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Endophytic Actinomycetes as potential agents to control common scab of potatoes

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ENDOPHYTIC ACTINOMYCETES AS A POTENTIAL AGENT TO CONTROL COMMON SCAB OF POTATOES

By

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Endophytic Actinomycetes as Potential Agents to Control Common Scab of Potatoes

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ABSTRACT

ENDOPHYTIC ACTINOMYCETES AS POTENTIAL AGENTS TO CONTROL COMMON SCAB OF POTATOES

By

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Potato marketability and tuber quality can be decreased upon infection by a pathogen, *Streptomyces scabies*. Current disease control consists of expensive irrigation systems and using hazardous chemicals, which exert limited disease control and are potential hazards to those exposed. Biological control offers a cost effective method for controlling disease. Antibiotic producing *Streptomyces* have indicated their ability to combat plant diseases. Some *Streptomyces* species colonize within plant tissues without causing disease symptoms (endophytes). This research sought for the presence of endophytic *Steptomyces* or closely related species in potato plants that were grown in fields containing pathogen-inhibiting *Streptomyces*. To establish genus level identification of the endophytes, the 16S rRNA gene was sequenced. To assess inhibition abilities of endophytes against the pathogen, double layer antibiotic assays were done by spot inoculating endophytes and overlaying agar with the pathogen. Multiple layer agar plates were used to test the paired interactions among endophyte isolates on their ability to use quorum sensing to enhance antibiotic production to inhibit the pathogen. In each assay zones of inhibition were measured. From distinct potato stem tissues, four putative,

Streptomyces strains were isolated. The 16S rRNA sequencing indicated a 99% match to *Microbispora*, closely related to the *Streptomyces* genus. Our results demonstrated that an isolate (called 1) could inhibit the pathogenic strain 87. Isolate 1 also produced zones of reduced growth of the pathogen in pairwise combinations with the other 3 isolates. This research is among the initial studies that are trying to utilize endophytes for biological control agents.

DEDICATION

This thesis is dedicated to my family for their support of my educational endeavors.

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Introduction

The Potato

Increasing healthy crop yields is an important task for the agricultural industry. Potatoes (*Solanum tuberosum*) are not only a regionally relevant food item for residents of the mid-Western United States, but also across the world due to their role as a staple food. The tuber of the potato is extremely nutritious and contains many vitamins and minerals, as well as antioxidants. Potato plants are highly productive and rich sources of protein (Al-Saikhan et al., 1995).

Tubers of potatoes are underground modified stems. Potatoes develop ideally during cold summer temperatures with plenty of soil moisture and tuber formation is favored during short days. Propagation of potatoes is done by planting whole tubers or pieces of tubers that have at least one "eye," termed "seed tubers." The eyes on the tuber's surface are the buds from which new aboveground stems will develop. The tuber is an underground stem that develops one to several stems which over time become the main stems on the new plant. As the plant develops and matures the underground stems extend outward and become new tubers. Depending on the variety, a potato has a life span of 80 to 150 days from planting to maturation. After maturation, stems begin to die

and the outer covering called the periderm thickens (Fernie and Willmitzer, 2001). The periderm protects the potato from invading microorganisms, debris, and abrasion. The outer region of the potato is also covered with pores, termed lenticels, that allow for gas exchange. Lenticels effectively permit gas exchange while being small enough to prevent most microorganism from invading the tuber (Fernie and Willmitzer, 2001).

Potatoes are susceptible to diseases that impact their marketability. To achieve greater agricultural productivity, that is also sustainable, the dependence on the microbial population associated with agricultural plants is becoming increasingly important (Conn and Franco, 2004). Research has looked into the pathogenic microorganisms as well as the beneficial microorganisms that have impacted crop yields. Examples of microbial agents that have been successful in inhibiting soil-borne pathogens include *Streptomyces* and *Bacillus* genera (Sessitsch et al., 2002).

Pathogenicity

Potato plants and other agricultural foods are susceptible to diseases that can be caused by pathogenic microorganisms located in the soil where they are grown. For instance, common scab of potatoes is most commonly caused by a pathogenic bacterium, *Streptomyces scabies* (Liu et al., 1995). Common scab causes the tubers of potatoes to exhibit lesions and deep pits on the surface. In some cases, a black border is visible between lenticels. Because the potato market is quality driven, their appearance affects their marketability (Lerat, 2009; Wharton et al., 2007) (Figure 1). Synthesis of phytotoxins, termed thaxtomins, is necessary for virulence of scab-causing strains. *S. scabies* produces five related phytotoxins; among these, thaxtomin A is the main source

of virulence in pathogenic *Streptomyces* spp. and has been shown to be 20 times more virulent in comparison to a similar compound thaxtomin B (Lerat, 2009; Loria, 1995) These phytotoxins produce the necrosis observed in scab infected tubers and hypertrophy. Hypertrophy in plant tissues results from an inhibition of cellulose synthesis (Lerat, 2009).

Figure 1. Potatoes exhibiting scab caused by *Streptomyces scabies* (Loria, 1991).

Currently, the practices to control common scab in potatoes have been to apply chemical treatments, and use irrigation systems, crop rotations, applications of green manures, and soil fumigation. However, the use of chemicals and soil fumigants impact the soils and are potential hazards to the farmers, consumers, and wildlife exposed (McKenna, 2001). Breeding for resistant varieties is unattainable for disease control and has yielded little success. Irrigation systems are costly to small farming operations, making them an impractical solution to combating the disease. Biofertilizers that contain antibiotic producing strains, lethal to the pathogen causing common scab (*S. scabies*), have also been used in disease controlling efforts (Liu et al., 1995).

Endophytes

Plants can be involved in a symbiotic relationship with microorganisms. In this relationship the microorganisms gain a place to reside, within the tissues of the plants, while the plants may gain protection from pathogens and access to nutrients that are made available to them by the microorganism. Additionally the microorganisms receive the environmental stability that rhizosphere or non-endophytic microbes lack (Sturz et al., 1999). Endophytes are bacteria or fungi that colonize the interior of plant hosts where nutrient sources are readily available, but do so without causing disease symptoms (Reiter et al., 2002; Zinniel et al., 2002). Endophytes can enter plant tissues through their stomata, damaged secondary root zones, aerial portions (stems, flowers, cotyledons, and germinating radicles (Zinniel et al., 2002). Following colonization the endophytes may spread to further regions of the plant body. The degree to which this occurs and the destined location varies with intrinsic factors of the host plant as well as the mode of colonization by the endophyte (Andreote et al., 2009). Because of their close, stable relationship with plant hosts and their ability to suppress disease while promoting plant growth, endophytes have the potential to be great sources of disease fighting agents (Conn and Franco, 2004; Zinniel et al., 2002).

Von Bodman et al. (2008) found that 61 of 192 endophytic bacteria isolated from stem tissues of potatoes were successful control agents against *Clavibacter michiganesis*, the pathogen that causes ring rot in potatoes. Endophytic bacteria have also been isolated in oak, where they have been active against *Ceratocystis fagacearum*, the pathogen causing oak wilt (Conn and Franco, 2004). Tall fescue harboring endophytic fungi,

Neotyphodium coenophialum, were shown to have a significant increase in response to water stress in deficient locations. This was attributed to the ability of tall fescue symbiotic with the endophytic fungi to have increased solute uptake allowing for greater control over osmotic adjustments, which becomes particularly important when maintaining turgor (Nagabhyru, 2013). Previously documented enhancements to host plants simply due to the presence of endophytes include: greater resistance to herbivores, insects, and microbial pathogens. Plants also exhibit increased tolerance to stress conditions with endophytes present. These mentioned enhancements are credited to the alkaloid production by the particular endophytes involved in this relationship (Bush, 1997). The following endophytic isolates have been located in wheat roots: *Microbispora* sp. strain EN2, *Streptomyces* sp. strain EN27, and *N. albus* EN46. Two species, *Streptomyces celluloseae* and *Streptomyces albidoflavus,* have not been reported to exhibit any endophytic links with plants (Conn and Franco, 2004). It is important to note that not all microbes will have the ability to colonize endophytically.

Streptomyces

One particular endophytic group of bacteria of interest is *Streptomyces* species. These bacteria are classified as aerobic, Gram- positive, filamentous soil bacteria belonging to the order Actinomycetales, family Streptomycetaceae, and genus *Streptomyces*. *Streptomyces* are considered soil saprophytes that play a critical role in nutrient cycling (Kinkel et al., 2012). *Streptomyces* have a complex lifecycle where vegetative hyphae emerge from germinating spores, termed conidia, and the hyphal filaments grow by tip extension (Figure 2). *Streptomyces* species have a wide geographic distribution. The same strains can be found in very different parts of the world, making

them an applicable species to study because of the commonalities among regions (Loria et al., 1997).

Streptomyces are useful to study because of their ability to produce antibiotics as well as other secondary metabolites (Davelos et al., 2004). Secondary metabolites are chemicals made by the cell that do not have a vital role in maintaining cellular growth or function (Keller and Surette, 2006). The majority of known antibiotics used in medicine today have come from soil-borne species of *Streptomyces* (Castillo et al., 2006).

Some of the substances produced by various *Streptomyces* species discourage the growth of other pathogenic *Streptomyces* species and other pathogenic microbes in general (Becker et al., 1997). Many strains of *Streptomyces* are non-pathogenic to humans and harmless to plants.

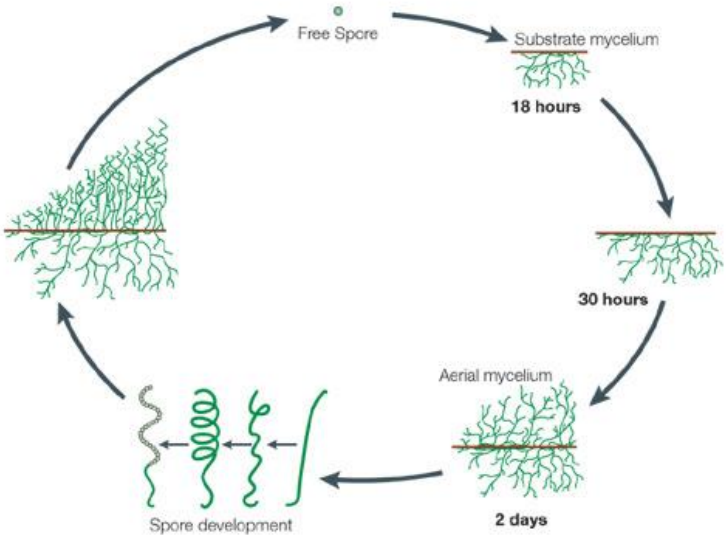


Figure 2. The complex lifecycle of *Streptomyces* species (Esther, 2005).

Biological control

Practices of disease control in the agricultural industry has focused on previously mentioned methods (e.g. fumigants, chemicals, resistant crops). There is an increasing demand for healthy foods due to the constant growth of the human population. This demand requires controlling and decreasing diseases existing in crops. There are strong concerns related to these disease control practices, among these are soil contamination, safety regulations for animals and humans in contact with the chemicals, and the development of resistant pathogens (Emmert and Handelsman, 1989). Therefore, there is an increasing demand for new methods and strategies to effectively control disease without using harmful chemicals. Microorganisms offer an ability to suppress disease in plants without the use of harsh, synthetic chemicals (Han et al., 2005). Microbe populations are extremely diverse, abundant, and share an intimate environment with the crops of interest (Emmert and Handelsman, 1989). For these reasons biological control or the use of non-pathogenic microbes to reduce or prevent the diseases caused by pathogenic microbes, has the potential to be a more forceful and direct approach to its counterpart methods (Whipps, 2001).

Streptomyces species have the ability to produce antibiotics, making them able to notably impact plant health (Davelos et al., 2004). In fact, two antibiotic producing strains of *Streptomyces* demonstrated suppression of common scab disease in a field study conducted in Minnesota (Liu et al., 1995). Soils that have become void of pathogenic strains are termed suppressive, while soils that have high densities of pathogens are termed conducive. Potato scab soils have been well documented among suppressive soils studied, which have indicated *Streptomyces* as playing a significant role in the suppression. The ability of *Streptomyces* to survive and increase their population in

the soils that they inhabit make them great candidates for biological control agents (Liu et al., 1995). Field studies of potato scab infected soils in Minnesota have revealed that populations of non-pathogenic *Streptomyces* have turned these conducive soils suppressive over a period of time (Lorang, 1989; Ndowora, 1996). From 1943 to 1965 a breeding plot, located in Minnesota, which was used for the selection of scab resistant plants demonstrated a decline in scab occurrence. By 1987 *Streptomyces* strains from tubers grown in that plot demonstrated the ability to suppress the growth of pathogenic *S. scabies* (Ndowora, 1996).

Gram-positive bacteria, such as *Streptomyces* and *Bacillus* species, offer the advantage of spores versus gram-negative bacteria, which do not produce spores. Spores can be dried and stored for years without losing viability, thereby making Gram-positive species advantageous in biological control practices (Emmert and Handelsman, 1989). Using microbes that are good antibiotic producers has recently been the course of action when choosing an agent for biological control. Antibiotic producing microbes allow for disease control, with their antibiotics being easily broken-down, essentially leaving no detrimental residues behind (Han et al., 2005). Although this is a viable method, there has been limited consistency in disease control and new options need to be explored.

It is known that endophytic microbes exist and currently there is a growing realization that they could potentially be a new and more effective option for use as biological control agents in agricultural settings due to their intimate, long-existing relationship with their plant partners. Understanding the interactions among endophytic microbes and their plant hosts will hopefully prove them to be an alternative source for controlling disease. Gaining knowledge of how they enter their plant hosts, what their

interactions are, and whether or not we can influence them for biocontrol purposes are all pertinent to advancing current agricultural disease control practices.

