR-like compounds are likely secreted to suppress host plant immune response as COR has been shown to inhibit jasmonic acid dependent gene

expression necessary for plant growth and defense against herbivory and necrotic pathogen attack (Bignell et al., 2010).

In addition to the COR-like phytotoxin and *tomA* enzyme, *Streptomyces scabies* also produces a melanin-like pigment (Beauséjour & Beaulieu, 2004). Production of melanin pigments by *Streptomyces scabies* appears to be tied to thaxtomin production. Significant proportions (~63%) of *S. scabies* mutants lacking the ability to produce melanin pigments were also inhibited in the production of thaxtomin with reductions ranging from 18%-88% (Beauséjour & Beaulieu, 2004). However, this did not seem to diminish the pathogenicity of these organisms (Beauséjour & Beaulieu, 2004). Although not necessary to the virulence of these bacteria, melanin-like pigments did offer protection from environmental stressors and, similarly to *tomA*, prevented the inhibition of spore formation by host defense mechanisms (Beauséjour & Beaulieu, 2004). Given the antioxidant nature of these pigments, it may also be that their production protects the bacteria from immune degradation by the host species after infection, as seen in other pigment-producing bacteria like *Staphylococcus aureus* (Liu et al., 2005).

Evidence suggests numerous factors are involved in the infective process of *Streptomyces scabies*. The Sanger Institute recently annotated the genome of *S. scabies*, finding it to contain 10,148,695 base pairs coding for thousands of genes along the linear chromosome of this organism (Sanger Institute, 2014). The function of many of these genes are still unknown and research will likely uncover novel means of virulence regulation. However, given the current information, thaxtomin production seems to be the single largest contributor to pathogenicity of *Streptomyces scabies* and the causative mechanism behind tuber lesion formation in common scab disease. As food scarcity issues continue to threaten global economies, the necessity of new measures to prevent crop disease continues to increase. *Streptomyces scabies* already poses a

worldwide threat to the production of potatoes, the world's fourth largest food crop. As with any other disease, the pathways involved in the infection process are complex and multifactorial.

CHAPTER THREE: PLANT MEDICINE

When host defense systems fail, opportunistic pathogens can capitalize on the situation and establish infection. It is generally understood that treatment of an infection often requires a two-part approach. The first part of treatment requires the destruction of the pathogen. In the realm of agriculture this often involves the use of pesticides and/or other chemical fumigants. The second phase of treatment revolves around strengthening the host to prevent a repeat and new or super infection during the healing process.

In current agricultural practices, chemical pathogen management strategies are a main stay in the standard treatment of crop diseases including potato scab. The use of these products has profoundly expanded since the 1960's, when many of these chemicals were identified and synthesized. Of the chemical pesticides and fumigants available, fludioxonil, pentachloronitrobenzene, and chloropicrin are the most established treatments of potato scab disease (North Dakota State Univ., 2018). However, the effectiveness of these methods is variable and there is still work needed to elucidate the long-term consequences of this approach. While standard and often effective in the year to year management of scab and other plant diseases, such approaches may convey broad-ranging health effects to the consumer, especially when multiple chemicals are used and thereby consumed in combination (Hohenadel et al., 2011, Blair et al., 2015). Of the research available, pesticide exposure has been strongly linked to a wide array of human health conditions including non-Hodgkin's lymphoma and other cancers,

reduced cognitive processing and development, Parkinson's disease, Alzheimer's disease, diabetes, and obesity (Blair et al., 2015).

Furthermore, the combined use of multiple pesticides may have broad ranging effects in the surrounding environment. While it is difficult to study the additive and/or synergistic effects of these chemicals *in vivo*, research has shown evidence to suggest combined pesticide exposure to bees can negatively affect foraging behavior and increase worker bee mortality rates (Gill et al., 2012). When evaluating their effect on soil health, research has demonstrated the use of such chemicals often comes with unwanted effects including negative environmental impacts, reduced soil fertility, and negatively altering the soil microbiome (Popp et al. 2013, Yousaf et al., 2013). This damage to the beneficial soil microbiome may push the soil ecosystem towards a pathogenically selective environment (van Elisas et al. 2012). Therein, while the treatment may have been effective that year, the reduced soil fertility and microbiome alterations may unfortunately limit the fitness of future crops in that field.

Alternative biocontrol methods have shown promise in both reducing and preventing common scab. Microbial inoculants, crop rotation, cover cropping, and green manure have all demonstrated efficacy in reducing scab occurrence (Dees & Wanner, 2012; Liu et al., 1995; Sturz et al., 1998; Wiggins & Kinkel, 2005). Of these methods, green manure applications of beneficial plants such as soybeans, alfalfa, and clover have not only demonstrated an ability to reduce scab occurrence, but have also proven to be an established method of improving soil fertility (Abawi & Widmer, 2000). These green manures have proved effective at improving both nitrogen cycling and microbial community richness and biodiversity (Longa et. al, 2017).

Early studies on potato scab disease investigated the relationships between the beneficial and pathogenic saprophytes and amounts of available soil organic material (Millard & Taylor,

1927). Original thoughts on the matter of organic material and disease suppression revolved around the availability of microbial “food” (i.e. the available green material in the soil) and the levels of disease. However, research completed by Millard and Taylor in the late 1920’s found this was not entirely true. Instead, they demonstrated levels of scab disease were more related to levels of competition between pathogenic and beneficial *Acintomyces* (former name for *Streptomyces*). Their research suggested that scab occurrence was reduced when the pathogenic species were outcompeted by other *Acintomyces* species (Millard & Taylor, 1927).

