

CHARACTERIZATION OF *BACILLUS AMYLOLIQUEFACIENS* STRAIN BAC03
IN DISEASE CONTROL AND PLANT GROWTH PROMOTION

By

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ABSTRACT

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Streptomyces spp. can cause scab symptoms on many plants, including potato and radish, which can result in significant economic losses for agricultural production. As no satisfactory control methods are currently available, biological control can be considered as a promising strategy for managing the disease. A field in Michigan showed naturally occurring suppressiveness to potato common scab. The disease suppression was potentially associated with a group of antimicrobial bacteria. A strain of *Bacillus*, BAC03 (Patent No. US 7,615,366 B2), was selected from the bacteria showing antimicrobial activity. In this study, BAC03 was characterized under laboratory, greenhouse, and field conditions for antimicrobial activity, common scab control, and plant growth promotion activity.

BAC03 was identified as *Bacillus amyloliquefaciens* by analyzing sequences of fragments of the *recA*, *recN*, *cheA*, and *gyrA* genes. BAC03 displayed antagonistic activities against *Streptomyces* spp. on agar plates using a co-culture method. An antimicrobial substance, extracted from BAC03 by ammonium sulfate precipitation, was identified as an LCI peptide using liquid chromatography-mass spectrometry. The antimicrobial activity of either BAC03 liquid culture or the ammonium sulfate precipitate fraction was stable under a wide range of temperatures, and pH levels, as well as following incubation with several chemicals, but was removed by proteinases tested.

In greenhouse assays, BAC03 applied as a drench to soil-less potting mix significantly reduced scab in both potato and radish ($P < 0.05$), while no effect was observed using a foliar

spray or a seed treatment. BAC03 applied before radish planting completely suppressed the disease, but the later BAC03 was applied the less effective it was. BAC03 at 10^5 CFU cm^{-3} of potting mix or higher concentrations was effective at reducing radish scab ($P < 0.5$). Increasing the frequency of BAC03 application did not increase efficacy for disease reduction. In population dynamics, as determined by qPCR, the amount of *S. scabies* when BAC03 was applied before radish planting was significantly lower ($P < 0.5$) than that in other treatments with BAC03 application at later stages.

The ability of BAC03 to promote plant growth was investigated in nine selected plants at the concentration of 10^5 CFU cm^{-3} potting mix under greenhouse conditions. Application of BAC03 at 10 days after planting produced higher radish biomass compared to applications at other stages ($P < 0.5$). Multiple applications of BAC03 increased the biomass of radish roots and leaves compared with a single application. BAC03 produced indole-3-acetic acid and ammonia and showed a 1-aminocyclopropane-1-carboxylate deaminase activity, which potentially could be related to plant growth promotion. Volatiles released from BAC03, with acetoin and 2,3-butanediol as the major components detected by gas chromatography–mass spectrometry analysis, might be responsible for inhibition of plant seed germination, plant seedling growth, and the growth of *S. scabies*.

In two Michigan fields in 2011 and 2012, BAC03 (10^6 CFU ml^{-1} , 1 L m^{-2}) was applied as a drench into the root zone soil of potato four times at 2-week intervals starting at one month post planting. The BAC03 treatments significantly ($P < 0.05$) reduced the severity of potato common scab in all tests and had enhanced potato tuber weight in 2011 only ($P < 0.05$) at two locations.

To my parents, Xianchun Meng and Li Wang,
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Figure S6.3. Frequency of antagonists from bulk soil (result was derived from combined data sampled on 4/10/07, 11/04/08, 7/03/09, and 5/13/10) in both disease suppressive (SS) and conducive (CS) soils. Bars over the values are standard deviation, and symbol “*” indicates significant differences between SS and CS at $\alpha = 0.05$ 137

Figure S6.4. Frequency of pathogenic *Streptomyces* strains isolated from bulk and rhizosphere soils in both disease suppressive (SS) and conducive (CS) soils. Bars over the values are standard deviation, and symbol “*” indicates significant differences between SS and CS at $\alpha = 0.05$. Result was derived from combined data sampled on 7/10/07 and 5/13/10. 138