Communication

In the past, bacterial communities have been studied as individual populations that act independently. Recently it has been indicated that this is not the case, and that there are significant interactions and communication occurring within populations. Bacteria are able to sense and respond to chemicals in their surrounding environment. Bacterial communication is known as quorum sensing. Quorum sensing is a system that allows bacteria to communicate with each other and gather information on the density of bacteria within their environment (Keller and Surette, 2006). Quorum sensing employs the use of multiple, easily diffusible, signaling molecules which allow microbes to behave as one large organism (Becker et al., 1997). It has been suggested that quorum sensing evolved to allow bacteria to coordinate the group's behavior, thereby taking on attributes of multicellular organisms (Keller and Surette, 2006).

Among the many microorganisms that are able to communicate via quorum sensing, is the well-studied *Vibrio fischeri*. *V. fischeri* has the ability for luminescence when in a symbiotic relationship with both fish and squid (Dunlap, 1999). Nealson et al. (1970) have reported that luminescence occurred at high cell densities. This cell-density control of luminescence is mediated by the accumulation of a diffusible chemical, called an autoinducer that *V. fischeri* produces. Autoinducers are chemicals that are produced by one microorganism and can affect the actions of surrounding microorganisms (Keller and

Surette, 2006). This same type of density-dependent gene expression has also been found in another luminous species, *Vibrio harveyi* (Dunlap, 1999).

Many other bacterial species including *Escherichia coli, Pseudomonas aeruginsoa,* and *Bacillus subtilis* make use of quorum sensing. (Bjarnsholt, 2007; Diggle et al., 2007; Sperandio, 2002). *E. coli* employ quorum sensing to regulate genes that encode for virulence including production of the Shiga toxin and motility via flagella (Sperandio, 2002). For *Pseudomonas aeruginosa,* a pathogen capable of infecting plants, insects, and animals, quorum sensing is critical for virulence. Quorum sensing controls behaviors including biofilm development, motility and swarming, as well as the production of extracellular virulence factors which cause bloodstream invasion and tissue damage. In *P. aeruginosa* quorum sensing is controlled by two pathways that regulate the synthesis of signaling molecules called N-acyl homoserine lactone (AHL). These two systems use different AHL signaling molecules. Six to ten percent of its genome is regulated by the very systems which control AHL, demonstrating the importance of quorum sensing to this microbes behavior (Bjarnsholt, 2007; Diggle et al., 2007).

Bacillus subtilis is a microbe that can produce endospores during unfavorable conditions in order to persist in that environment. After an assessment of alternative responses to the stressors has been done, many of the cells will undergo sporulation. In a case of 168 strains studied, 50%-70% of the cells sporulated under environmental stress. Research has been conducted and devoted to understanding the decision making system that allows microbes to decide to sporulate or to wait. It is presently understood that the decision to sporulate is preceded by cell to cell communication using peptide pheromones as signaling molecules to assess cell density (Schultz et al., 2009).

Streptomyces use gamma-butyrolactones (GBLs) as quorum sensing signals which coordinate population behaviors and stimulate the synthesis of secondary metabolites as well as morphological differentiation (Wang et al., 2011). *Streptomyces* also sporulate to persist in their habitat as nutrients deplete or under unfavorable conditions (Recio et al., 2004). Antibiotic production is an important means of protection for *Streptomyces* against competing organisms; thus strains that are able to do so are more likely to persist in the environment (Becker et al., 1997). While bacteria may use quorum sensing as an assessment of recognition of self with in a mixed population, it seems that they also must be able to detect the presence of the other surrounding species in this mix (Federle and Bassler, 2003). In *Streptomyces*, diffusible signaling molecules which induce antibiotic synthesis have been documented indicating that most antibiotics are produced when cells are at an increased density. The presence of a suppressive strain and a pathogenic strain prompted the synthesis of antibiotics by another suppressive strain, suggestive of interspecies communication (Becker et al., 1997). Becker et al. (1997) demonstrated that a suppressive strain produced antibiotics earlier and at a lower density upon the addition of another, distinct suppressive strain's exudates to culture media. In the absence of the exudates, antibiotic production was induced later and at high cell densities indicating that the strains are able to communicate and impact antibiotic production in each other (Becker et al., 1997).

There is a myriad of interactions occurring among species of microbes within the soil system. Gaining knowledge of how these microbes interact with each other, as well as the plants they share the soil with is pertinent to advancing biological control options. Quorum sensing employed by *Streptomyces* species has the potential for a unique

approach for determining the appropriate species to use in biological control. Understanding the ability of *Streptomyces* of different species to communicate could allow us to determine a combination of species that work synergistically to be effective biological control agents. In this study I assessed the different combinations of endophytes that are most successful in communicating with each other to enhance their antibiotic production. The placement of non-pathogenic endophytic bacteria directly into plant tissues has the potential to be a successful and sustainable avenue to controlling diseases in the agricultural industry.

Aims & Goals

The increasing demand for higher crop yields in combination with the current limited disease control methods indicates that new methods and strategies for effective disease control need to be explored. The long term goals of this area of research are to develop a non-synthetic disease control system for farmers and to enhance our current understanding of the interactions among microbes and the plants that exist with them in the soil system. The focus of this work is to explore the use of *Streptomyces* endophytes as tools for biological control by first isolating them from stem tissues and then testing each isolate against one another as well as the pathogen. Gaining knowledge of what interactions are occurring and the impacts of quorum sensing on antibiotic production are pertinent to advancing current disease control practices.

Experimental Design

Using potato stem and tubers obtained from 60 plants collected from a biocontrol experiment station in Becker, MN in 2012, and 60 stems obtained from an experimental

field in Becker, MN in 2013, endophytic microbes were isolated for use in Petri plate assays, and 16S rRNA sequencing.

To obtain information on the identity (phenotypes and genotypes) of the isolates, the 16S rRNA gene was sequenced. Sequencing provided insight on the identity of the organisms.

Antibiotic assays were performed to determine antibiosis abilities of endophytic isolates against *Streptomyces scabies*, pathogenic strain 87 as well as against each other. The assay was done with all pair wise combinations of the four isolates. Zones of inhibition were measured to determine antibiotic sensitivity and resistance abilities of the isolates to the pathogen and to each other.

Quorum sensing assays were performed to determine the ability of the isolates to communicate with one another in a manner that works synergistically against the pathogenic strain 87. The assay was done with all pair wise combinations of the four isolates. Zones of inhibition were measured to determine inhibition abilities of the isolates to the pathogen.

Methods

Isolation of endophytes

Endophytic bacteria were isolated from potato stem tissues that were collected from Pingping Sun's 25 treatment biocontrol experiment conducted in Becker, MN in 2012 and an experimental field in Becker, MN in 2013. Samples obtained from each

experiment were treated differently as indicated below. Isolated endophytes were imaged using an Olympus SZ6045 (Model: LMS-226) dissecting scope with a camera attached.

Tissues obtained in 2012

The outside of the stem tissue was surface sterilized aseptically under a biological safety cabinet in three solutions (2 minutes in 20% bleach, 1 minute in 70% ethanol, and 1 minute in sterile water). Stems were checked for sterility by pressing and rolling the tissues onto oatmeal agar and incubating for 24 hours at 28 C. Samples with growth within that 24 hour time period were discarded. Two methods of isolation were used. Using a sterile scalpel, cross-sections of tissue were thinly sliced (3-5 mm) and the cross-sectioned tissue was laid out onto oatmeal agar plates (OA) and incubated at 28º C to get the endophytic isolates to grow out onto the agar. The second method involved a macerate of tissue to obtain endophytes. The stem tissues were massed at 1 g and then surface sterilized. The tissue was placed in a sterile container with 50 mL of sterile water and hand blended in a pulsating manner. The blender was sterilized by soaking in 70% ethanol for 3 hours prior to use and then allowing the ethanol to dry off before use. The blender used was Miallegro®, model MiTutto® Turbo (550 watts). Serial dilutions of this macerate were made (10^{-1}-10^{-5}) and transferred to oatmeal agar plates (OA), starch casein plates (SCA), and water agar plates (WA).

Tissues obtained in 2013

The stem tissues obtained in 2013 were surface sterilized in three solutions (6 minutes in 5% bleach, 3 minutes in 70% ethanol, and 3 minutes in sterile water). Stems were checked for sterility by pressing and rolling the tissues onto oatmeal agar and

incubating for 24 hours at 28°C. Samples with growth were discarded. Two methods of isolation were used. Using a sterile scalpel, cross-sections of tissue were thinly sliced (3-5 mm) and the cross-sectioned tissue was laid out on OA plates and incubated at 28º C to get the endophytic isolates to grow out onto the agar. The second method involved a macerate of tissue to obtain endophytes. The stem tissues were massed at 2 g and then surface sterilized. The tissue was placed in a sterile container with 50 mL of sterile water and hand blended in a pulsating manner. Serial dilutions of this macerate were made (10^{-1}-10^{-5}) and transferred to OA amended with antibiotics to inhibit fungal and contaminating bacterial growth.

Non-*Streptomyces* endophytes

Bacterial colonies obtained that were not identified as *Streptomyces* spp. were imaged using a dissecting scope with a camera attached. Any plates containing 30-300 colony forming units (cfu) were counted and recorded. All other plates were discarded.

Making stock cultures of isolated *Streptomyces* endophytes

Stock cultures of putative *Streptomyces* strains were made by streaking the isolated strains onto OA, WA, and OA amended with antibiotics. Plated cultures were placed into metal trays with an open container of water to keep the agar from drying out, and covered with another inverted metal tray. Cultures were incubated at 28°C until vigorous sporulation was observed. *Streptomyces* spores were scraped off of five streak plates using sterile cotton swabs and transferred into a sterile test tube containing 3-5 mL of 20% glycerol. The swab was twirled vigorously to dislodge spores and then vortexed for 30 seconds. The stock cultures were stored in a -80°C freezer and streaked onto plates

after 24 hours to ensure viability of the cells. This technique was performed for isolated endophytes 1, 5, and 9 as they each sporulated in a reasonable time. For isolated endophyte 8, sporulation was slow; therefore, the above was performed except that colonies, rather than spores, were scraped off streak plates and stored in 20% glycerol.

Isolation Media

Oatmeal Agar (OA)

Oatmeal agar (OA) is one type of media that favors *Streptomyces* growth. OA is often amended with antibiotics that suppress fungal and bacterial growth, both of which allows for identification of targeted *Streptomyces* spp.

Twenty g of oatmeal (Gerber's ™ Baby Oatmeal) was added to 1.0 L of distilled water into a 2.0 L Erlenmeyer flask containing a magnetic stir bar, while heating on a hotplate to melt the agar. One g of casamino acids (caa) was added and dissolved. Fifteen g of granulated agar was then added to the flask. The OA was autoclaved for 25 minutes.

To amend the OA with antibiotics, before autoclaving 500 mL of OA was added to two, 1.0 L bottles, labeled, and autoclaved for 25 minutes. The bottles were then placed in a water bath to cool the agar to 50°C. Nystatin (10,000 ppm), Cycloheximide (100,000 ppm), Penicillin (10,000 ppm), and Polymyxin B (10,000 ppm) were added and the agar was poured into petri plates.

Antibiotics

Nystatin-fungicide

Nystatin was added to OA at a concentration of 100 mg/L by adding 10 mL of a stock solution to one L of agar. The stock solution was made by mixing 10 g of nystatin into 100 mL of dimethyl sulfoxide (DMSO) under a fume hood. The solution was not filter sterilized, but was stored at 4°C until used.

Cycloheximide-fungicide

Cycloheximide solution was added to OA at a concentration of 100 mg/L by adding one mL of stock solution to one L of the agar. The stock solution of cycloheximide was made by adding 4 g of cycloheximide at room temperature to a mixture of 20 mL of 95% ethanol and 20 mL of water. The stock solution was filter sterilized using a 0.45 µm nylon acrodisc syringe filter and was stored at 4°C until used.

Polymyixin B- antibacterial

Polymyxin B stock solution was added to OA at a concentration of 10 mg/mL by adding one g polymyxin to 100 mL of distilled water. This was then filter sterilized and stored at 4°C until used.

Penicillin-antibacterial

Penicillin solution was added to OA at a concentration of 1.0 mg/mL by adding 0.1 mL of stock solution to 1.0 L of agar. The stock solution was made by adding 0.5 g of penicillin at room temperature to 50 mL of distilled water. This was then filter sterilized and stored at 4°C until used.

Starch Casein Agar

Starch casein agar was used in this study to target *Streptomyces* spp. and decrease the amount of non-*Streptomyces* spp. growing on the agar. Ten g of starch, 0.30 g of casein, 0.02 g $CaCO_3$, 15 g granulated agar, 100 mL of solution A, 100 mL of solution B, were added to 800 mL of distilled water, mixing with a stir bar on a hotplate in a two-liter Erlenmeyer flask. Solution A was made with 20 g KNO_3, 20 g NaCl, and 20 g K_2HPO_4 in 1.0 L of distilled water. Solution B was made with 0.5 g $MgSO_4 \cdot 7H_20$, 0.1 g $FeSO_4 \cdot 7H_2O$, and 0.01 g $ZnCl_2$ in 1.0 L of distilled water. The media was then autoclaved for 25 minutes.

Other Media

Water Agar

Water agar was used as an overlay in the antibiotic assay involving pathogenic strain 87 with the isolates. Fifteen g of granulated agar was added to 1.0 L of distilled water into a 2.0 L Erlenmeyer flask containing a magnetic stir bar, while heating on a hotplate to melt the agar and mix the ingredients. The media was then autoclaved for 25 minutes.

Spore Agar

Spore agar is a medium that is rich in nutrients and is used to promote sporulation of bacteria. Ten g of glucose, 2.0 g tryptose, 1.0 g beef extract, 1.0 g yeast extract, and 1.0 µg of $FeS0_4$ were added to 1.0 L of distilled water into a 2.0 L Erlenmeyer flask containing a magnetic stir bar, while heating on a hotplate to melt the agar and mix the ingredients. The media was then autoclaved for 25 minutes.