This early works supports more current research completed by Dr. Donna Becker and Dr. Linda Kinkel, who have studied the scab resistant crop fields of Minnesota. Their research validated the work by Millard and Taylor and found significantly higher proportions of antagonist *Streptomyces sp.* in scab-resistant crop fields (Kinkel et al., 2011). Their research found higher populations of antagonistic *Streptomyces spp.* residing in soil communities were correlated with suppression of pathogenic virulence and disease (Kinkel et al., 2011). It is therefore likely that in order for a treatment to be sustainably beneficial, it must cause a more permanent shift in the soil microbiome towards antagonistic *Streptomyces* species.

Applications of these green manures may induce these shifts in *Streptomyces* populations and support the growth of antagonistic species over pathogenic microbes (Wiggins & Kinkel, 2005). Medicinally bioactive plants in particular promote a distinct and highly specific microbiome in part due to numerous secreted bioactive secondary metabolites (Qi et al., 2012). Furthermore, not only do these medicinal plants produce a number of antibiotic phytochemicals capable of altering *Streptomyces* populations, but the endophytic bacteria they harbor are also capable of producing a variety of their own novel antibiotics and biochemicals, which may aid in this effect (Strobel et. al, 2004).

Research by Dr. Ushiki in the 1990's showed the potential for the extracts of medicinal plants to inhibit the growth of *S. scabies* and use as an alternative in the treatment of potato scab disease (Ushiki et al., 1996). Takenaka et al. (1997) also showed rosemary extract was capable of significantly reducing potato scab disease. Additionally, soil incorporation of some aromatic plants as green manures has shown potential in reducing potato scab occurrence (Boydston & Hang, 1995; Cheah et al., 2001; Griffin et al., 2009; Sturz et al., 1998). Given this information and the known benefit of green manuring, there is a likelihood that plant species, especially those with antibacterial properties, could be specifically selected to reduce pathogen presence and simultaneously promote the growth of antagonistic species.

INVESTIGATIONS

The challenge arises in selecting the proper plant species for use. Although shown effective in *in vitro* studies, certain plant species may carry negative effects in regards to potato production. For example, Takenaka et al. (1997) showed rosemary extract was capable of significantly reducing potato scab disease. However, the use of this plant was found to induce strong sprout suppression of the potato tuber (Vokou et al., 1993). Feasibility of use is a major factor that must be addressed. Some species which demonstrated scab reduction qualities *in vitro* required long propagation and extended greenhouse cultivation to be available at time of potato plantings or proved hard to find in adequate quantities at such time.

While some research is available, published literature on the use of medicinal plant species as green manures is still lacking. Small scale agriculture tends to be more experimental in its approach to disease management. In this realm, there is growing anecdotal evidence for the use of medicinal and aromatic plant species in pest management processes. Comfrey (*Symphytum officinale*) and calendula (*Calendula officinalis*) are two specifically that receive a lot of attention in this regard. Directed study on the use of these species can add to this body of literature.

Comfrey (*Symphytum officinale*) is a herbaceous perennial known for its deep taproot that “mines” leached nutrients and water from the deeper soil layers. In the past, this plant has been evaluated for use as a forage crop and for its benefit as a botanical medicine in the topical treatment of wounds, burns, and other injuries (Teynor et. al, 1997). While it is gaining popularity in home gardening as a green manure plant, little research is available in the literature for its use as a green manure crop. The leaves of this plant are high in minerals including calcium, shown in studies to help prevent potato scab disease (Palta, 2010; Staiger, 2012). Once

established, comfrey produces abundant foliage with a high nitrogen content shown in study to improve crop yields (Teynor et. al, 1997, Sincik et al., 2008). Extracts of comfrey are found to include triterpene saponins, tannins, alkaloids, amino acids, flavonoids, triterpenes, terpenoids, tannins, saponins, sterols, mucopolysaccharides and other hydroxycinnamic acid derivatives (Salehi et. al, 2019). Included in these, extracts also show a high content of allantoin, ellagic acid and rosmarinic acid (Savić et. al, 2015). Allantoin is well studied and shows potent anti-inflammatory properties and epithelial growth stimulation in study (Shestopalov et. al, 2006). Allantoin has also demonstrated an ability to alter the antibiotic production of certain *Streptomyces* species (Navone et. al, 2013). This plant grows wild on the site of study and is generally available in sufficient quantities at the time potatoes are planted, making it a viable option as a green manure treatment.

Calendula (Calendula officinalis), regarded in folk medicine as a treatment for common skin diseases, is an annual plant whose anti-inflammatory and antibacterial property are well studied in the literature (Fronza et al, 2009; Ukiya et al., 2006). This plant is used as a botanical traditional medicine, especially for wound healing, and has demonstrated effect with anti-HIV, cytotoxic, anti-inflammatory, and hepatoprotective properties (Muley et al., 2009). Evaluations have identified numerous compounds in plant material including triterpenoids, flavonoids, coumarines, quinones, volatile oil, carotenoids and amino acids (Muley et al., 2009). Of note, calendula extracts consistently demonstrate potent antibacterial properties against numerous groups of pathogenic bacterial (Shahen et. al, 2019). Calendula matures in a short time, approximately 60 days in green house conditions, as compared to other options. For this reason, it could be planted early in the spring and be available for harvest at planting. Both calendula and

comfrey are often used in combination to treat wounds and skin conditions and are believed to be synergistic in their effects, further justifying these as a choice for trial.