CHAPTER 1: LITERATURE REVIEW

POTATO PRODUCTION

Potato crop

The potato (*Solanum tuberosum* L.) originated in the Andes Mountains of South America, and has been cultivated for about 10,000 years (Salaman, 1949). Since it was introduced into Europe in the sixteenth century, the potato has gradually become a major food source in many European countries (Brown, 1993). Potatoes are now grown in more than 100 countries, with the top four producers being China, Russia, Ukraine, and Poland (Hijmans, 2001). Potato is the world's number one non-grain food commodity, and the fourth most important food crop following wheat (*Triticum aestivum* L.), maize (*Zea mays* L.), and rice (*Oryza sativa* L.) (Johnson and Powelson, 2008). According to a USDA report in September, 2013, commercial potato production in the US was 463 million hundredweight (cwt) in 2012, with a harvested area of 1.13 million acres, average yield of 409 cwt per acre, value of \$3.73 billion, and quantity of potatoes sold at 430 million cwt (93% of the production) (NASS, 2013). Potatoes are utilized as table stock, in processed food, and as propagation materials (seed tubers) (NASS, 2013) (Table 1.1). The two states with the largest potato production in the US are Idaho and Washington, which account for almost half of the national production, while Michigan ranks 9th in production in the US (NASS, 2013).

The potato belongs to the Solanaceae family that includes more than 90 genera (Burton, 1989). *Solanum tuberosum* is the most commonly grown potato worldwide (Harris, 1992). The species includes two subspecies: *S. tuberosum* subsp. *tuberosum* and *S. tuberosum* subsp. *andigena* (Burton, 1989; Johnson and Powelson, 2008). The genetic backgrounds of wild and cultivated potatoes are complex due to interspecific hybridization at the diploid and polyploid

levels (Rodriguez *et al.*, 2010). Chromosome numbers may vary from diploid, triploid, tetraploid, pentaploid to hexaploid (Spooner and Bamberg, 1994; Gebhardt and Valkonen, 2001).

The potato is classified as an annual, herbaceous, dicotyledonous plant. However, it can grow as a perennial because it can be vegetative propagated from the potato tuber (Burton, 1989). Potato tubers are formed from the tips of stolons as a lateral proliferation of storage tissue via cell division and enlargement (Burton, 1989). This process usually starts five to seven weeks post planting or when the potato plants are 15 to 20 cm high (Johnson and Powelson, 2008). Tuberization mainly depends on translocation and storage of carbohydrate from other parts of the plant, but is not dependent upon flowering (Stevenson *et al.*, 2001).

Table 1.1. Potato utilization in the United States in 2012 (NASS, 2013)

Purpose	Category	Consumption (x 1,000 cwt)	Share (%)
Sale	Table stock	118.5	25.6
	Processing	283.2	61.2
	Chips and shoestrings		
	Dehydrated		
	Frozen French fries		
	Other frozen products		
	Canned products		
	Other canned products (hash, stews, soups)		
	Starch, flour, and others		
	Livestock feed	4.1	0.9
	Seed	23.7	5.1
Non-sale	Seeds	33.2	7.2
	Household use		
	Shrinkage and loss		
Total		462.8	100

Potato diseases

The potato plant is a host for many pathogenic organisms, including bacteria, fungi, viruses, viroids, nematodes, and phytoplasmas (Stevenson *et al.*, 2001; Fiers *et al.*, 2012). These pathogens can cause damage on all parts of the potato plant (Burton, 1989). Potato growth is also threatened by insects, weeds, and mites (Johnson and Powelson, 2008). Abiotic factors can cause nonparasitic diseases. These abiotic factors include excess or insufficient plant nutrients, excess moisture, low oxygen levels, and high temperature (Stevenson *et al.*, 2001).

Potatoes are commercially grown using cut potato tuber pieces (Johnson and Powelson, 2008). This practice results in wounds that are favorable for pathogen infection during the stage of seed preparation and the early stage of growth after planting (Tsror *et al.*, 1999). These infections may lead to potato seed piece decay which can be caused by *Fusarium* spp. and *Pythium* spp., as well as by some soft rot bacteria, resulting in delayed emergence and poor stand establishment (Shashirekha and Narasimham, 1989). The infection is primarily from inoculum that is spread during seed handling and seed cutting, and occasionally from contact with inoculum in the soil (Toth *et al.*, 2003; Wharton and Kirk, 2007). The decayed plant tissues and debris in the soil can serve as the media for pathogen survival (Wharton and Kirk, 2007).