Soy-flour Mannitol Agar

Soy-flour mannitol agar was also used in this study to promote sporulation because of its rich sources of nutrients. Twenty g of agar, 20.0 g of soy-flour, and 20 g of mannitol were added to 1.0 L of distilled water into a 2.0 L Erlenmeyer flask containing a magnetic stir bar, while heating on a hotplate to melt the agar and mix the ingredients. The media was then autoclaved for 25 minutes.

Colony Polymerase Chain Reactions (PCR)

Colony PCR was modeled after a protocol obtained from Dr. Kurt Galbreath of Northern Michigan University. Using a pipette tip, one *Streptomyces* bacterial colony was removed/plucked off of a pure culture plate. The colony was placed into a PCR tube with 10 µL of distilled water and crushed with the pipette tip to break up the colony into pieces. The PCR tube was labeled and heated in a thermocycler to 99°C and then removed and frozen at -20°C for 30 minutes to disrupt the cells. Again using a pipette tip the cells were crushed again to ensure they were well dispensed. A master mix was made using 14.9 µL of distilled water, 2 µL of buffer, 0.8 µL of primer pA (5´-AGATTTTGATCCTGGCTCAG-3´) 0.8 µL of primer pH (5´AAGGAGGTGATCCAGCCGCA-3´) 0.4 µL of dNTP's and 0.1 µL of *Taq* giving the master mix a total of 19 µL per reaction. Primers pA and pH were selected to target the 16S rRNA gene as previously performed (Davelos et al., 2004).

One µL of each bacterial sample was added to a PCR tube containing the above master mix. One tube contained one µL of distilled water with master mix to act as a negative control. Reaction tubes were placed in the thermocylcer for 35 cycles

(denaturation 30 sec at 94°C, annealing 30 sec at 55°C, extension 1 min at 72°C) The PCR tubes were stored at -20°C until running the samples onto a gel.

Gel Electrophoresis

Loading samples and running the gel

A previously made 1% agarose gel was used to run the samples on. One µL of loading dye and 5 µL of sample were pipetted onto parafilm and mixed by pipetting up and down until I was able to visualize the dye was well mixed. Six µL of dye/sample solution was pipetted to each lane. Four µL of ladder (HyLadder1.5 kb, Denville Scientific) was added to the last lane. The gel was run for 30 minutes at 110 volts (V) in Tris-borate EDTA buffer (TBE buffer). The power source was turned off and the gel carefully removed from the gel box.

Vizualization of bands

To visualize the bands on the gel, it was placed in a plastic container with 5 µL of ethidium bromide (EtBr) added to 100 mL of distilled water for a final concentration of 5×10^{-5} µL/mL. The gel was soaked for 45 minutes and then placed under UV light to visualize the DNA bands. The gel was imaged under UV light using a BIO RAD Molecular Imager®, Gel Doc™ XRT imaging system.

DNA extraction from gel

Bands for each sample were cut from the gel using a clean razor blade. DNA was extracted from the gel using a DNA purification kit by Denville Scientific (South Plainfield, NJ) and following manufactures instructions.

DNA sequencing

Ten µL of the samples was diluted to ~4.0 ng/µL using a NanoDrop 2000c spectrophotometer (Thermo Scientific Inc.) and 5.0 µL of each primer (pA and pH) was added at the concentration of 25.0 pmol/µL and sent to Genewiz (South Plainfield, NJ) for sequencing. Sequences were put into Genbank and BLAST was used to assess their identity (Altschul et al., 1990).

Antibiotic assay

Due to the pathogenic strain growing much faster than the isolated endophytes, two methods were used to assay the isolated endophytes against *S. scabies,* in an attempt to allow for the endophytes to be established before introducing the pathogen.

Antibiotic co-plate assay of isolated endophytes against S. scabies: method 1

One hundred µL of *S. scabies* spore suspension (10^8 spores/mL in 20% glycerol) was pipetted onto OA and spread using a glass spreader that had been soaked in 70% ethanol and burned off. The inoculum was allowed to soak into the agar for 30 minutes. Spore suspensions (10^8 spores/mL in 20% glycerol) of each individual isolate (10 µL) were spot inoculated onto this same OA plate on top of the spread *S. scabies* and allowed to soak into the media (Figure 3). This was done in replicates of 5 for each isolate inoculated. Plates were inverted and incubated at 28º C for one week. Zones of inhibition were measured in millimeters after incubation using a ruler. Asymmetrical zones were compensated for by measuring the zone diagonally.

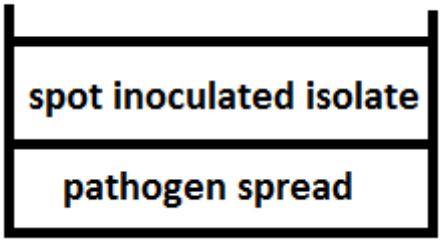


Figure 3. Antibiotic co-plate assay of isolated endophytes against *S. scabies*: method 1. This figure depicts the first of two methods used to assess the isolates against the pathogen.

Antibiotic co-plate assay of isolated endophytes against S. scabies: method 2

Ten µL of the isolated endophytes spore suspensions (10^8 spores/mL in 20% glycerol) were spot inoculated onto OA. The plates were inverted and incubated at 28°C for five days. At this point 15 mL of WA was overlaid onto the inoculated plates. The agar was allowed to solidify and then 100 µL of *S. scabies* (10^8 spores/mL in 20% glycerol) was pipetted onto the WA overlay, and spread using a glass spreader that had been soaked in 70% ethanol and burned off (Figure 4). The plates were then inverted and incubated at 28°C for four days. Zones of inhibition were measured in millimeters after incubation using a ruler. Asymmetrical zones were compensated for by measuring the zone diagonally.

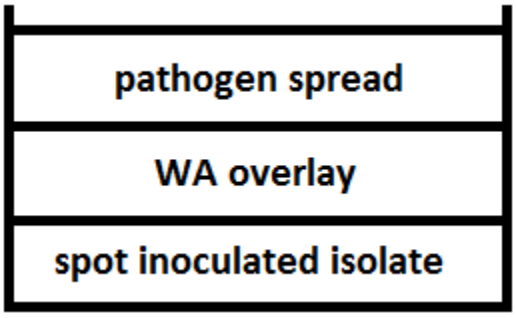


Figure 4. Antibiotic co-plate assay of isolated endophytes against *S. scabies*: method 2. This figure depicts the second of two methods used to assess the isolates against the pathogen.

Antibiotic co-plate assay of isolates against each other

One hundred µL of an isolate spore suspension (10^8 spores/mL in 20% glycerol) was pipetted onto OA and spread using a sterile glass spreader and allowed to soak into the agar for 30 minutes. Spore suspensions (10^8 spores/mL in 20% glycerol) of another isolate (10 µL) was spot inoculated onto this same OA plate on top of the previously spread isolate and allowed to soak into the media (Figure 5). Plates were inverted and incubated at 28º C for one week. Zones of inhibition were measured in millimeters after incubation using a ruler. Asymmetrical zones were compensated for by measuring the zone diagonally. This was done in replicates of 5 for each isolate inoculated in pairwise combinations for assessment of each isolate against each other.

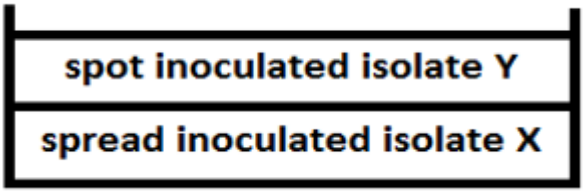


Figure 5. Antibiotic co-plate assay of isolates against each other. This figure indicates the layering method that was used to perform this assay.

Quorum sensing assay

To assess antibiotic production and communication interactions by the pathogen and isolates, a coplate assay was performed with all pairwise combinations of isolates. Spore suspensions (10^8 spores/mL in 20% glycerol) of individual isolates (10 µL) were spot inoculated onto OA plates and incubated for 5 days at 28º C. The plates were thinly overlaid with 15 mL of 1% OA and a second isolate (10 µL) was spot inoculated directly on top of or above the first isolate and incubated for 5 days at 28º C. The plates were then thinly overlaid with 15 mL of 1% WA and 100 µL of the pathogen was spread across the overlay and incubated for 3 days at 28º C (Figure 6). Zones of inhibition were measured in millimeters after incubation using a ruler. Asymmetrical zones were compensated for by measuring the zone diagonally.

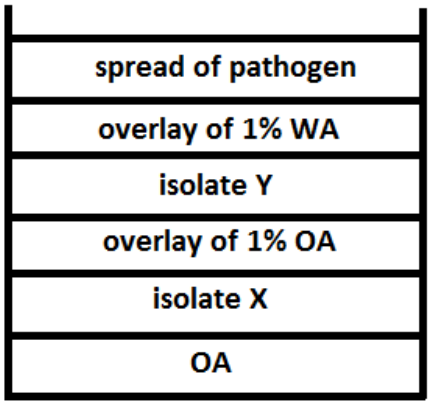


Figure 6. Quorum Sensing Assay. This figure indicates the multilayer agar plate method used in the quorum sensing assay.

Statistical Analysis

IBM SPSS Statistics 21 was the software used to analyze all data. Univariate ANOVAs were used to compare the mean differences in maximum zones of inhibition among treatments (isolate 1 as bottom strain and isolates 5, 8, 9 as top strains, against 87 in the quorum sensing assay). Post-hoc Tukey tests were done for pairwise comparisons between treatments. Kruskal Wallis non parametric tests for some treatments were used because the data did not meet normality (isolate 5 as bottom strain and isolates 1, 8, 9 as top strains against 87; isolate 8 as bottom strain and isolates 1, 5, 9 as top strains against 87; isolate 9 as bottom strain and isolates 1, 5, 8 as top strains against 87). These tests were used to determine significance (α=0.05) of inoculated combinations of pair-wise isolates to reduce growth of the pathogenic strain 87.

Results

Isolation of endophytes

Twenty-five stem tissue samples were processed from the 2012 field samples while all 63 provided samples were processed from the 2013 field season samples. Four morphologically distinct strains of endophytic Actinomycetes were isolated from the two sources of stem tissues. Strains called 1 and 8 were obtained from tissues of 2012; each strain was isolated from a different plant, within a different treatment block of the experimental station. Strains 1 and 8 were isolated using the macerate technique from dilution plate at 10^{-2} of SCA (Figures 7 & 9; Table 1). Strains 5 and 9 were obtained from tissues of 2013, each from a different plant in the experimental field as well as a different treatment in the experimental field. Isolate 5 was obtained using the macerate

technique from a 10^{-2} dilution plate of OA amended with fungicides and antibiotics. Isolate 9 was obtained by cutting stem cross sections and laying onto OA amended with fungicides and antibiotics (Figures 8 & 10; Table 1).

Numerous non-Actinomycete endophytes were also isolated from the two sources of stem tissues. This ranged from silver fungal endophytes to several red, yellow, white and orange bacterial colonies. The bacterial colonies mentioned were obtained frequently and from tissues from both sample years. Red and yellow colonies were not able to be counted as there were too few on each plate. Orange colonies isolated on dilution plates 10^{-2} were found to have 10^5 colony forming units (cfu). White colonies were too numerous to count and were observed to be spreading across the plates in dilutions from 10^{-1} to 10^{-3} (Figure 19).

16sRNA sequencing

Sequencing of the four isolates (1.5 kb region of target gene, 16S rRNA) indicated that all four isolates are Actinomycetes, with 99% of their DNA being the same as for *Microbispora* spp. and this was the only genera indicated by BLAST. *Microbispora* are classified as aerobic, gram-positive, filamentous soil bacteria belonging to the order Actinomycetales, family Streptosporangiaceae, and genus *Microbispora* (Nakajima, et al., 1999). The microbes I was interested in isolating are classified as aerobic, gram- positive, filamentous soil bacteria belonging to the order Actinomycetales, family Streptomycetaceae, and genus *Streptomyces* (Kinkel et al., 2012). See appendix for sequence data.

Antibiotic assay

Isolate 1 inhibited isolate 5 during the antibiotic assay (average zone of inhibition=18.3 mm). No other isolates were found to be inhibitory to one another in any pairwise combinations tested. Isolate 1 inhibited the pathogenic strain 87 (average zone of inhibition=11.6 mm). No other isolates were found to be inhibitory to the pathogenic strain in either method stated. Strain 9 was able to grow in the presence of strain 87, while strains 5 and 8 were not (Figure 32).

Quorum sensing assay

Controls (control 1= bottom strain only; control 2= top strain only) of this assay showed no zones of reduced growth, inhibition or sporulation, except when isolate 1 was inoculated as the top strain only. Zones of inhibition were measured when the isolates were able to completely kill or eliminate the pathogen (no aerial hyphae or spores from pathogen present in zone). Zones of reduced growth were measured when the isolates were able to notably decrease the sporulation/aerial hyphae of the pathogen within the measurable zone. Isolate 1 was able to induce larger zones of reduced growth in all other strains when it was inoculated as the bottom strain in the pairwise combinations. Isolate 1 and 5 in pairwise combinations had the largest visible zones of reduced growth as well as reduced sporulation of pathogenic strain 87 (Table 2; Figure 33, 34, 42, 43). Isolates 1 and 8 tested in pairwise combinations produced zones of reduced growth against pathogenic strain 87 (Table 2; Figures 35, 36, 42, 44). Pairwise combinations of isolates 1 and 9 produced zones of reduced growth of the pathogenic strain 87 (Table 2; Figures 37, 38, 42, 45). Isolate 5 tested with isolate 9 indicated a slight amount of reduced growth of pathogenic strain 87 (Figure 39). The combination of isolate 9 inoculated first followed

by inoculation with isolate 8 also produced zones of reduced growth (Table 2; Figures 40, 45).