METHODS

Prior to field trials, it was important to determine that it was indeed *Streptomyces scabies* that was causing the disease issues on the farm. To do so, infected potato tubers and soil samples were taken from scab conducive soils from the Seeds and Spores Family Farm in Marquette, Michigan, where eventual field trials would occur. Nine soil samples, each approximately 15 g in volume, were taken in total. Three samples came from each of the three scab conducive fields where scab positive potatoes were harvested that season. Each sample was taken using a trowel from a random, dispersed location within the scab conducive fields. Samples were stored in air tight bags under refrigeration at 4 °C overnight.

The samples taken from each of the three fields were mixed and 10 grams of mixed soil from each field was diluted into 1 liter of sterile distilled water (dH₂O). One hundred mL of each solution was extracted into 250 ml Erlenmeyer flasks and shaken overnight on a platform shaker rotating at 245 rpm at room temperature to ensure the separation of bacteria from soil particles. Next, 100 µL samples from each beaker were plated onto Oatmeal Agar augmented with antibiotics (See appendix A) to encourage the growth of *Streptomyces* spp. over other soil bacteria. Samples were incubated at 28 °C for 5-7 days until there were positive morphological signs of spore formation. Isolates from each plate were sub-cultured and spread plated onto Oatmeal agar plates, augmented with L-Tyrosine (See appendix A) to indicate the putative presence of *S. scabies*. This amino acid is used in the production of a melanin-like pigment by

the pathogen. Studies have shown melanin-like pigment production of *Streptomyces* species on agar augmented with L-tyrosine to be indicative of pathogenicity (Keinath & Loria, 1989).

To isolate *Streptomyces* from potato tuber samples, three potato tubers from random locations of scab conducive fields were used that showed positive signs of scab formation. The potato tubers were surface sterilized with 70% ethanol solutions for one minute and allowed to dry. All scab lesions were carefully cut from root tubers, blended into 100 mL of dH₂O, and serially diluted to 10⁻⁴. Serial dilutions were streak plated onto water agar (see Appendix A) to encourage the growth of *Streptomyces* spp. versus other possible microbes and incubated at 28 °C for 5-7 days until signs of sporulation appeared. Samples were then sub-cultured onto Oatmeal Agar plates augmented with L-Tyrosine and again incubated at 28 °C for 5-7 days until sporulation. Isolates from the cultures were transferred and incubated on fresh oatmeal agar plates (see Appendix A) until pure cultures were achieved.

In preparation for the first season of green manure trials, calendula (*Calendula officinalis resina*) from Johnny's Seed Co. (Fairfield, Maine) was grown in greenhouse conditions at Northern Michigan University. Seeds were planted three seeds per each cell in standard 32 cell seedling trays. The soil used was a 50:50 mixture of Happy Frog Potting Soil and FoxFarm Ocean Forest Smart Naturals Potting Soil. The plants were allowed to grow without thinning, as not all germinated, and to promote competition that may induce increased production of secondary metabolites (Broz et al., 2010). Plants were harvested after 55 days growth by cutting at the base of the plant stem at soil level. Comfrey (*Symphytum officinale*) was harvested wild in similar fashion onsite at Seeds and Spores Family Farm (Marquette, Mi). Each was harvested just before final maturity (both just starting to flower) due to the semi-random nature of timing for the season.

Harvest of the medicinal plants occurred on the morning potatoes were planted. Each species was harvested and stored separately in the shade until planting. Plant material was rough chopped (similar to tillage). To mimic real world application of green manures, the actual amount of green material added per length was decided based on ease of home gardening application. Similar to other antibiotic use, there is likely a dosage threshold for therapeutic effect and, quite possibly, toxicity. Plant material was added to the trench rows in quantity sufficient to cover the base of the row in a single layer of plant material, as would a home gardener. Total amounts of each plant material varied greatly due to the significant difference in plant matter density. Three treatments were completed and included calendula, comfrey, and a mixed 1:1 by volume ratio of the two. Given the varying amounts of plant material available, row length of each treatment varied between groups. After the laying of the green manure, Yukon Gold potatoes from Fedco Seed Potatoes (Clinton, Maine) were cut and planted at 20 cm spacing per standard protocol. In total, treatments consisted of 13.1 m of calendula at 0.28 kg/m, 13.4 m of comfrey at 0.65 kg/m, and 9.1 m of an approximate per volume 1:1 calendula/comfrey mixture at 0.48 kg/m. The remainder of the three rows of Yukon Gold, 92.8 m total, was left untreated as control. All plots, treatment and control, were equally fertilized with 6-1-1 with zinc + boron, at approximately $.15 \text{ kg/m}^2$, which could have promoted increased fertility leading to less scab overall.

Potatoes were harvested at 107 days from planting. Potatoes were rough cleaned and weighed for total weights per harvest group. Potatoes were then rinsed, counted, and rated for scab individually according to a standardized scab rating scale (see Table 2). Soil samples from three random points in each treatment group were taken, as was a sample from the soil prior to

planting. Soil samples were sent to A&G Great Lakes Laboratories in Ft. Wayne, IN for analysis (Table 5).

Differences in scab occurrence based on rating were analyzed between the groups using one-way analysis of variance (ANOVA) and post-hoc Tukey examination. Differences in average weight of harvested tuber per row length, number of tubers per row length, and % organic matter pre and post treatment were also analyzed descriptively due to the limitations of the study. Data analysis was completed using IBM SPSS v24 (Armonk, NY).