During the potato growth period from planting to early tuber bulking of potato, bacterial and fungal diseases are a major concern (Johnson and Powelson, 2008). There are several bacterial diseases that impact potato production. For example, soft rot or blackleg is caused by *Pectobacterium* spp. and *Dickeya* spp., which produce pectolytic enzymes that break down the pectins that are required for cell wall structure and the middle lamella (Davidson, 1948). Once potato plants are infected, plants tissues start to decay and discoloration progresses from the base of the stem to vascular tissues. Tuber soft rot symptoms range from a slight vascular

discoloration to complete decay (Davidson, 1948; Czajkowski *et al.*, 2011). Another bacterial disease, potato ring rot is caused by *Clavibacter michiganensis* subsp. *sepedonicus* (Deboer and Slack, 1984). The pathogen invades the host xylem, and interrupts the transport of water and dissolved minerals, resulting in wilting of stems and leaves, along with chlorosis and necrosis of leaves (Deboer and Slack, 1984). Brown rot (*Ralstonia solanacearum*), also known as bacterial wilt, causes symptoms similar to ring rot, but brown rot can result in oozing of bacterial slime from the potato tuber eyes (Messiha *et al.*, 2009).

As well as bacterial disease, diseases caused by fungi and fungal-like organisms also can be an issue. For example, potato early dying (*Verticillium dahliae* and *V. albo-atrum*) is a syndrome consisting of premature vine death and declining yields (Powelson and Rowe, 1993). Although *Verticillium* spp. are the primary pathogens, other organisms, such as root-lesion nematodes, have been associated with this disease (Rowe and Powelson, 2002). Rhizoctonia cankers (*Rhizoctonia solani*) affects potato growth and tuber quality, resulting in canker, stolon pruning, and sclerotia formation on the surface of potato tubers (Stevenson *et al.*, 2001). Early blight (*Alternaria solani*) causes circular dark spots with concentric rings on leaves. Tuber infection and subsequent lesion formation in storage can affect the quality of potato tubers (Leiminger and Hausladen, 2012). Late blight (*Phytophthora infestans*) is the most destructive disease of potato (Judelson, 1997). Under favorable conditions, it only takes two to three weeks from disease initiation to the potato being completely defoliated (Ersek, 1979). Late blight infected potato tubers are also susceptible to other diseases, which can result in further problems during storage (Secor and Gudmestad, 1999). In 1845 and the following year, outbreaks of late blight destroyed the potato crop in northern Europe, resulting in 1.5 million Irish people dying of famine and disease, and driving a large emigration from Europe to North America (Salaman,

1949). Late blight continues to be a threat in potato production across the world (Swiezynski and Zimnoch-Guzowska, 2001).

Wounds or bruises occurring during harvesting or handling help pathogens easily enter into potato tubers and these can cause diseases in storage (Stevenson *et al.*, 2001). Storage pathogens include *Fusarium* spp. (Fusarium dry rot), *Pythium* spp. (Pythium leak), *Pectobacterium* spp. (soft rot), and *Phytophthora* spp. (pink rot, late blight) (Secor and Gudmestad, 1999).

POTATO COMMON SCAB

Common scab is an important and prevalent soil-borne disease of potato (Loria *et al.*, 1997; Loria *et al.*, 2006). It was first identified in North America in the late 19th century (Thaxter, 1892), and is now found all over the world wherever potatoes are grown (Lindholm *et al.*, 1997; Park *et al.*, 2003b; Wanner, 2006; Flores-Gonzalez *et al.*, 2008). Yield reductions due to the disease are usually minor, but poor quality and bad appearance of the tuber caused by common scab can result in significant economic losses (Larkin and Griffin, 2007; Hiltunen *et al.*, 2009; Dees and Wanner, 2012).