Statistical Analysis

A one-way ANOVA showed that mean zones of inhibition among treatments differed. For the treatment of isolate 1 as bottom strain there was a significant difference in the mean inhibition among top strains 5, 8, and 9 against 87 ($F= 4.955$; d.f.=2,12; $P=0.027$). The Tukey post hoc test used for the treatment of isolate 1 as bottom strain and isolates 5, 8, 9 as top strains against 87 showed a difference in inhibition between isolates 5 and 8 only ($P=0.035$) (Figure 41).

Treatments which consisted of isolate 5 as bottom strain and isolates 1, 8, 9 as top strains tested against 87 were found to be significant when a Kruskal Wallis was run ($H=13.325$; d.f.=2; $P=0.001$). Treatments consisting of isolate 8 as bottom strain and isolates 1, 5, 9 as top strains tested against 87 compared the differences in means among treatments and was found to be statistically significant when a Kruskal Wallis was run ($H=15$; d.f.=2; $P=0.001$). Treatments consisting of isolate 9 as bottom strain and isolates 1, 5, 8 as top strains tested against 87 showed a significant difference in mean zones of inhibition ($H=15$; d.f.=10.718; $P=0.005$). All treatments which contained isolate 1 were found to show a difference in inhibition between isolates 5, 8, and 9 (Figure 41).

Table 1. Phenotypes of endophytes and pathogenic strain 87.

Strain	Origin	Colony Color	Spore Color	Diffusible Pigment Color
1	**2012 tissues; Becker, MN**	**Red**	**Light Pink**	**None**
5	**2013 tissues; Becker, MN**	**Orange/Tan**	**Light Pink**	**None**
8	**2012 tissues; Becker, MN**	**Dark Brown**	**White**	**Light Pink**
9	**2013 tissues; Becker, MN**	**Dark Brown**	**Dark Pink**	**Dark Brown/Black**
87	**Becker, MN**	**Dark Brown**	**Dark Grey**	**Brown/Black**

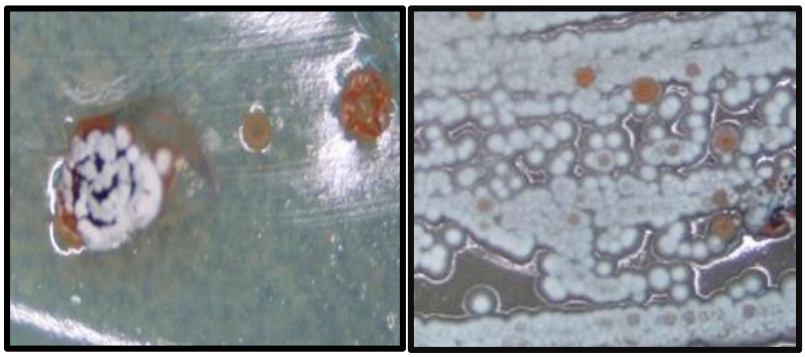


Figure 7. Isolate #1. Isolated from block 5, 22 of tissues obtained in 2012. Culture media is OA amended with antibiotics and fungicides. Colonies are dry and crumbly, tan in color, with pink colored spores.

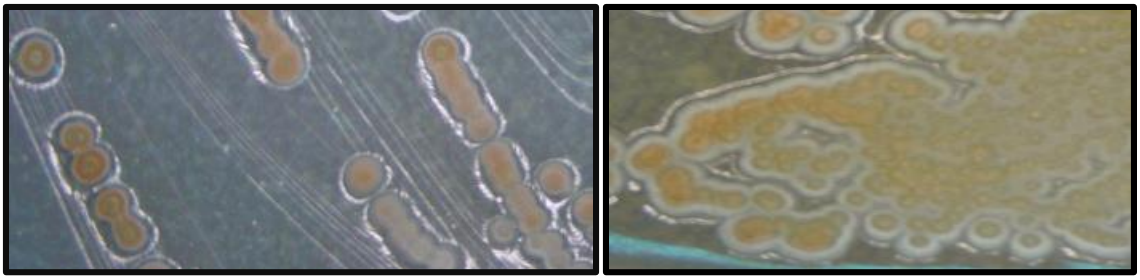


Figure 8. Isolate #5. Isolated from block 5, treatment 2 of tissues obtained in 2013. Culture media is OA amended with antibiotics and fungicides. Pink spores present**.**

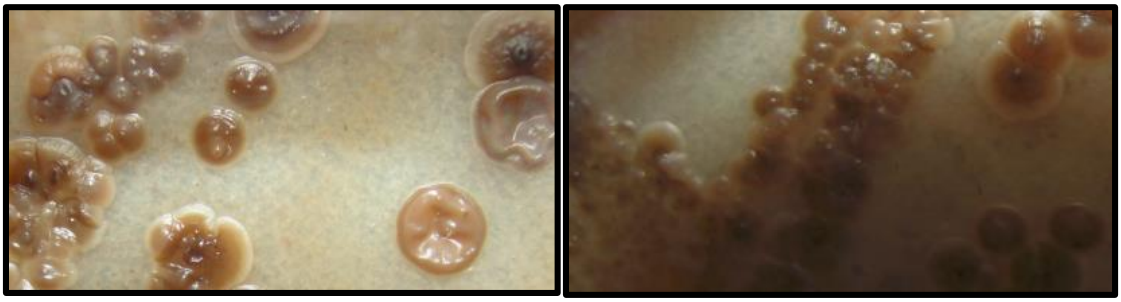


Figure 9. Isolate #8. Isolated from block 5 treatment 22 of tissues obtained in 2012. Culture media is OA. No spores indicated. White aerial hyphae present. Colonies are dark brown with a light pink pigment that diffused out into the agar.

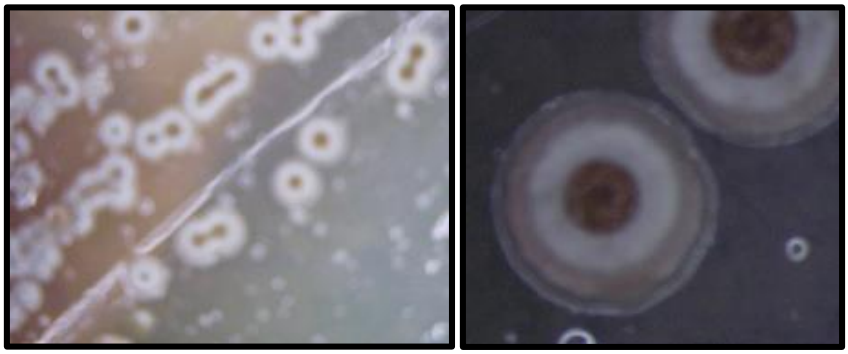


Figure 10. Isolate #9. Isolated from block 5 treatment 5 stem cutting of tissues obtained in 2013. Culture media is OA amended with antibiotics and fungicides. Colonies are dark brown with dark pink spores and has a brown pigment produced out into the agar.

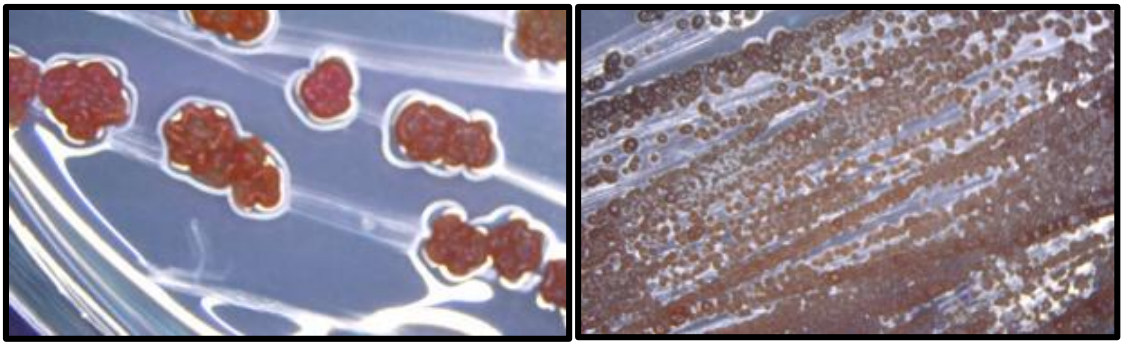


Figure 11. Isolate #1 plated on spore agar. Colonies are red, dry, raised, and crumbly. No spores were present.

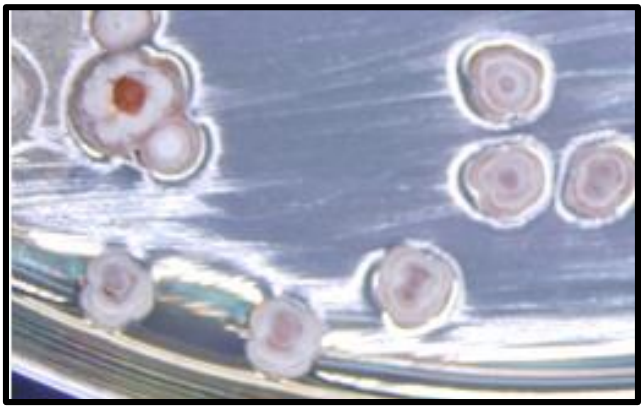


Figure 12. Isolate #5 plated on spore agar. Light pink spores present. Raised, dry appearing colonies.

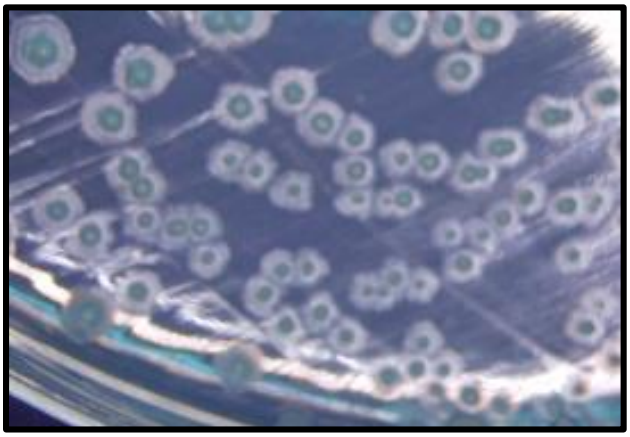


Figure 13. Isolate #8 plated on spore agar. No spores present. Raised, dry appearing colonies.

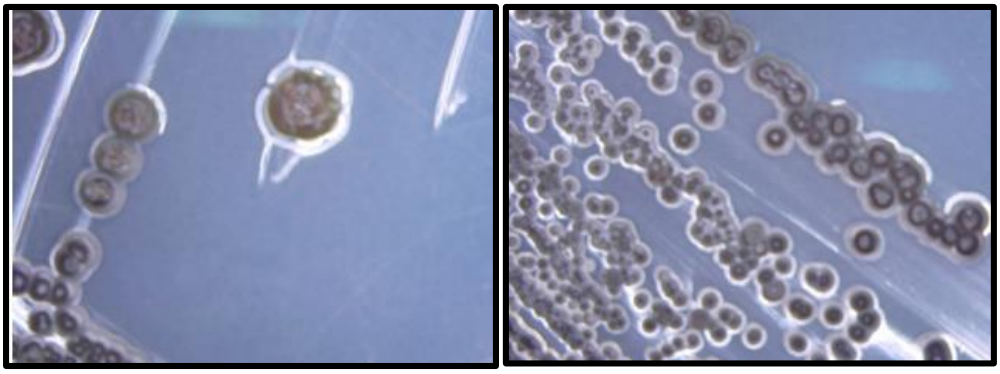


Figure 14. Isolate #9 plated on spore agar. No spores present. Raised, dry, crumbly colonies.

Figure 15. Isolate #1 plated on soy-flour mannitol agar. No spores present.

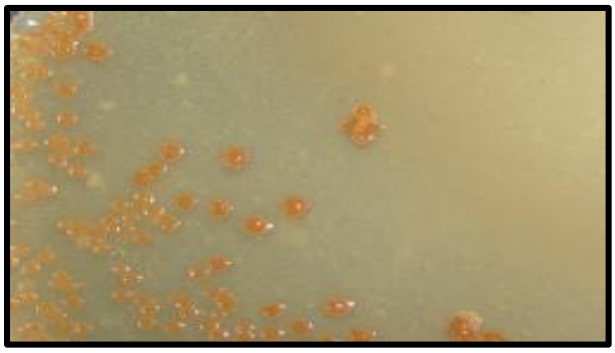


Figure 16. Isolate #5 plated on soy-flour mannitol agar. No spores present. Clearing of agar surrounding colonies was observed.

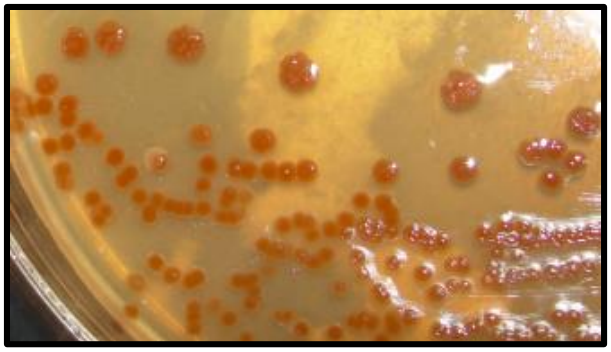


Figure 17. Isolate #8 plated on soy-flour agar. No spores present. Clearing of agar surrounding colonies was observed.