During the second season of trials, comfrey (*Symphytum officinale*) was used alone as a green manure treatment. This choice was made given the data from previous season demonstrating decreased scab occurrence and improved harvest yields. As well, comfrey grows wild on site which made for low input, easy obtainability, and minimal economic cost, as compared to that necessary to cultivate calendula in a greenhouse environment prior to planting. The comfrey plant sprouts and matures earlier in the season than the potatoes and plant material is generally available in sufficient quantity at the time of planting. This makes it a viable choice for year to year use and would be more likely chosen by smaller agricultural ventures. The comfrey used was again harvested wild onsite at Seeds and Spores Family Farm (Marquette, MI).

Harvest of comfrey occurred the morning of potato planting. Plant material was rough chopped and added to the trench rows in a similar manner to the previous season's trial, in quantity sufficient to cover the base of the row in a single layer of plant material. The second year's trial occurred on an adjacent field to the previous year to allow for crop rotation, but had grown potatoes previously and shown signs of scab disease. Upon application of the green manure, Yukon Gold potatoes from Fedco Seed Potatoes (Clinton, Maine) were again cut and

planted at 20 cm (8") spacing per standard protocol. In total, treatments consisted of 18 m of chopped comfrey at a similar approximate 0.65 kg/m. These treatments were split and placed at random in the area of study in three treatment sites each 6 m in length. The rest of the field was left untreated. The entire plot was again fertilized with 6-1-1 with zinc + boron, at approximately 0.15 kg/m². At maturity, potatoes were harvested and grouped in 2 m sections. Two sections were divided from each of the three treatment zones (n=6) and six 2 m sections were taken at random from the remainder of the rows (n=6) as control. The potatoes were then rinsed, counted, weighed individually, and rated for scab using the same standardized scab rating scale as used previously (Driscoll et al., 2009).

Differences in scab occurrence based on rating were analyzed between the control and treated groups using unpaired T-test analysis. Differences in average weight of harvested tuber and number of tubers per row length were also analyzed.

RESULTS

Five (5) putative *Streptomyces* isolates were obtained from the soil samples (1-4) and potato scab lesions (5). The first *Strep.* (1) isolate became grey in appearance during sporulation and produced a yellow pigment when plated on the oatmeal agar plates augmented with L-Tyrosine. *Streptomyces* isolate 2 also developed a whitish/grey appearance during sporulation but produced a melanin-like, dark pigment when cultured on the OA+L-Tyrosine media (See Figure 1 for comparison of pigment producing and non-pigment producing isolates). *Streptomyces* isolate 3 was another soil isolate that produced a yellow pigment on the L-Tyrosine augmented OA but was white in appearance upon sporulation. The last soil isolate (4) produced no pigment when plated on the OA+L-Tyrosine Media and grew white in appearance upon

sporulation. *Streptomyces* isolate 5 was the only isolate obtained from the potato scab lesion. This isolate also developed a grey/white appearance during sporulation and produced a melanin-like pigment when cultured on the OA+L-Tyrosine media. See Table 1 for a listing of the putative *Streptomyces* isolates and their characteristics.

Potatoes were rated for scab using a standardized scale from Driscoll et al. (2009) (Table 2). In the first pilot season trial, a statistically significant difference in scab occurrence was found between treatments as determined by one-way ANOVA ($F(3,1865) = 31.216, p = 0.000$) (Table 3). A Tukey post-hoc test revealed that the levels of scab occurrence after treatments of calendula ($1.70 \pm 0.86, p = 0.000$) or comfrey ($1.72 \pm 0.82, p = 0.000$) were significantly lower than the control group (2.16 ± 0.90) while the mixed treatment of calendula and comfrey showed no significant difference in scab occurrence from the control group ($2.30 \pm 0.91, p = 0.316$) (Table 3). Interestingly, there was no significant statistical difference in scab reduction between calendula and comfrey treatments ($p = 0.997$) (Table 3). The treatment of calendula and comfrey, thereby, showed a 21.3% and 20.4% reduction in scab occurrence respectively for an average reduction of approximately 20.9% overall. Furthermore, the use of the comfrey green manure showed a 24% increase in average harvested weight per length over the control group (1.68 kg/m vs 1.35 kg/m) and a 20% increase in harvested weight per length over the average of all groups (1.68 kg/m vs. 1.40 kg/m) (Table 4). This was attributed not to larger tuber sizes but due to a greater number of tubers harvested per meter. Though limited, the data showed 3.4 extra tubers harvested each meter for comfrey groups over the control groups (17.4 tubers/m vs. 14 tubers/m) and an extra 2 tubers per meter for the comfrey groups over the average of all control + treatment groups (17.4 tubers/m vs. 15.4 tubers/m) (Table 4).

Soil analysis from the first season found little correlation between decreased scab occurrence and soil variables. Calendula and comfrey treatments did however show the lowest pH of the analysis (6.5 and 6.7 respectively) and a decrease from pre-treatment and control groups (Table 5). Soil acidification is one method of scab disease control. This reduction in pH may have contributed to the decreased levels of scab disease post-treatment. This was unlikely however, as other treatments showed a pH at similar levels (pH 6.8 and 6.9). Other analyses varied. Notably, calendula treatment showed increased levels of potassium post treatment with considerably higher levels noted for both the calendula and mixture treatments over other groups (Table 5). Though often used as a control method, evidence for increased levels of calcium provided by comfrey as responsible for changes in scab rates was lacking in this study as calcium levels post comfrey treatment were less than control and pre-treatment quantities (Table 5) (Palta, 2010; Staiger, 2012). Of other note, zinc levels were considerably lower for all groups than those seen pre-treatment (Table 5).