Pathogen(s)

Potato common scab is caused by a group of pathogenic *Streptomyces* spp. (Loria *et al.*, 1997; Wharton *et al.*, 2011). *Streptomyces* are Gram-positive actinobacteria with high G+C content in the genomic DNA and form filamentous mycelia (Schaad *et al.*, 2001). The family generally has a saprophytic life cycle and produces a number of hydrolytic enzymes which help nutrient acquisition through degrading organic materials (Loria *et al.*, 1997; Loria *et al.*, 2008).

Of the over 900 species in the *Streptomyces* genus identified, only over a dozen are plant

pathogens (Bignell *et al.*, 2010a). Potato common scab has long been known to be caused by *S. scabies*, which is the most common *Streptomyces* spp. causing scab in most places (Loria *et al.*, 1997). Several other pathogenic species have been reported to be responsible for potato common scab (Lindholm *et al.*, 1997; Song *et al.*, 2004; Wanner, 2006). For example, *Streptomyces stelliscabiei* has been reported from France and the USA (Bouček-Mechiche *et al.*, 2000; Wanner, 2006; Jiang *et al.*, 2012). *Streptomyces europaeiscabiei* was found in northern Europe (Flores-Gonzalez *et al.*, 2008). *Streptomyces turgidiscabies* has been found causing potato scab in Japan and Finland (Miyajima *et al.*, 1998; Kreuze *et al.*, 1999), and *S. acidiscabies* was reported in Maine in the US, and subsequently found in Korea, and Japan (Lambert and Loria, 1989; Tóth *et al.*, 2001; Song *et al.*, 2004). In addition, three new species that can cause scab have been described in Korea (Park *et al.*, 2003a; Park *et al.*, 2003b). Recently, a new group of scab-causing *Streptomyces* was identified in Idaho (Wanner, 2006), and one isolate (DS3024) that caused common scab in Michigan was reported not related to any known *Streptomyces* spp. (Hao *et al.*, 2009). These may suggest that more unknown pathogenic *Streptomyces* spp. exist.

Symptoms and disease cycle

Symptoms include superficial or russet scab (cork-like lesions darker than the healthy skin), raised or erumpent scab (corky growth protruding from the periderm), and sunken or pitted scab (penetrates into the tuber and produces sunken lesions) (Loria *et al.*, 1997; Stevenson *et al.*, 2001). More than one type of lesion may be present on a single tuber. The type(s) of lesion(s) formed on a tuber is believed to be dependent on a combination of host resistance of the potato cultivar used, the aggressiveness of the infecting species, time of infection, and environmental conditions (Loria *et al.*, 1997; Wharton *et al.*, 2011).

Pathogenic *Streptomyces* spp. can be soil-borne or seed-tubers borne, and can be spread from field to field by wind, rain, and irrigation, as well as by residual soil attached to seed tubers and farm equipment (Johnson and Powelson, 2008). To survive from one season to another, spores and mycelia of the pathogen stay alive in soil or in residues of hosts, and organic matter in soil. Vegetative mycelia of the pathogen develop specialized aerial filaments, and eventually produce spiral spore chains through fragmentation with cross wall. Individual spores are obtained by breaking off from the tip of the spore chains. The disease infection starts when mature spores land on the plant host. The spores germinate and form germ tubes, which penetrate into the potato tuber through immature lenticels and stomata on the epidermis (before the periderm differentiates), as well as through wounds on the surface of the potato tuber. Spores can infect developing tubers in soil within a wide range of temperatures (from 10° to 27°C), but the optimal temperature for pathogen infection ranges from 20 to 22°C (Stevenson *et al.*, 2001).

Once the pathogens have invaded into the tuber tissue, they can grow through a few layers of cells and make the cells to die. The *Streptomyces* were feed off the dead cells by retrieving nutrients from them. Living potato cells around the infection areas divide rapidly to form several suberized layers (Wharton *et al.*, 2011). When the first corky layer is penetrated by the pathogen, a new layer forms underneath by repeating the cycle. The lesion can be enlarged throughout the plant growth period with the expansion of the potato tuber. Sometimes circular or irregular lesions on mature tubers may coalesce to form large scab areas during maturation (Stevenson *et al.*, 2001; Wharton *et al.*, 2011).

Host range of *Streptomyces* spp.