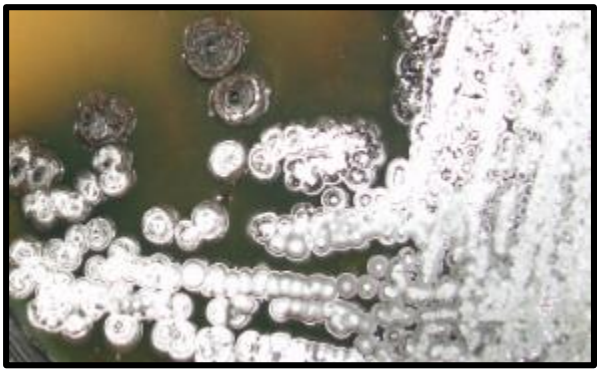


Figure 18. Isolate #9 plated on soy-flour agar. Spores present are light pink-white in color. Obvious darkening of agar. Black pigment heavily produced.

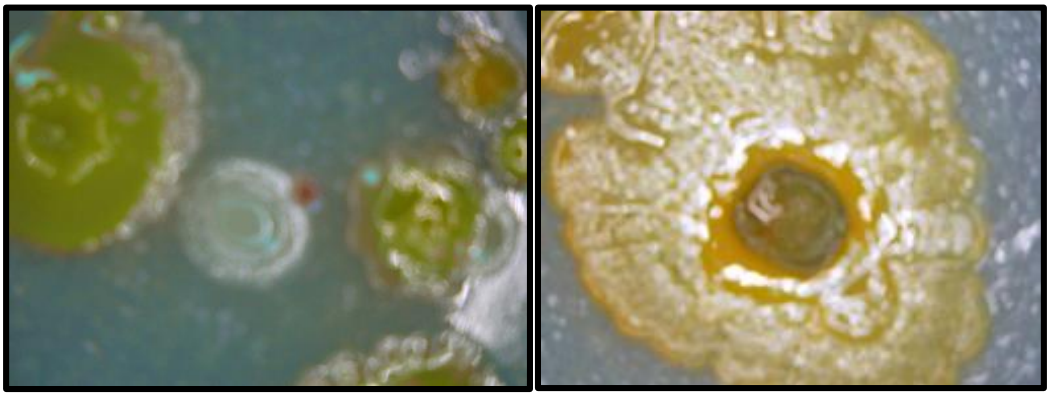


Figure 19. Contaminating bacteria encountered. This plate is from block 5 treatment 2 showing abundant red, yellow, white, and orange endophytic colonies observed on numerous plates (left). Stem cutting shown at the center of orange contaminating endophytic bacteria (right).

Figure 20. Antibiotic assay isolate 5 against isolate 1. Isolate 1 was spread across the plate. Isolate 5 was spot inoculated on top. Aerial hyphae was produced by each isolate as indicated by the white areas. No zones of inhibition observed.

Figure 21. Antibiotic assay of 9 against 5. No aerial hyphae or zones of inhibition present. Isolate 5 was spread across the agar and 9 was spot inoculated on top.

Figure 22. Antibiotic assay of 5 against 9. Isolate 9 was spread across the agar and 5 was spot inoculated on top. No zones of inhibition. Aerial hyphae was produced by isolate 5.

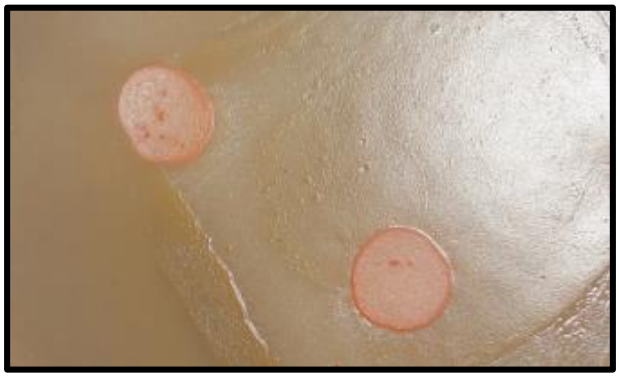


Figure 23. Antibiotic assay of isolate 1 against isolate 9. Isolate 9 was spread across the agar, isolate 1 was spot inoculated on top. No zones of inhibition produced. Isolate 1 shows aerial hyphae (white area).

Figure 24. Antibiotic assay of isolate 9 against isolate 1. Isolate 1 was spread across the agar, isolate 9 was spot inoculated on top. No zones of inhibition produced. Isolate 1 shows aerial hyphae (white area).

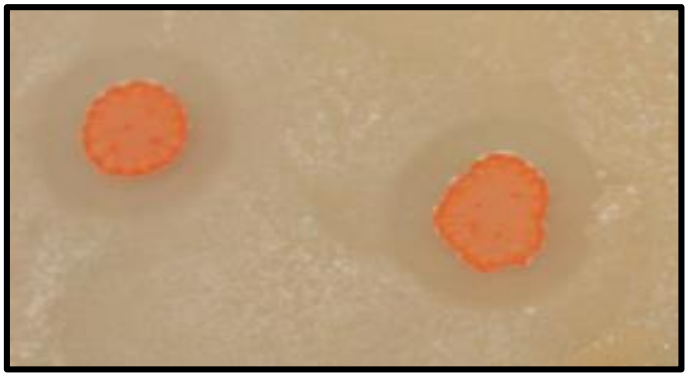


Figure 25. Antibiotic assay of isolate 1 against isolate 5. Isolate 5 was spread across the agar and isolate 1 was spot inoculated on top. Clear zones of inhibition are present. Isolate 5 shows aerial hyphae production (white areas).

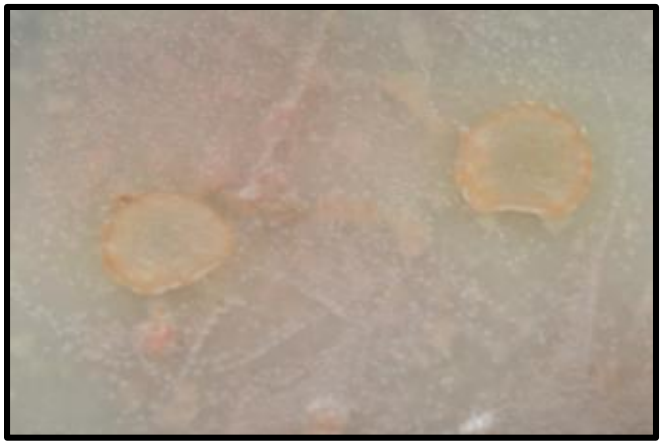


Figure 26. Antibiotic assay of isolate 9 against isolate 8. Isolate 8 was spread on the agar, isolate 9 was spot inoculated. No zones of inhibition present. Isolate 8 produced a light pink pigment.

Figure 27. Antibiotic assay of isolate 5 against isolate 8. Isolate 8 was spread on the agar, isolate 5 was spot inoculated. No zones of inhibition present. Isolate 8 produced a light pink pigment.

Figure 28. Antibiotic assay of isolate 8 against isolate 5. Isolate 5 was spread on the agar and spread, isolate 8 was spot inoculated. No zones of inhibition present.

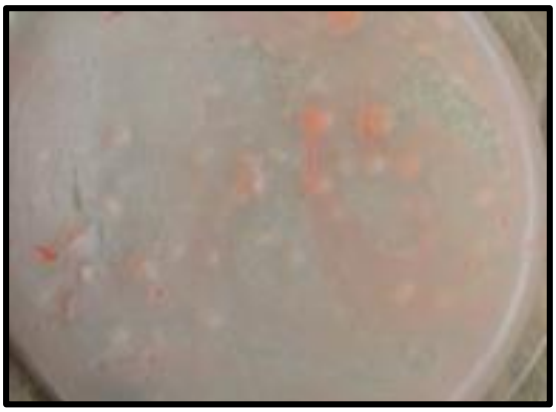


Figure 29. Antibiotic assay of isolate 8 against isolate 1. Isolate 1 was spread on the agar, isolate 8 was spot inoculated. No zones of inhibition present.

Figure 30. Antibiotic assay of isolate 1 against isolate 8. Isolate 8 produced a light pink pigment. Zones of inhibition present.

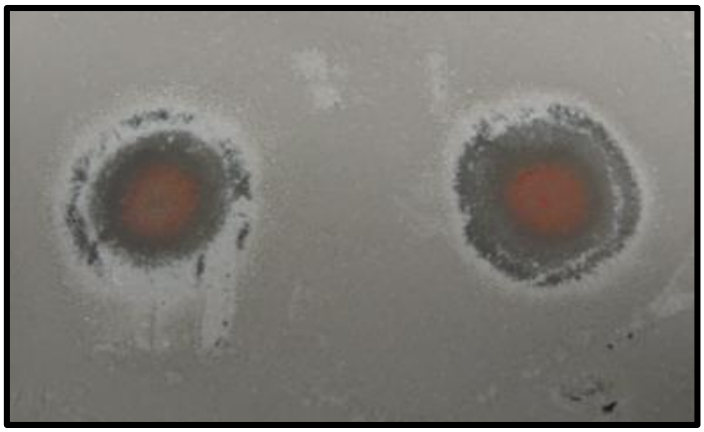


Figure 31. Antibiotic assay of isolate 1 against *S. scabies* strain 87. Clear zones of inhibition present. Strain 87 is shown sporulating.

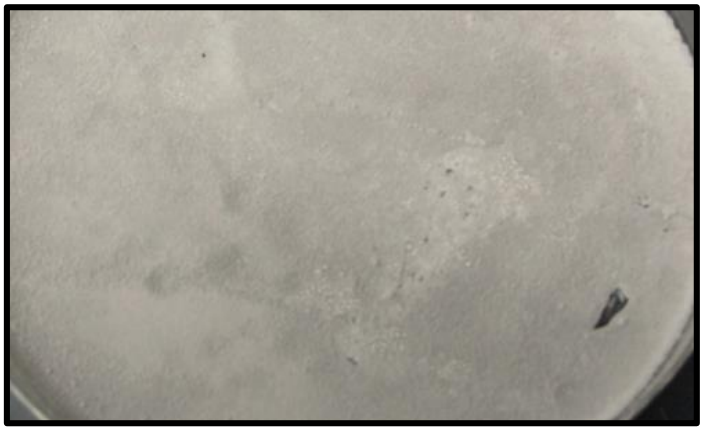


Figure 32. Representative antibiotic assay of isolates 5, 8, and 9 against strain 87. No zones of inhibition or growth detected.

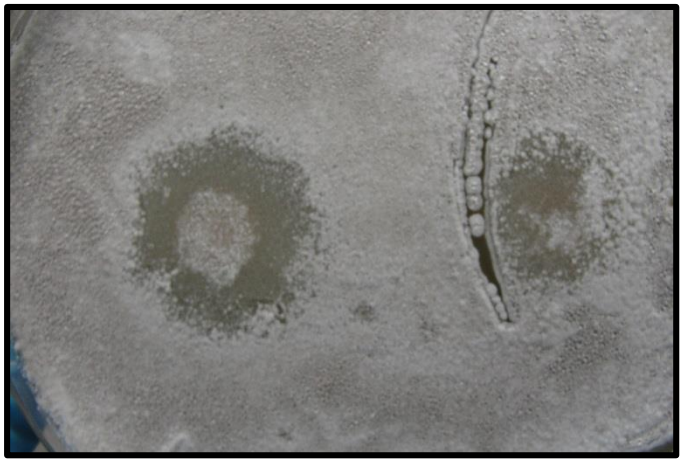


Figure 33. Quorum sensing assay isolates 1 and 5. Isolate 1 was first spot inoculated, isolate 5 was inoculated second, and strain 87 was spread. Zones of reduced pathogenic growth of strain 87 shown.

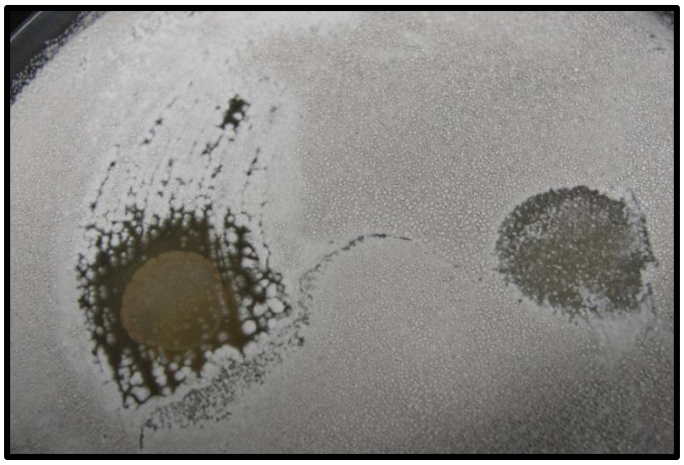


Figure 34. Quorum sensing assay isolates 5 and 1. Isolate 5 was first spot inoculated, isolate 1 was inoculated second, and strain 87 was spread. Zones of reduced pathogenic growth of strain 87 shown.

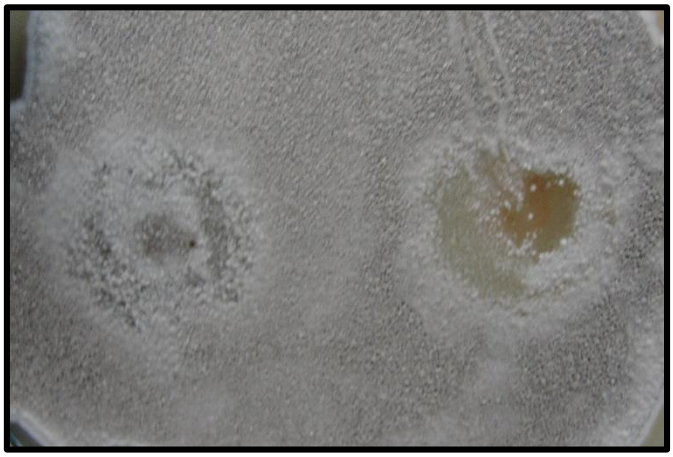


Figure 35. Quorum sensing assay isolates 1 and 8. Isolate 1 was first spot inoculated, isolate 8 was inoculated second, and strain 87 was spread. Zones of reduced pathogenic growth of strain 87 shown.