In the second field trial the next growing season, comfrey showed noted reductions in scab disease occurrence. Levels of scab occurrence after treatments of comfrey (1.55 ± 0.60 , $p = 0.000$) were significantly lower than the control group (1.91 ± 0.76) (Figure 2). Percent reduction of disease was similar to that seen in the pilot study with treatments showing an 18.8% drop in disease rates overall. Unfortunately, during the second season, comfrey failed to show a statistically significant increased total harvest. Though the differences were not statistically different, green manure treatment with comfrey did demonstrate improved average harvest weight per row length (1.12 ± 0.38 kg/m vs. 1.05 ± 0.40 kg/m, $p=0.804$) and an increase in the average number of tubers harvested per length at 16.5 ± 5.97 tubers/m vs. 14.6 ± 4.68 tubers/m for control plots ($p=0.628$) (Figure 2). Difference in average weight of each tuber was also

limited between treatment and control groups with 67.7 ± 32.0 g/tuber vs. 72.8 ± 36.4 g/tuber respectively ($p=0.200$) (Figure 2). It may be that the addition of green manure failed to improve harvest yields or the limitations of the size of the study failed to show significance in the results. Further research is needed to elucidate these findings.

DISCUSSION

Of the five *Streptomyces* species isolated, isolates 2 and 5 displayed the melanin-like pigment production indicative of the pathogenicity of *S. scabies* (Figure 1) (Beauséjour & Beaulieu, 2004). These two isolates showed similar growth characteristics regarding sporulation. One of these isolates was isolated directly from a scab lesion (Table 1). Given their similar growth characteristics and their ability to grow on an oatmeal agar supplemented with antibiotics, the plausibility that these were indeed *S. scabies* was high. This information, along with the yearly observation of potato scab lesions at the farm, gave high likelihood that the pathogen at hand was *Streptomyces scabies*.

As potential treatments against this pathogen, both calendula and comfrey treatments showed significant scab reductions in the first season of trial. The treatments both reduced the occurrence and severity of scab by more than 20% (21.3% and 20.4% respectively). A follow-up study using comfrey as a green manure showed similar results. While further study is necessary to prove its effectiveness in larger-scale trials, the perennial nature, minimal input requirements, and obtainability of this plant at time of potato planting make it a viable choice for, at minimum, small farms in the treatment of scab disease. The use of comfrey green manure during the pilot trial exhibited a 24% increase in harvested weight per length over the control group and a 20%

increase in harvested weight per length over the average of all groups. These results were not verified by statistical analysis the second season. However, though not statistically significant, mean harvest weight and number of tubers harvested per meter did show similar, though limited trends. An improved harvest yield and greater percentage of marketable tubers both add value to this treatment option and further study should look to investigate these results.

In the first season, differences in amount of green manure treatment between treatment groups may have led to these effects. However, soil analysis post-treatment actually showed higher amounts of percent organic matter (or at minimal no significant difference in percent organic matter) following the calendula treatment (lowest overall amount of green manure by weight) over the comfrey treatment (4.2% vs. 3.8%). This suggests there is an alternate reasoning behind the increase in production after comfrey green manure application. The high nitrogen content of comfrey may have been responsible as nitrogen amendment is shown to improve crop yields but more research is necessary to state this definitively (Teynor et. al, 1997, Sincik et al., 2008).

The use of standard green manures to treat potato scab disease has been well established and documented. Unfortunately, one of the main issues with this method of disease suppression is inconsistency (Wiggins & Kinkel, 2005). There is minimal research however, on the use of medicinal plants as green manure currently available for which to compare this study to. Research by Ushiki et al. (1996) did find that plant material from geraniums (*Geranium pratense*) was capable of reducing scab occurrence. However, this study failed to show statistically significant results. While there are likely numerous possible reasons for the discrepancy between this and the results found in this study, two in particular may address differences noted between the results generated in this study using calendula and comfrey as

compared to those by Ushiki et al. (1996). These two factors are often addressed to successfully eliminate a pathogen and include the selection of the correct antibiotic for the pathogen and the correct dosage of the antibiotic to reach therapeutic effect.

It may be that these factors are similarly applicable in the use of antibiotic green manures in the treatment of soil disease. Abstraction in effect between this study and that by Ushiki et al. (1996) may highlight the importance and challenge of choosing the correct “antibiotic” for treatment of the soil pathogen. In addition, correct dosing or quantity of material per area is likely necessary for proper and efficient treatment. Further study into the use of these green manures at varying concentrations could elucidate these requirements.

Nonetheless, these factors may only in part describe the reasoning behind discrepancy and the effectiveness shown by comfrey and calendula in this study. While the antibiotic compounds inherent in the plant material studied may have directly inhibited growth specifically of pathogenic *Streptomyces* species, there is significant evidence to suggest it is more likely due to shifts in microbial community composition. A study by Cohen et al. (2005) found that pathogen suppression of *rhizoctonia* root rot in orchard soils with *Brassica napus* seed meal was more likely the result of altered communities of both pathogenic and saprophytic soil microorganisms rather than direct inhibition of pathogen via bioactive, antibiotic glucosinolate products. Interestingly, in this study it was found specifically that *Streptomyces* species populations increased significantly in response to the brassica soil amendment. More specifically, it was the beneficial, antagonistic species that thrived with the treatment that led to reduction in disease prevalence (Cohen et al., 2005).