Potato is not the only host for *Streptomyces* spp. The pathogens that cause common scab also can infect many other plants (Goyer and Beaulieu, 1997). Pathogenic *Streptomyces* spp. are

not host specific (Goyer and Beaulieu, 1997), enabling them to induce scab lesions on various root and tuber crops, including beet (*Beta vulgaris* L.), radish (*Raphanus sativus* L.), rutabaga (*Brassica napobrassica* L.), turnip (*Brassica rapa* L.), carrot (*Daucus carota* L.), and parsnip (*Pastinaca sativa* L.) (Goyer and Beaulieu, 1997). In addition, scab-causing *Streptomyces* isolates can cause disease in seedlings of both monocotyledonous and dicotyledonous plants, resulting in root and shoot stunting, browning, tissue necrosis, and seedling death (Scheible *et al.*, 2003). This occurs because the target of a pathogenicity determinant (thaxtomin, described later) is the cellulose (Fry and Loria, 2002). However, the range of hosts where disease has been reported in the field is limited, which may be due to the population density of the pathogen and interactions with other microbes (Loria *et al.*, 2006).

Pathogenicity determinants

Regardless of the species, all pathogenic *Streptomyces* produce a group of phytotoxins, called thaxtomins (King *et al.*, 1989; Loria *et al.*, 2006). Thaxtomins have been confirmed as a pathogenicity determinant for *Streptomyces* spp. (Kers *et al.*, 2005; Loria *et al.*, 2006). The structure and biological activity of these secondary metabolites were originally described by Lawrence and King (King *et al.*, 1989; Lawrence *et al.*, 1990). Thaxtomin A, a cyclic dipeptide (consisting of cyclized nitro-tryptophan and phenylalanine), is the predominant form produced by scab-causing *Streptomyces* spp. among 11 members of the thaxtomin family (Loria *et al.*, 2008).

The primary mode of action of thaxtomin A is to inhibit cellulose biosynthesis in expanding plant tissues, but it can also elicit cell hypertrophy, root and shoot stunting, tissue necrosis, programmed cell death, and alterations in plant Ca⁺ and H⁺ ion influx (Loria *et al.*,

1997; Loria *et al.*, 2006; Loria *et al.*, 2008). They may also facilitate penetration or nutrient acquisition through the inhibition of cellulose synthesis (Bignell *et al.*, 2010a).

Genes for the biosynthesis of thaxtomins are clustered in a large chromosome region that functions as a pathogenicity island (PAI) that can be horizontally transferred to a non-pathogenic isolate to create a new pathogenic strain (Kers *et al.*, 2005). Genes related to thaxtomin biosynthesis are clustered in one section of the PAI where they are called the ‘toxicogenic region’ (Lerat *et al.*, 2009). This includes the *txtA* and *txtB* genes responsible for production of the non-ribosomal peptide synthetases TxtA and TxtB (Healy *et al.*, 2000), which are required for production of the cyclic dipeptide backbone; the *txtC* gene, responsible for a P450 monooxygenase TxtC (Healy *et al.*, 2002), required for post-cyclization hydroxylation reactions; the *nos* gene responsible for a nitric oxide synthase NOS (Kers *et al.*, 2004), which is required for site-specific nitration; and the *txtR* gene, responsible for a transcriptional regulator protein TxtR (Joshi *et al.*, 2007b).

In addition to genes responsible for toxin production there is another segment of the PAI, called the ‘colonization region’, which contains genes such as *nec1* and *tomA* (Kers *et al.*, 2005). Nec1 (encoded by *nec1*) is a secreted protein required for colonization by the pathogen and may suppress plant defense during infection (Joshi *et al.*, 2007a). TomA (encode by *tomA*), a homologue of tomatinase enzymes, can detoxify some phytoanticipins that function in plant defense response against pathogens (Kers *et al.*, 2005). Unlike the *txtAB* genes, *nec1* and *tomA* are not fully conserved in all pathogenic isolates, which indicates that they are not essential to pathogenicity, but may play a significant role in virulence (Loria *et al.*, 2006).