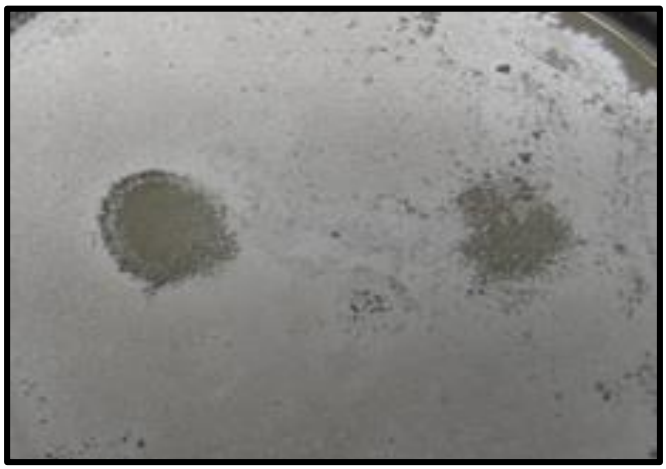


Figure 36. Quorum sensing assay isolates 8 and 1. Isolate 8 was first spot inoculated, isolate 1 was inoculated second, and strain 87 was spread. Zones of reduced pathogenic growth of strain 87 shown.

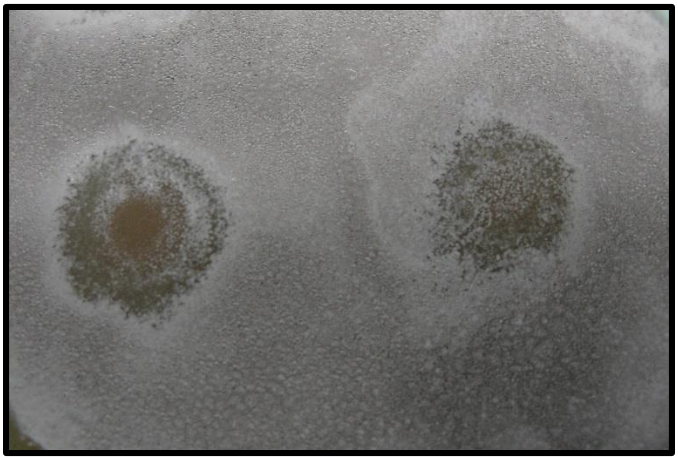


Figure 37. Quorum sensing assay isolates 1 and 9. Isolate 1 was first spot inoculated, isolate 9 was inoculated second, and strain 87 was spread. Zones of reduced pathogenic growth of strain 87 shown.

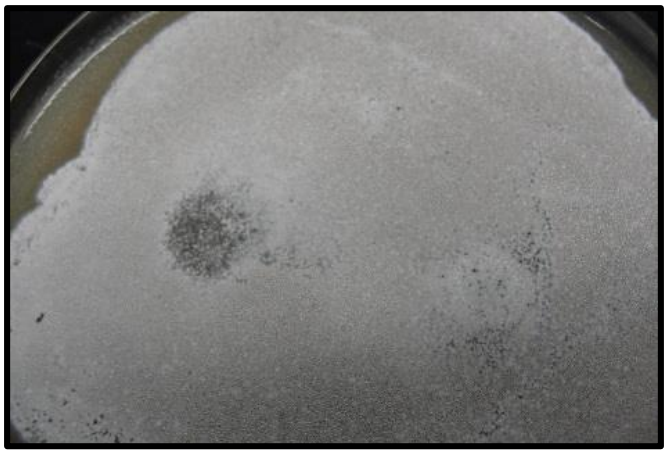


Figure 38. Quorum sensing assay isolates 9 and 1. Isolate 9 was first spot inoculated, isolate 1 was inoculated second, and strain 87 was spread. Zones of reduced pathogenic growth of strain 87 shown.

Figure 39. Quorum sensing assay isolates 5 and 9. Isolate 5 was first spot inoculated, isolate 9 was inoculated second, and strain 87 was spread. Areas of reduced pathogenic growth of strain 87 shown.

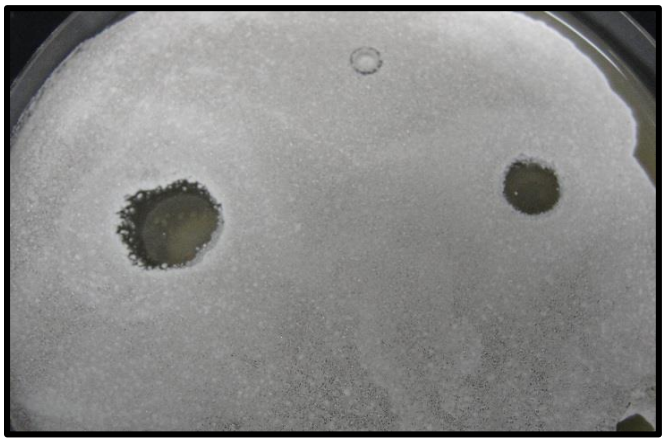
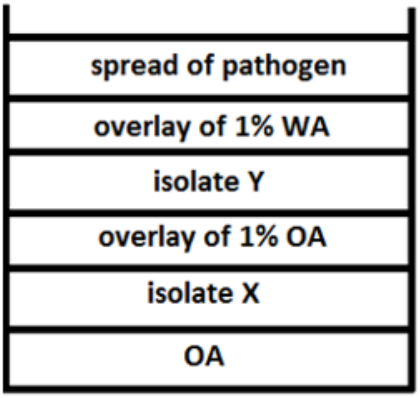


Figure 40. Quorum sensing assay isolates 9 and 8. Isolate 9 was first spot inoculated, isolate 8 was inoculated second, and strain 87 was spread. Areas of reduced pathogenic growth of strain 87 shown.



1st bottom isolate	2nd top isolate	Pathogen	Average Zone of Reduced Growth (mm)
1	5	*S. scabies*	16.8[a]
1	8	*S. scabies*	10.4[b]
1	9	*S. scabies*	16.7[a,b]
5	1	*S. scabies*	13.1[c]
5	8	*S. scabies*	0[d]
5	9	*S. scabies*	0[d]
8	1	*S. scabies*	9.9[e]
8	5	*S. scabies*	0[f]
8	9	*S. scabies*	0[f]
9	1	*S. scabies*	5.3[g]
9	5	*S. scabies*	0[h]
9	8	*S. scabies*	6.8[g]

Figure 41. Zones of Reduced Growth. Zones of reduced growth are indicated for each pairwise combination of isolates against the pathogen, *S. scabies* strain 87 in quorum sensing assays. There were two controls for this assay: control 1= bottom strain only; control 2= top strain only. Control 2 for treatments involving isolate 1 showed max zones of 12 mm. All other controls for all pairwise combinations showed no inhibition of the pathogen. Treatments with matching superscripts were not significantly different; treatments with different superscripts were significantly different.

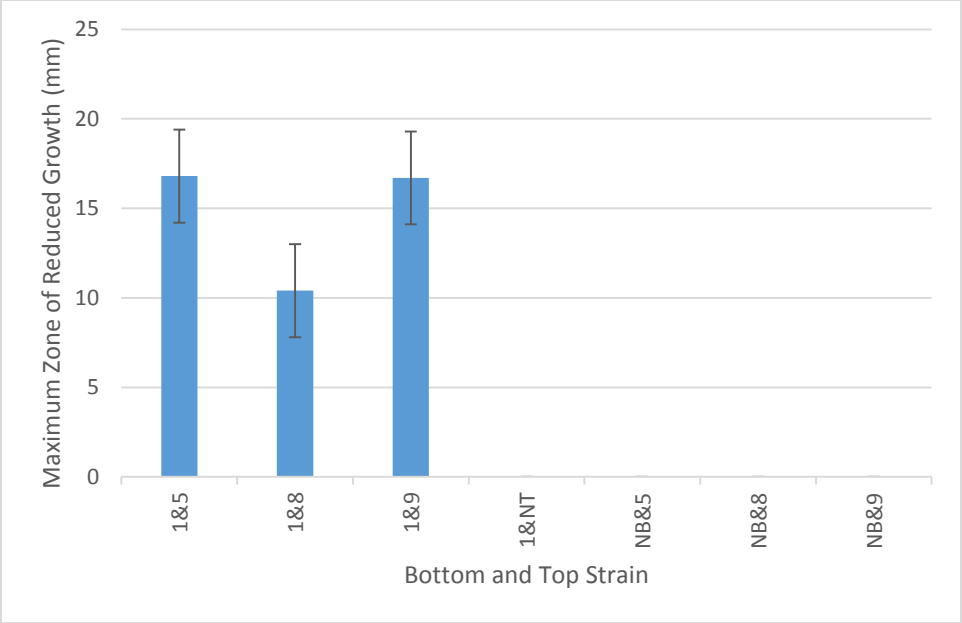


Figure 42. Quorum Sensing Interactions for Isolate 1 as the Bottom Strain. Max zones of reduced growth are indicated for pairwise combinations where isolate 1 was the bottom strain. Controls included are NT=no top strain inoculated, and NB=no bottom strain inoculated. Standard error bars shown.

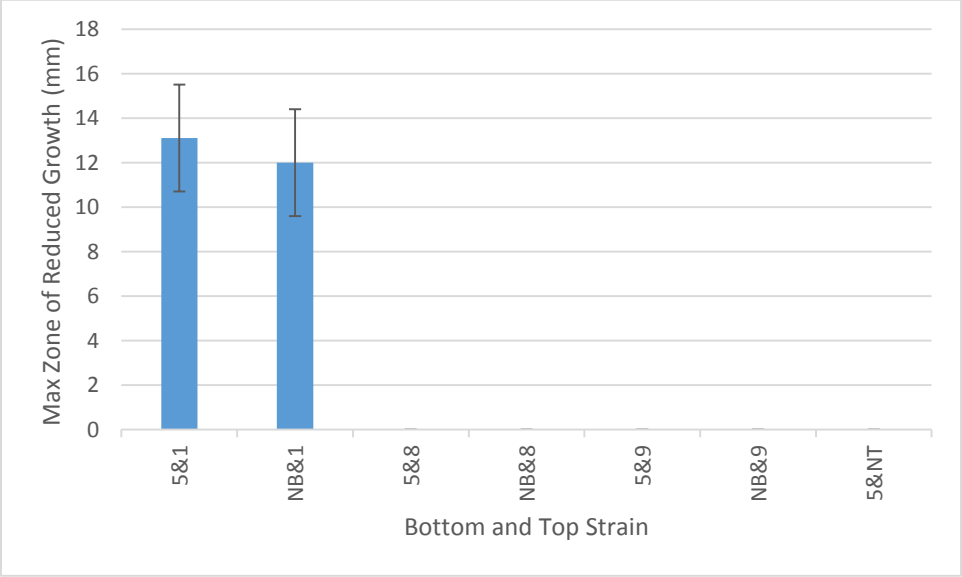


Figure 43. Quorum Sensing Interactions for Isolate 5 as the Bottom Strain. Max zones of reduced growth are indicated for pairwise combinations where isolate 5 was the bottom strain. Controls included are NT=no top strain inoculated, and NB=no bottom strain inoculated. Standard error bars shown.

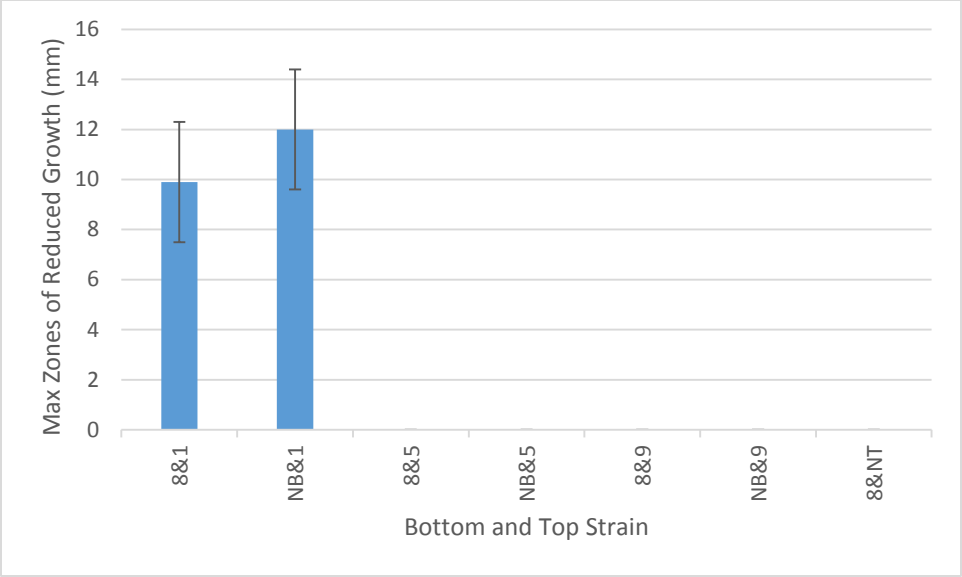


Figure 44. Quorum Sensing Interactions for Isolate 8 as the Bottom Strain. Max zones of reduced growth are indicated for pairwise combinations where isolate 8 was the bottom strain. Controls included are NT=no top strain inoculated, and NB=no bottom strain inoculated. Standard error bars shown.