In terms of the control of potato scab disease by calendula and comfrey in this study similar alterations of bacteria communities are likely involved. Conventional applications of

green manure are shown to increase inhibitory activity of antagonistic *Streptomyces* (Wiggins & Kinkel, 2005). It has been found that scab resistant soil systems are generally composed of higher antagonistic to pathogenic ratios of *Streptomyces* species (Kinkel et al., 2012). It may be that the addition of the organic matter from green manure applications of calendula and comfrey similarly promoted antagonistic species. Yet, simple numbers of antagonist are likely insufficient to explain long-term resistance in scab-resistant soil systems as sole applications of antagonist *Streptomyces* inoculants often fails to yield consistent results in disease reductions (Compant et al., 2005).

While the addition of bulk green material may have supported the growth of antagonist *Streptomyces* species, other factors are likely involved. In the 1920's, Millard and Taylor (1927) criticized the “preferential food theory” as the mechanism of soil disease resistance. It is therefore probable the secondary biometabolites incorporated along with the bulk fibrous material likely aided in the effects seen. Allantoin, as noted previously, is a major constituent of comfrey and was shown in one study to alter the antibiotic production of certain *Streptomyces* species (Navone et. al, 2013). This supports theories of disease management described in Kinkel et al. (2011) that “...strategies should aim to initiate or enhance an antagonistic coevolutionary arms race” between *Streptomyces* species. The addition of selected bioactive plant metabolites that alter metabolism of antagonistic species also coincides with research by Becker et al. (1997) which described the role of bacterial signaling variation between scab-resistant and scab-conducive soil systems as a factor in the development of resistant soil systems. Research has revealed that plants produce a number of secondary metabolites, specifically root exudates, to alter the growth and development of specific bacterial species directly through the alteration and or exploitation of bacterial signaling molecules (Fray, 2002; Bais et al., 2006). Future study

could look to elucidate these interactions and work to select plant species specifically for the secondary metabolites capable of altering these signals.

CONCLUSIONS

If to be more widely accepted and practiced in lieu of other, often less desirable disease control methods, the results of this treatment need to prove consistent over time. In the future, research could improve the current study for broader application and better clarify the results gathered in a number of ways. Side by side comparisons of comfrey and calendula green manures to traditional green manures would help identify potential differences in treatment effectiveness. Having known this would be a multi-year project, tracking of *Streptomyces* populations and density could have eluded to increased populations of beneficial *Streptomyces* species being responsible for changes in disease occurrence. Additionally, year to year tracking of populations in treated soils could help demonstrate long-term effect of single treatments. This data could be augmented by comparisons between densities of melanin-pigment producing to non-pigment producing species. This study was also limited by availability of resources. Though appropriate for the pilot nature of this work, larger treatment trials are needed to truly interpret results. Though attempts were made to minimize abstractions, treatment effect was limited to certain field locations, wherein large applications would better account for variations of nutrient availability and moisture content between locations, even within a given plot.

Yet, the results of this two-year study of medicinal plants, specifically comfrey as a potential green manure treatment of potato scab disease proved promising. While the nature of this investigation was that of a pilot study, there appears ample evidence which warrants the

further exploration of this treatment option. An average 19.6% reduction in scab disease over the two seasons would be significant, to say the least, especially in terms of a multibillion-dollar industry. If a paired yearly increase in total harvest could be found it could make these results that much more attractive. Furthermore, this treatment option qualifies as organic, providing added label value if substituted for conventional treatment methods. Financial motivation is strong in all industries. While a cost-benefit analysis would be needed to ascertain the value of these treatments on larger scales, if proven consistent, the results found in this study merit such investigation.

With all research comes more questions and more opportunities for investigation. While this research described plant species with antibacterial properties specifically as being medicinal, in actuality it is likely that many plants are medicinal when applied under the right circumstance. As research continues to explore and expand the understanding of plant-microbe interactions and the bioactivity of plant metabolites, there is further opportunity to connect this knowledge to the application of soil disease management.

APPENDIX A: MEDIA PREPARATION

Media

Oatmeal Agar (OA)

Gerber's brand Baby oatmeal flakes (20 g) were added to 1 L of distilled water in a 2 L Erlenmeyer flask. The agar (15 g) and casamino acids (1.0 g) were added and mixed using a magnetic stir bar and the solution was brought to near boiling. The agar was autoclaved for 40 minutes at 121 °C. Agar was poured into Fisherbrand™ 100 x 15 mm petri plates as needed.

Oatmeal Agar plus Tyrosine (OAT)

The recipe for Oatmeal Agar OA was followed with the addition of 5.0 g L-Tyrosine.

Oatmeal Agar plus Tyrosine and Antibiotics (OATA)

Oatmeal Agar plus Tyrosine was prepared as noted above.

The antibiotics (Cycloheximide (100,000 ppm), Penicillin (10,000 ppm), Polymyxin B (10,000 ppm), and Nystatin (10,000 ppm) – See antibiotics below) were added after the agar was sterilized and cooled but prior to media setting (~50 °C). OATA was poured into Fisherbrand™ 100 x 15 mm petri plates as needed.

Oatmeal Broth (OB)

OB is prepared by mixing the Gerber brand baby oatmeal (20 g) and casamino acids (1.0 g) with distilled water in a 2 L Erlenmeyer flask. A magnetic stir bar is used to thoroughly mix the ingredients. The broth is autoclaved for 40 minutes at 121 °C.