In recent years, several complete *Streptomyces* genomes have been made available, such as *S. scabies* (Bignell *et al.*, 2010b), *S. turgidiscabies* (Huguet-Tapia *et al.*, 2011), *S. coelicolor*

(Bentley *et al.*, 2002), *S. avermitilis* (Ikeda *et al.*, 2003), *S. griseus* (Ohnishi *et al.*, 2008), and several other *Streptomyces* species (online link: <http://www.ncbi.nlm.nih.gov/genome/?term=streptomyces>). Comparative genomic analyses may facilitate identification of genes responsible for plant pathogenicity in *Streptomyces* spp. For example, comparison of genome information revealed many genes in *S. scabies* that may relate with virulence or with plant-microbe interactions since they are conserved in other plant or animal pathogens (Loria *et al.*, 2006; Bignell *et al.*, 2010a). The genome of *S. scabies* strain 87-22 contains a gene cluster highly similar to the gene cluster for coronafacic acid (CFA), which is an important virulence determinant in *Pseudomonas syringae* (Bignell *et al.*, 2010b). Based on colonization and mutational studies, the CFA-like cluster was confirmed to be important for the virulence of *S. scabies*, but it is not conserved in *S. turgidiscabies* or *S. acidiscabies* (Bignell *et al.*, 2010b). In addition, researcher found genomes of *Streptomyces* spp. have several other potential virulence genes encoding homologues of concanamycins (Natsume *et al.*, 1996), expansin-like proteins (Bignell *et al.*, 2010a), cutinase (Fett *et al.*, 1992), phytohormones (Elshanshoury, 1991), which work as virulence or plant-microbial interaction-associated factors in other plant or animal pathogens. However, these genes are not conserved in all *Streptomyces* spp. (Bignell *et al.*, 2010a), indicating they may not be essential for the pathogenicity. Their functions need to be further investigated by confirmation of production.

MANAGEMENT OF POTATO COMMON SCAB

Potato common scab is difficult to manage because factors contributing to disease occurrence and severity are poorly understood (Hiltunen *et al.*, 2009; Dees and Wanner, 2012).

Though many strategies have been recommended and examined for common scab control, inconsistent and/or inadequate results have been obtained in some cases (Lazarovits *et al.*, 1999; Abbasi *et al.*, 2006). The most important factors that impact scab and its management include: 1) the pathogens: a diverse genetic background results the pathogen to have different level of virulence (Wanner and Haynes, 2009) and the organisms can live in the soil for a long time even without hosts (Johnson and Powelson, 2008); 2) the host: lack of information of the relationship between common scab severity and plant physiology and genetic background (Dees and Wanner, 2012); 3) environmental factors: pH, soil moisture, and interaction with soil microbial organisms all contribute to common scab outbreaks (Lazarovits *et al.*, 2007). Therefore, management of the disease usually depends on a combination of practices, but without complete control. Major strategies include:

Cultivar resistance

Using disease-resistant cultivars is an ideal choice for controlling soil-borne diseases (Fiers *et al.*, 2012). Although some potato varieties have moderate levels of disease tolerance, there are no completely resistant cultivars commercially available for scab control (Wanner and Haynes, 2009). Varieties ‘Ontario’ and ‘Krantz’ are moderately tolerant, but are not widely grown (Goth *et al.*, 1995). ‘Superior’, ‘Russet Burbank’ and ‘Pike’ are three common varieties that can be infected but the severity of common scab is low (Haynes *et al.*, 2010).

There are many challenges in developing common scab-resistant cultivars. For example, the complexity of the potato genetic background and disease severity variable are the top concerns (Dees and Wanner, 2012). Although resistance genes to common scab have been found in haploid or diploid potatoes (Cipar and Lawrence, 1972), these genes cannot work in commercial cultivars that are usually tetraploid (Goth *et al.*, 1993). In the case that disease

resistance is controlled by a recessive gene, the chance of getting a putative recessive phenotype in a tetraploid following the crossing process is extremely low, and the tetraploid nature results in a very slow breeding process (Driscoll *et al.*, 2009). Factors contributing to common scab development are complex, resulting in inconsistent selection of resistance phenotypes (Wharton *et al.*, 2011). For instance, with the same pathogen inoculation condition, scab symptoms varied from none to severe on tubers of a single potato plant (Dees and Wanner, 2012).