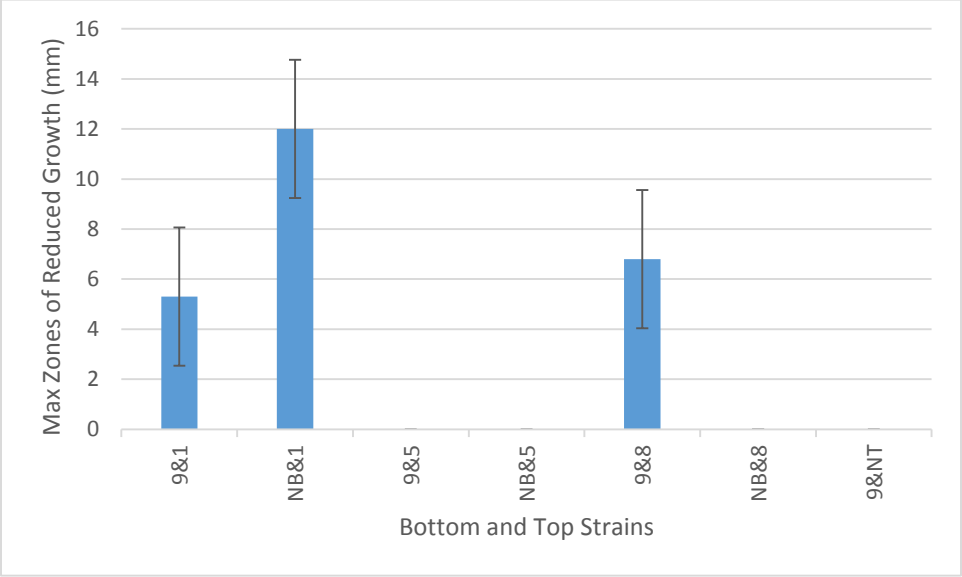


Figure 45. Quorum Sensing Interactions for Isolate 9 as the Bottom Strain. Max zones of reduced growth are indicated for pairwise combinations where isolate 1 was the bottom strain. Controls included are NT=no top strain inoculated, and NB=no bottom strain inoculated. Standard error bars shown.

Discussion

I aimed to assess whether or not endophytic *Streptomyces* bacteria could be obtained from potato stem tissues and, if so, to test each isolates ability to inhibit the potato scab pathogen and to assess their communication abilities with one another. Each of the four isolates were recovered from stem potato tissues obtained from experimental fields in Becker, MN. Pathogenic *Streptomyces scabies* was present in the soil in these fields. Different combinations of pathogenic-inhibiting *Streptomyces* strains were inoculated into these fields upon planting of the seed tubers. Stem tissue isolates were noticed on the isolation media due to their characteristic dry, crumbly appearance and raised morphologies on the plates. Characteristic growth morphologies consistent with Actinomycetes include regions of white at colony margin edges termed aerial hyphae and fuzzy, often pigmented, growth covering colonies, termed spores (Waksman and Henrici, 1943). Isolate 1 was obtained from an experimental field containing five different strain combinations of pathogen-inhibiting *Streptomyces* in the soil system, while isolate 8 was obtained where only one pathogen-inhibiting *Streptomyces* isolate was in the soil system. Isolates 5 and 9 were obtained from newly harvested stem tissues of different plants from an experimental field inoculated with pathogen-inhibiting *Streptomyces* and pathogenic *Streptomyces scabies*. Numerous other endophytic bacteria were also isolated from these samples. Isolates 1 and 8 were obtained from frozen, necrotic tissues which had been stored for several months at -80°C. The condition of the stem tissues was sub-optimal, yet I was successful in isolating these two endophytic Actinomycete strains as well as numerous other endophytic bacteria. This is promising evidence as to their ability to survive and persist in harsh environmental conditions, indicating their promise in disease

management against pathogens as well as being well suited to survive in farming locations that experience severe weather. None of the four isolates obtained were from the study being conducted by the University of Minnesota (Kinkel, personal communication, 2014); however this does not rule out the possibility that they had gone endophytic and further experimental investigations are necessary.

Sequencing of the isolates allowed us to positively identify them as belonging to the order Actinomycetales. This order contains numerous filamentous bacteria including *Streptomyces* spp. (Waksman and Henrici, 1943). Though the sequencing indicated that the isolates have 99% of their 16S rRNA region identically matching with *Microbispora* spp., 16S rRNA this does not allow us to rule out the possibility that they are in fact *Streptomyces* spp. *Microbispora* are classified as aerobic, gram-positive, filamentous soil bacteria belonging to the order Actinomycetales, family Streptosporangiaceae, and genus *Microbispora* (Nakajima et al., 1999) whereas the bacteria we were interested in isolating are classified as aerobic, gram- positive, filamentous soil bacteria belonging to the order Actinomycetales, family Streptomycetaceae, and genus *Streptomyces* (Kinkel et al., 2012). A more thorough analysis of their sequences would allow for identification of the isolates to the species level.

Isolate 1 was found to be inhibitory against pathogenic strain 87 as well as against isolate 5. Zones of inhibition for isolate 1 against strain 87 were completely clear indicating its potential as a tool in disease management of potato scab caused by strain 87. Though isolate 1 was the only strain to be inhibitory in the antibiotic assays it is important to note we only tested strains on OA media. Also notable is that the isolates

were only tested *in vitro*, while *in vivo* the results may be quite different as the stem tissues likely provide a more optimal environment than do OA media plates.

Quorum sensing assays allowed for the assessment of each isolate's ability to communicate with one another as indicated by the presence or absence of zones of reduced growth of *S. scabies* strain 87. I aimed to assess the isolate's abilities to produce chemical signals or triggers through quorum sensing while in close proximities in order to enhance inhibition of the pathogen. Controls for this assay showed no zones of inhibition when strains were inoculated on the bottom only. However the test plates demonstrated zones of reduced growth. When the isolates were tested alone against the pathogen, only isolate 1 was able to inhibit, but when paired, (Figure 41) they were able to reduce growth of pathogen. Thus, it appears that quorum sensing could be involved to establish the zones of reduced growth when they are paired and tested against the pathogenic strain. Pairwise combinations of isolate 1 with each of the other isolated endophytes indicated regions of reduced growth of the pathogen (Figures 41, 42). Our findings indicate that when isolate 1 is inoculated as the bottom strain it is able to trigger a chemical response in each of the other endophytes. The data presented indicate isolate 1's potential to be of use in the biological control of *S. scabies*. Controls for this assay (isolate 1 as the top strain only) demonstrated clear kill zones similar to those exhibited in the antibiotic plates. However, zones of reduced growth in the pairwise combinations were found to be larger on average than the zones of inhibition.

Each of the endophytes were isolated from different stem tissues which belonged to different plants. This has the potential to notably impact their interactions and their quorum sensing with each other. Since the endophytes were not existing within the same

tissue of a particular plant this could help to explain the lack of kill zones against strain 87 in the quorum sensing assays as they may not have been co-existing *in vivo*. Possibly if multiple endophytes are isolated from the same tissues this would improve their ability to communicate and inhibit *S. scabies*. Endophytes that co-existed in the same tissues provides the opportunity for them to have coevolved, therefore potentially improving their abilities to use quorum sensing to inhibit pathogens.

The use of endophytic *Streptomyces* strains as biological control agents holds a hopeful place for the agricultural and environmental industries. The potential to decrease the use of harsh synthetic materials to control diseases associated with agricultural practices is appealing to many. Endophytic Actinomycete microorganisms are highly attractive biological control agents due to their ability to produce a broad range of biologically active substances including: secondary metabolites, enzymes, antitumor agents, antibiotics, vitamins, and plant growth factors (Becker et al., 1999, Guo et al., 2008). Scientists estimate nearly one million different endophytic microbes are likely to exist, with only a handful being isolated this implies there is a great opportunity for finding novel targets for the medical and agricultural industry (Liu, et al., 2012; Guo et al., 2008). In addition to the diversity and abundance of bioactive compounds produced, endophytes also show great promise of control plant pathogens as they will be less likely to be killed off by pathogenic strains and other competing microbes due to their location within the plant body (Kinkel et al., 2012). Strobel et al. (1996) have been successful in isolating numerous endophytes and their secondary metabolites which have been used in human medicine as well. This includes isolation of the fungal endophyte, *Pestalotiopsis microspora*, obtained from the bark of *Taxus wallachiana*, which produces the taxol drug

currently administered to cancer patients (Strobel et al., 1996). Many other endophytes that have been isolated and characterized as effective agents in agricultural settings have been found to be useful and effective for drugs in human medicine as well, further demonstrating the attractiveness for exploring this area as a source of bioactive compounds (Guo et al., 2008).

Endophytic microbes are abundant, extremely diverse, and have been located in every plant species assessed to date, indicating their ability to proliferate and persist in this intimate relationship (Guo et al., 2008). The potential for endophytic microorganisms to be effective tools in controlling plant diseases has recently been strengthened as they are believed to be responsible for promotion of plant growth as well as pathogen inhibition (Kinkel et al., 2012). Formulating a long-term approach to manage disease will involve understanding the ecology and evolution of the plant-microbe system. Plant hosts are not passive in this relationship, but are likely playing a vital role driving the selection of microbes inhabiting their tissues. Often novel interactions between colonizing microbes occur in the rhizopshere. Here intimate relationships between plants and microbes are abundant and can result in symbiosis where the microbe is incorporated by the plant. This has the potential to increase the health and fitness of the plants involved (Hartmann et al., 2009). The ability for endophytes to have long-term relationships with their plant hosts is of importance when thinking of good biocontol agents. In agricultural practices endophytes existing in potato seed tubers planted into fields may then potentially move to colonize other parts of the plants which develop from this seed tuber. We can relate this concept to plants grown from seeds as well; the seeds harbor the endophytes that co-exist with the plant from the initial germination phase and can migrate

to other tissues of the plant. The strategy of searching for endophytes in longer-lived tissues (e.g., stems) rather than shorter-lived regions (e.g., tubers that do not form until late in the growth cycle of the plant), may offer the plant a better, more stable means of protection due to the extended time for the plant and microbe to coevolve.

The use of non-pathogenic *Streptomyces* to decrease disease seems to be promising as demonstrated by numerous scientists. Studies have shown that soils inoculated with potato scab suppressive soils demonstrate a decline in pathogenic populations over a four-year period (Liu et al., 1995). It has also been shown that among *Streptomyces* species recovered from scab resistant cultivars, high proportions of non-pathogenic *Streptomyces* were found that coincided with a decrease in potato scab disease severity (Wanner, 2007). Furthermore, studies of endophytic *Streptomyces* strains have demonstrated their ability to be successful control agents against pathogens causing ring rot in potatoes (von Bodman et al., 2008). Conn and Franco (2004) have isolated endophytes from oak and demonstrated their ability to be effective against the pathogen causing oak wilt. It seems reasonable that adding more combinations of non-pathogenic strains to a soil system will aid in the plants ability to ward off pathogens. It also seems logical that increasing the amount of non-pathogenic strains in a soil system would have the potential to increase the amount of non-pathogenic *Streptomyces* strains that could become endophytic.

Current methods of choosing a biocontrol agent have weighed heavily on the microbe's antibiotic production as well as their ability to produce secondary metabolites (Bakker, et al., 2010; Kinkel et al., 2012). This has provided some improvements to disease control in various management systems (Becker, et al. 1997; Conn et al., 2008;

Kinkel et al., 2012; Liu et al., 1995). Liu et al. (1995) have shown that suppressive strains inoculated at 1% (volume) were effective in decreasing potato scab by 80% in field-pot trials. However, this is a highly complex system of plant-microbe and microbe-microbe interactions which leads to the concept that several methods of biocontrol may need to be employed together to ultimately be effective against plant diseases. It is unlikely that simply by inoculating seeds or, in the case of potatoes, seed tubers, with endophytic isolates and then planting them into soil will be a stand-alone option to combat disease. To consider effective methods we need to understand the ecology and evolution at play. For example studies by Kinkel et al. (2012) have suggested plants may play a significant role in the development of a disease suppressive system. Data from their studies indicate that *Streptomyces* isolated from monoculture fields (rather than from a field with diverse plant species) have coevolved to be non-pathogenic and highly antagonistic towards the potato scab pathogen. Work by Becker et al. (1997) showed that, in addition to quorum sensing occurring among biocontrol strains which enhanced antibiotic production, a pathogenic strain of *Streptomyces* (RB4) induced biocontrol strains to produce antibiotics *in vitro* that were inhibitory to that same pathogen. Thus, the pathogenic strain was able to induce a chemical response in the suppressives, suggestive of quorum sensing by the pathogen to the suppressive strains as well as demonstrating the concept of interspecies communication. Taken together, these studies (and others) demonstrate the complexity of plant-microbe and microbe-microbe interactions.

In addition to better understanding quorum sensing among biocontrol strains to enhance disease suppression, quorum quenching has the potential to be used for biocontrol purposes. Quorum quenching employs the use of chemical manipulations

which alter the actions and gene expression of surrounding microorganisms. Unlike quorum sensing, quorum quenching negatively impacts the fitness of the receiver (Keller and Surette, 2006). *Streptomyces* spp. have been shown to synthesize AHL-degrading enzymes that attack the AHL molecules produced by target pathogenic strains, rendering the pathogen's quorum sensing system defective (Mahmoudi et al., 2011; Park et al., 2005). Thus, finding biocontrol strains that can use quorum sensing to enhance their antibiotic levels while producing quorum quenching chemicals that attack the pathogen's ability to communicate amongst themselves would conceivably be advantageous for disease suppression.

This study focused on studying endophytic bacteria located in potato stem tissues. Further isolation and identification of endophytic bacteria continues to hold the promise for finding successful biological control agents for the management and control of potato scab disease. Studies should be continued to assess the ability of non-pathogenic *Streptomyces* and closely-related species to communicate via quorum sensing to increase the timing and level of antibiotics produced. The ultimate goal for finding an optimal disease management system to control common scab disease should include biocontrol agents that are able to quorum sense with other non-pathogenic strains, quorum quench pathogenic signals, exist in a highly resistant spore state, produce a vast array of secondary metabolites, and be abundant in the environment.