Water Agar

One liter of distilled water was and agar (15 g) were added in a 2 L Erlenmeyer flask and mixed using a magnetic stir bar as the solution was brought to near boiling. The agar was autoclaved for 40 minutes at 121 °C. Agar was poured into Fisherbrand™ 100 x 15 mm petri plates as needed.

Antibiotics

Cycloheximide (10,000 ppm)

Cycloheximide solution is added to OATA at a concentration of 100 mg/L by adding one mL of stock solution to one L of agar. Cycloheximide stock solution is made by adding one ml of original solution to 99 mL of distilled water. The solution is filter sterilized using a 0.45 µm syringe filter.

Nystatin (10,000 ppm)

Nystatin 10 g/L stock solution was mixed into dimethyl sulfoxide (DMSO) immediately before use. Nystatin solution was filter sterilized with a 0.45 µm nylon Acrodisc™ syringe filter and added directly to the agar prior to pouring.

Penicillin (10,000 ppm)

Penicillin (10 g) was mixed into distilled water. The solution was filter sterilized using a Millipore® apparatus with a 0.2 µL filter pad. The sterilized solution was stored at 4 °C until use.

Polymyxin B (10,000 ppm)

Polymyxin B (10 g) was mixed into distilled water and filter sterilized using a Millipore® apparatus with a 0.2 µL filter pad. The filtered solution was stored at 4 °C until use.

TABLES AND FIGURES

Isolate #	Location	Color Characteristics of Sporulation	Pigment Production on OA + L-Tyr.	Antagonistic/ Pathogenic (Putative)
1	Soil	Grey	Yellow	Antagonistic
2	Soil	White-Grey	Melanin-like (Black)	Pathogenic
3	Soil	White	Yellow	Antagonistic
4	Soil	White	No Pigment	Antagonistic
5	Lesion	White-Grey	Melanin-like (Black)	Pathogenic

Table 1: Characteristics of isolated *Streptomyces* species from soil and potato scab lesions. Putative identification of antagonistic or pathogenic based on presence or lack thereof of melanin-like pigment production on OA + L-Tyrosine.

Scab Rating	Scab Lesion Percent % Surface Area	Pitted Lesion Percent % Surface Area	Depth of Pitted Lesions mm
0	0	0	0
1	1 to 10	0	0
2	11 to 25	0	0
3	26 to 50	1 to 5	< 1
4	> 50	6 to 25	> 1
5	> 50	> 25	> 1

Table 2: Scab rating system used to evaluate tubers in field trials. Rating system from Driscoll, J. et al., 2009.

Anova: Single Factor						
SUMMARY						
Groups	Count	Sum	Average	Variance		
Control	1299	2812	2.164742109	0.809510931		
Calendula	196	334	1.704081633	0.742752486		
Comfrey	234	403	1.722222222	0.664997616		
Mixture	140	322	2.3	0.830215827		
ANOVA						
Source of Variation	SS	df	MS	F	P-value	Fcrit
Between Groups	73.60921278	3	24.53640426	31.21602486	1.09761E-19	2.60967377
Within Groups	1465.926368	1865	0.7860195			
Total	1539.535581	1868				
Tukey-Kramer Procedure						
		Num df	Qu	3.258		
			4	Den df	1865	
Comparison	Absolute Difference	Critical Range	Results			
Control-Calendula	0.460660477	0.156510	Sgnificant Difference			
Control-Comfrey	0.442519887	0.145048	Sgnificant Difference			
Control-Mixture	0.135257891	0.181683	No Sgnificant Difference			
Calendula-Comfrey	0.01814059	0.197766	No Sgnificant Difference			
Calendula-Mixture	0.595918367	0.226011	Sgnificant Difference			
Comfrey-Mixture	0.577777778	0.218231	Sgnificant Difference			

Table 3: Statistical analysis of pilot trial potato scab occurrence. Data shows ANOVA and Tukey-Kramer procedural analysis of potato scab ratings per treatment group.

Treatment	Control	Calendula	Comfrey	Mixture
Total Tuber Count	1299.0	196.0	234.0	140.0
Total Length of Row (m)	92.9	13.1	13.4	9.2
Avg # Tubers per m	14.0	14.9	17.4	15.3
Avg Harvest kg per m	1.4	1.3	1.7	1.2
Avg Wt. per Tuber (kg)	0.1	0.1	0.1	0.1
Avg Scab Occurrence	2.2	1.7	1.7	2.3

Table 4: Summary data of potatoes harvested by treatment in pilot field trials. Note that total length and harvest of control group was far larger than treatment groups.