Chemical control

The most widely used chemical in controlling potato common scab is pentachloronitrobenzene (PCNB), which was first studied in the 1950s in the US (Hooker, 1954). Davis reported PCNB suppressed potato common scab only at high concentrations (Davis *et al.*, 1976). However, tuber size and yield are reduced under high concentrations of PCNB (Wharton *et al.*, 2011). High cost and negative environmental impacts (Caseley, 1969) led to some chemical products being removed from the market. PCNB was withheld by the EPA in 2010 (EPA website: www.epa.gov).

Irrigation and pH

Since the 1920's, irrigation has been applied for potato common scab management (Lapwood *et al.*, 1970). Maintaining soil moisture at or near field capacity during the tuberization is efficient in reducing common scab symptoms (Davis *et al.*, 1976; Lambert and Manzer, 1991). This effect might be due to irrigation lowering *Streptomyces* spp. populations in tuber lenticels by increasing other bacterial populations (Stevenson *et al.*, 2001). However, studies show that irrigation is not always consistent in the management of potato common scab (Wilson *et al.*, 2001; Larkin *et al.*, 2011). A reason for these results may relate to the soil type since the water holding capacity varies in different soils (Johnson and Powelson, 2008).

Although using irrigation can suppress common scab, it may favor the development of other potato diseases such as pink rot or *Pythium leak* (Wharton *et al.*, 2011).

Lowering soil pH is another traditional method for common scab control (Lambert and Manzer, 1991; Waterer, 2002). A pH below 5.2 was shown to inhibit scab incidence effectively (Wharton *et al.*, 2011). In order to maintain this level of soil pH, acid-forming fertilizers such as ammonium sulfate or ammonium phosphate are applied to the soil (Lacey and Wilson, 2001). However, this strategy is not applicable in all situations because some pathogenic *Streptomyces* spp. are tolerant to acid condition such as *S. acidiscabies* and *S. turgidiscabies*. More species have been reported to cause disease in soils with pH as low as 3.8 (Lambert and Loria, 1989; Lindholm *et al.*, 1997). Alternatively, soils at a pH of 8.5 has been shown to suppress common scab (Waterer, 2002). Overall, irrigation and lowering soil pH are recommended methods for potato common scab management, especially in combination with other management methods, such as using crop rotation, resistant cultivars, and chemical control (Loria, 2001; Wharton *et al.*, 2011).

Cultural practices

Cultural practices for common scab management include crop rotation, green manures, cover crops, and organic soil amendments (Larkin and Griffin, 2007; Larkin *et al.*, 2011). The principle of these strategies is to manipulate soil fertility and soil structure, to replenish nutrients, and to avoid pathogens specific for one host, or to change residential soil microbial communities by using exudates from plant root or organic amendment (Johnson and Powelson, 2008).

Streptomyces spp. can survive as saprophytes in the soil and plant residues, which brings a big challenge for using crop rotation to control of potato common scab (Peters *et al.*, 2004). However, crop rotation combined with other cultural practices could provide good results for

scab control (Larkin, 2008). For example, Larkin et al. (2011) reported that common scab was reduced by 25 to 40 % using a combination of rotation with different crops for up to 3 years, along with green manures, as well as cover crop approaches.

Organic soil amendments usually use green manure and industrial or agricultural waste products (Lazarovits *et al.*, 1999). Many have been tested for common scab disease control. These include fish emulsion (Abbasi *et al.*, 2006); soymeal, meat and bone meal (Lazarovits *et al.*, 1999); lopsided oat green manure (Sakuma *et al.*, 2011); and mustard green manure (Larkin and Griffin, 2007). However, the results were not consistent among different seasons or locations.

Biological control

One approach of the biological control is to introduce specific microorganisms in order to establish a population of beneficial microorganisms (Kim *et al.*, 2011). Several groups of microorganisms have displayed promising activities for common scab control, such as *Pseudomonas* spp. (Al-Mughrabi, 2010; Singhai *et al.*, 2011), *Bacillus* spp. (Han *et al.*, 2005; Larkin and Tavantzis, 2013), and non-pathogenic *Streptomyces* spp. (Liu *et al.*, 1995; Hiltunen *et al.*, 2009). Biological control agents have multiple potential modes of action, including (but not limited to) secreting antibiotics detrimental to pathogens, repelling pathogens based on competitive activity, or inducing disease resistance in the host plant (Lugtenberg and Kamilova, 2009). St-Onge (2011) reported *Pseudomonas* sp. LBUM 223 produces phenazine-1-carboxylic acid (PCA), an antibiotic compound, which inhibited the growth of *S. scabies in vitro* and represses the gene expression of thaxtomin A (St-Onge *et al.*, 2011). Strain *Bacillus* sp. “sunhua” was shown to inhibit scab in the greenhouse, decreasing the infection rate from 75% to 35% but without field validation (Han *et al.*, 2005). Use of non-pathogenic strains of *Streptomyces* in