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APPENDIX A

Sequence File

Sequence File : forward pA isolate 1

NNNNNNNNNNNNNNNNCNNANNNTGCNNGTCGAGCGGAAGGCCCTTCGGG
GTACTCGAGCGGCGAACGGGTGAGTAACAC
GTGAGTAACCTGCCCCTGACTCTGGGATAAGCCTGGGAAACCGGGTCTAATA
CCGGATATGACACTCCTCCGCATGGTGT
GGGTGTGGAAAGTTTTTTCGGTTGGGGATGGACTCGCGGCCTATCAGCTTGT
TGGTGGGGTGATGGCCTACCAAGGCGAC
GACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCGCAATGGGCGAAAGCCTGACGCAGCGACGCCGCGTGGGGGA
TGACGGCCTTCGGGTTGTAAACCTCTTTC
AGCAGGGACGAAGTTGACGTGTACCTGTAGAAGAAGCGCCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGCG
CGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGTGGCTTGTTGC
GTCTGCCGTGAAAGCCCGTGGCTTAACT
ACGGGTCTGCGGTGGATACGGGCAGGCTAGAGGCTGGTAGGGGCAAGCGGA
ATTCCTGGTGTAGCGGTGAAATGCGCAGA
TATCAGGAGGAACACCGGTGGCGAANGCGGCTTGCTGGGCCAGTTCTGACG
CTGAGGAGCGAAAGCGTGGGGAGCGAACA
GGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTGGG
GGTCTTCCACGATCTCTGTGCCGTANCT
AACGCATTAAGCGCCCCGCCTGGGGAGTANGGCCGCAAGGCTAAAACTCAA
AGGAATTGANGGGGCCCGCACAAGCGGCG
GAGCATGTTGCTTAATTCGACGCAACGCNANAACCTTACCAAGGTTTGACNT
ACACCGGAAACNCTCAGAGATGGGTGCC
TNCTTTGNCTGNTGNACNNNGNGCANGNTGTNNTCAGCTCGTGNCNNNGAN
ANNNTGGGNNAAGTCCCNNAACNNNCNNC
NN

Sequence File : forward pA isolate 5

NNNNNNNNNNNNNNCNNNNNNNTGCAGTCGAGCGGAAGGCCCTTCGGGGT
ACTCGAGCGGCGAACGGGTGAGTAACACGT
GAGTAACCTGCCCCTGACTCTGGGATAAGCCTGGGAAACCGGGTCTAATACC
GGATACGACCATTTCTCGCATGTGATGG
TGGTGGAAAGTTTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTTGTTG
GTGGGGTGATGGCCTACCAAGGCGACGA
CGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCANTGGGG
AATATTGCGCAATGGGCGGAAGCCTGACGCATTGACGCCGCGTGGGGGATG
ANAGCCNTNCCTTCGTACAGTAACCCNCT
CTNGNANNANNNNNANANGTTANTGTCNNTNNATNNC

Sequence File : forward pA isolate 8

NNNNNNNNNNNNNNNNNNGCNNNNNNTGCAGTCGAGCGGAAGGCCCTTCG
GGGTACTCGAGCGGCGAACGGGTGAGTAAC
ANGTGAGTAACCTGCCCCTGACTCTGGGATAAGCCTGGGAAACTGGGTCTA
ATACCGGATACGACCATTTCTCGCATGTG
ATGGTGGTGGAAAGTTTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTT
GTTGGTGGGGTGATGGCCTACCAAGGCG
ACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCGCAATGGGCGGAAGCCTGACGCAGCGACGCCGCGTGGGG
GATGACGGCCTTCGGGTTGTAAACCTCTT
TCAGCAGGGACGAAGTTGACGTGTACCTGTAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGTGGCTTGTC
GCGTCTGCCGTGAAAGCCCGTGGCTTAA
CTACGGGTCTGCGGTGGATACGGGCAGGCTAGAGGCTGGTAGGGGCAAGCG
GAATTCCTGGTGTAGCGGTGAAATGCGCA
GATATCAGGAGGAACACCGGTGGCGAAGGCGGCTTGCTGGGCCAGTTCTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAA
CAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTG
GGGGTCTTCCACGATCTCTGTGCCGTAG
CTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAACTCA
AAGGAATTGANGGGGCCCGCACAAGCGGC
GGAGCATGTTGCTTAATTCGACNCACGCGANNAACCTTACCAGGTTTGANNN
CNCCNGNAAACNNTCAGAGATGGNNCTC
TTTGNCTGNGTNCNGNGGNGCATGNTGTCGTCANNTCNNGNNNNNNNATGN
TNGGNTNANTCCCGNANCNANNNN

Sequence File : forward pA isolate 9

NNNNNNNNNNNNNNNNNNCTTANNNTGCAAGTCGAGCGGAAGGCCCTTCG
GGGTACTCGAGCGGCGAACGGGTGAGTAAC
ACGTGAGTAACCTGCCCCTGACTCTGGGATAAGCCTGGGAAACCGGGTCTA
ATACCGGATACGACCATTTCTCGCATGGG
ATGGTGGTGGAAAGTTTTTTCGGTTGGGGATGGGCTCGCGGCCTATCAGCTT
GTTGGTGGGGTGATGGCCTACCAAGGCG
ACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACA
CGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCGCAATGGGCGGAAGCCTGACGCAGCGACGCCGCGTGGGG
GATGACGGCCTTCGGGTTGTAAACCTCTT
TCAGCAGGGACGAAGTTGACGTGTACCTGTAGAAGAAGCGCCGGCTAACTA
CGTGCCAGCAGCCGCGGTAATACGTAGGG
CGCGAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGTGGCTTGTC
GCGTCTGCCGTGAAAGCCCGTGGCTTAA
CTACGGGTCTGCGGTGGATACGGGCAGGCTAGAGGCTGGTAGGGGCAAGCG
GAATTCCTGGTGTAGCGGTGAAATGCGCA
GATATCAGGAGGAACACCGGTGGCGAAGGCGGCTTGCTGGGCCAGTTCTGA
CGCTGAGGAGCGAAAGCGTGGGGAGCGAA
CAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCTAGGTGTG
GGGGTCTTCCACGACTTCTGTGCCGTAN
CTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTC
AAAGGAATTGACGGGGGCCCGCACAAGCG
GCGGAGCATGNTGCTTATTTCGACGCACGCGANNAACCTTACCNAGGTTTGA
CNTACANCGGAAANNNTNNGANANNGGA
NNCCTCCTTTNGACTGNNTACNNNGNGCATGNNTGTCGTCNNCNNCNNGNN
NNNNTNTNGNNNAGTCCCGCANNANCNCN
NCNTTNNNCNTNNNTGCNGCANNCCCTTTGGGGNNGGNGNNNNNN

Sequence File : reverse pH isolate 1

NNNNNNNNNNNNNNNNNNNACGACTTCGTNNNNTCGCCAGCCCCACCTTC
GACCGCTCCCCCCACAAGTGGTTGGGCCA
CGGGCTTCGGGTGTTGCCGACTTTCGTGACGTGACGGGCGGTGTGTACAAGG
CCCGGGAACGTATTCACCGCAGCGTTGC
TGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCCC
AATCCGAACTGAGACCGGCTTTTAGGGA
TTCGCTCCACCTCACGGTATCGCAACCCTCTGTACCGGCCATTGTAGCATGTT
TGCAGCCCAAGACATAAGGGGCATGAT
GACTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATG
AGTCCCCACCACCCCAAAGGGCGTGCTG
GCAACATGGAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCAC
GACACGAGCTGACGACAGCCATGCACCA
CCTGTACACCAGTCCAAAGGAGGCACCCATCTCTGAGTGTTTCCGGTGTATG

TCAAACCTTGGTAAGGTTCTTCGCGTTG
CGTCGAATTAAGCAACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCT
TTGAGTTTTAGCCTTGCGGCCGTACTCC
CCAGGCGGGGCGCTTAATGCGTTAGCTACGGCACAGAGATCGTGGAAGACC
CCCACACCTAGCGCCCAACGTTTACAGCG
TGNACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGC
GTCAGAACTGGCCCAGCAAGCCGCCTT
CGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAAT
TCCGCTTGCCCCTACCAGCCTCTAGCCT
GCCCGTATCCANCGCAGACCCGTAGTTAAGCNNCGGGCTTTCACGGCAGAC
GCAACANNNCNACGAGCTCTTTNCGCCNN
NNNNNNANGCTNGNNNCCTACGTATTACCNNGNTGCTGNACGTANTANCNN
NNNTNNTTCNNNNGGNNNNNN

Sequence File : reverse pH isolate 5

NNNNNNNNNNNNNNNNNNNANGACTTCGTCCCAATCGCCAGCCCCACCTT
CGACCGCTCCCCCCACAAGTGGTTGGGCC
ACGGGCTTCGGGTGTTGCCGACTTTCGTGACGTGACGGGCGGTGTGTACAAG
GCCCGGGAACGTATTCACCGCAGCGTTG
CTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCC
CAATCCGAACTGAGACCGGCTTTTAGGG
ATTCGCTCCACCTCACGGTATCGCAACCCTCTGTACCGACCATTGTAGCATG
TTTGCAGCCCAAGACATAAGGGGCATGA
TGACTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATG
AGTCCCCACCACCCCGAAAGGCGTGCT
GGCAACATGGAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAGCCATGCACC
ACCTGTACACCAGTCCAAAGGAGGCACCCATCTCTGAGTGTTTCCGGTGTAT
GTCAAACCTTGGTAAGGTTCTTCGCGTT
GCGTCGAATTAAGCAACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTTAGCCTTGCGGCCGTACTC
CCCAGGCGGGGCGCTTAATGCGTTAGCTACGGCACAGAGATCGTGGAAGAN
CCCCACACCTAGCGCCCAACGTTTACAGC
GTGGNCTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAN
CGTCANAAATGGGCCCAGCCAGCCGCC
TTCGCNCCGGAGTTCCTCCCNGAAATCNGCNCNNTATCACCAGCTACACCAG
AAATTCCNNTTGCCCCTTNCNNNNNCAT
ANCTGNCNNGNNTCNANGNNANACCCGNANTTANGNCNANGNNNTTNNAC
GNATA

Sequence File : reverse pH isolate 8

NNNNNNNNNNNNNNNNNNNNNCGACTTCGTCNNNTCGCCAGCCCCACCTT
CGACCGCTCCCCCCACAAGTGGTTGGGCC
ACGGGCTTCGGGTGTTGCCGACTTTCGTGACGTGACGGGCGGTGTGTACAAG
GCCCGGGAACGTATTCACCGCAGCGTTG
CTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCC
CAATCCGAACTGAGACCGGCTTTTAGGG
ATTCGCTCCACCTCACGGTATCGCAACCCTCTGTACCGGCCATTGTAGCATG
TTTGCAGCCCAAGACATAAGGGGCATGA
TGACTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATG
AGTCCCCACCACCCCGAAAGGCGTGCT
GGCAACATGGAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAGCCATGCACC
ACCTGTACACCAGTCCAAAGGAGGCACCCATCTCTGAGTGTTTCCGGTGTAT
GTCAAACCTTGGTAAGGTTCTTCGCGTT
GCGTCGAATTAAGCAACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCC
TTTGAGTTTTAGCCTTGCGGCCGTACTC
CCCAGGCGGGGCGCTTAATGCGTTAGCTACGGCACAGAGATCGTGGAAGAC
CCCCACACCTAGCGCCCAACGTTTACAGC
GTGGACTACCANGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAG
CGTCAGAACTGGCCCAGCAAGCCGCCT
TCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCANCGCTACACCAGGAA
TTCCGCTTGCCCCTACCAGCCTCTAGCC
TGCCCGTATCCACGCAGACCCGTAGTTAAGCCACGGGCTTTCACGGCAGANG
CGANAGCCACCTACGAGCTCNTTACGCC
CAATAANTNCNGANACGCTTGNNNCTACGTATTACCGNGNTGCTGNNCGTA
GTTANCNGNNNNTTCTTNNNNNNGGTANN
NGTCAANTN

Sequence File : reverse pH isolate 9

NNNNNNNNNNNNNNNNNNNNNNNACTTCGTCCAATCGCCAGCCCCACCTT
CGACCGCTCCCCCCACAAGTGGTTGGGCC
ACGGGCTTCGGGTGTTGCCGACTTTCGTGACGTGACGGGCGGTGTGTACAAG
GCCCGGGAACGTATTCACCGCAGCGTTG
CTGATCTGCGATTACTAGCGACTCCGACTTCATGGGGTCGAGTTGCAGACCC
CAATCCGAACTGAGACCGGCTTTTAGGG
ATTCGCTCCACCTCACGGTATCGCAACCCTCTGTACCGACCATTGTAGCATG
TTTGCAGCCCAAGACATAAGGGGCATGA
TGACTTGACGTCATCCCCACCTTCCTCCGAGTTGACCCCGGCAGTCTCCCATG
AGTCCCCACCACCCCAAAGGGCGTGCT
GGCAACATGGAACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTC
ACGACACGAGCTGACGACAGCCATGCACC
ACCTGTACACCAGTCCAAAGGAGGGCTCTGTCTCCNAANGTTTCCGGTGTAT
GTCAAACCTTGGTAAGGTTCTTCGCGTT
GCGTCGAATTAAGCAACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCC

TTTGAGTTTTAGCCTTGCGGCCGTACTC
CCCAGGCGGGGCGCTTAATGCGTTAGCTACGGCACAGAAGTCGTGGAAGAN
CCCCACACCTAGCGCCCAACGTTTACAGC
GTGGACTACCANGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAG
CGTCAGAACTGGCCCAGCAAGCCGCCT
TCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGAATT
CCGCTTGCCCCTACCAGCCTCTAGCCT
GCCCGTATCCACCGCANANCCGTAGTTAAGCCACGGGCTTTCACGGNAGAC
GCGANNGCCACNACNAGCTCTTTACGNCC
ANANTCNNNANNANNCTNGNNNNCCTACGTATTACNGCGGCNNNNGGNAN
GTNNTN