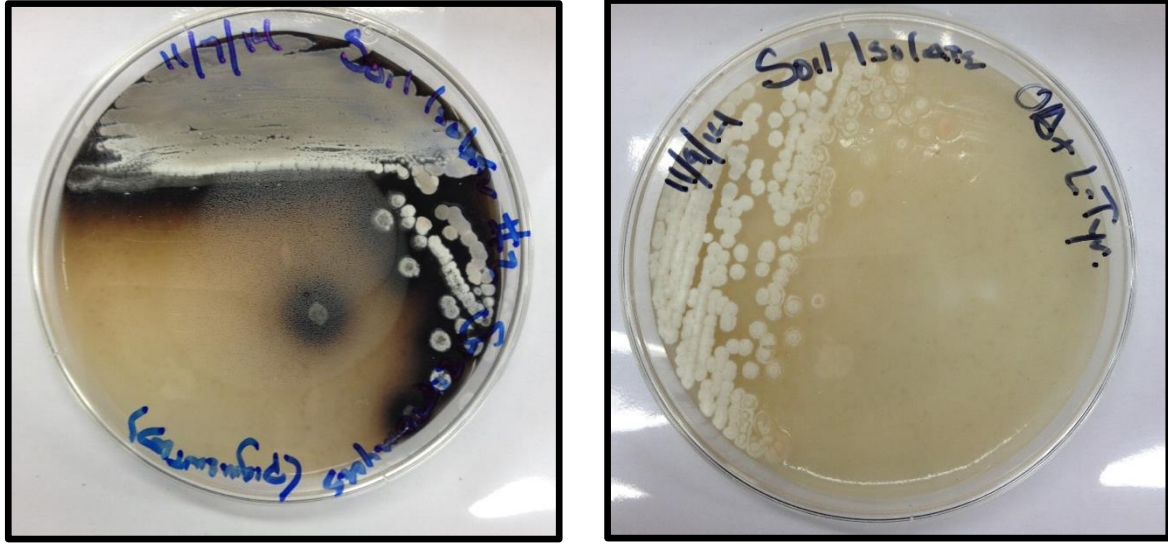


Figure 1: Streak Plates of *Streptomyces* isolates #2 (left) and #4 (right). Soil isolate #2 demonstrates the melanin-like pigment indicative the pathogenicity of *S. scabies*.

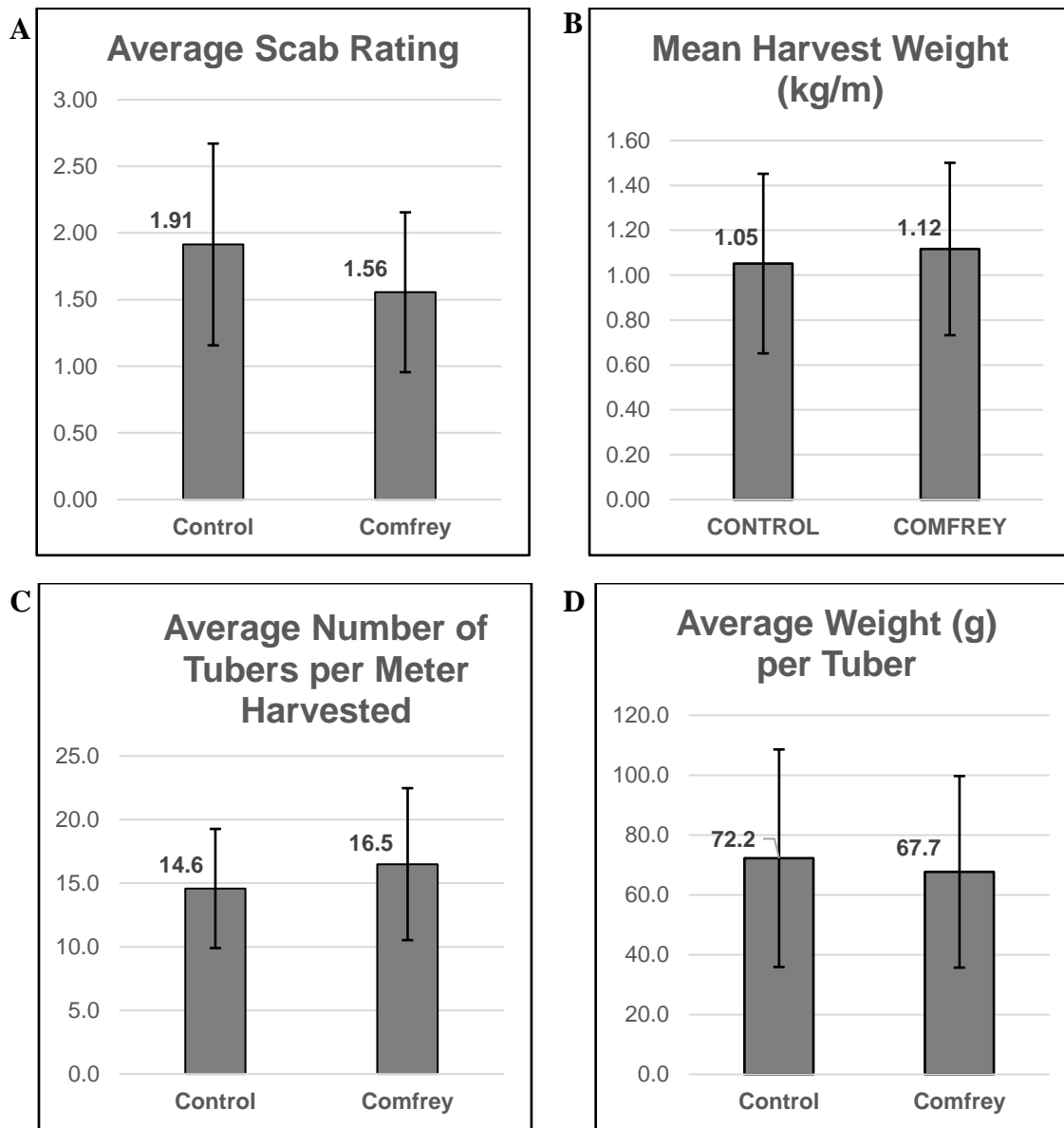


Figure 2: Graph comparison between control and comfrey treatment groups season two of **A.** Average scab rating, **B.** Mean harvest weight (kg/m), **C.** Number of tubers harvested per meter, and **D.** Weight (g) per tuber. Significant difference was found between average scab ratings of treatment and control groups.

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