inhibition of pathogenic *Streptomyces* and reduction in scab have also been demonstrated (Liu *et al.*, 1995; Hiltunen *et al.*, 2009).

PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR)

Plants have to cope with both biotic and abiotic stresses. These impacts are from both aboveground and belowground factors, and can be beneficial, neutral, or adverse for plant growth (Spaepen *et al.*, 2009). Below ground, some impacts come from the activities occurring in the region of the soil layer close to plant roots, called the rhizosphere (“the soil compartment influenced by the roots of growing plants”) (Hiltner, 1904; Kloepper *et al.*, 1980). As exudates secreted by plant roots in this area serve as nutrients, the rhizosphere is highly attractive to a great number of microorganisms, where their populations are 100- to 1,000-fold higher than that in the bulk soil (Lynch and Whipps, 1990).

Certain bacterial species associated with and/or living in the rhizosphere can improve plant growth, and were termed as plant growth-promoting rhizobacteria (PGPR) by Kloepper and Schroth (1980). To date, over two dozen bacterial genera have been reported to have plant growth-promoting activities. These bacterial genera include *Azotobacter*, *Azospirillum*, *Bacillus*, *Pseudomonas*, *Enterobacter*, *Klebsiella*, *Burkholderia*, *Serratia*, *Alcaligenes*, *Arthobacte*, *Micrococcus*, and new genera and species of PGPR are still being discovered (Bloemberg and Lugtenberg, 2001; Kim *et al.*, 2011).

The biological control agents is considered to be an attractive crop-management practice since it could substantially reduce the use of chemical pesticides. Use of some synthetic chemicals have caused serious problems to human beings and the environment, and raised the risk of fungicide or pesticide resistance (Ishii, 2006). In addition to decreasing chemical use,

application of PGPR can increase crop yield by helping plants to take in nutrients, thereby helping to reduce fertilizer inputs (Spaepen *et al.*, 2009).

***Bacillus* spp. as biocontrol agents and commercial products**

Members of the genus *Bacillus* are Gram positive, aerobic, rod-shaped, and endospore-forming bacteria, which belong to class I (Bacilli) of the phylum *Firmicute* (Schaad *et al.*, 2001). They survive in a wide variety of ecological environments including during adverse conditions in the form of their robust endospores (Nicholson *et al.*, 2000). The resistance of the endospores to heat, desiccation, disinfectants, and other sterilizing conditions favors the transportation and shelf life of these bacteria (Ongena and Jacques, 2008).

Bacillus-based bio-pesticides represent the most important class of microbial products commercially available, including bio-insecticides, bio-fungicides, and bio-fertilizers (McSpadden Gardener and Fravel, 2002a; Fravel, 2005). Bacilli are the most widely used bacteria in the biopesticides market in North America (Borriss, 2011). Among these products, bio-insecticides are the vast majority, with a share of up to 79% of the biopesticides market (Perez-Garcia *et al.*, 2011). The most popular microorganism for commercial bio-insecticides is *Bacillus thuringiensis*, which has been developed into more than 100 products (Roh *et al.*, 2007). It was discovered in 1901 and is used to control insect pests in agriculture and medicine (Roh *et al.*, 2007). This species produces a specific endotoxin (Cry protein), which has insecticidal properties. The genes responsible for the Cry protein have been successfully transferred into different crop plants, offering certain level of insect resistance (Roh *et al.*, 2007; Kumar *et al.*, 2008). In addition to the bio-insecticides, *Bacillus subtilis* and its close relatives have been frequently used as bio-fertilizers and bio-fungicides (Borriss, 2011) (Table 1.2). One obstacle to commercial development is a lack of consistent effect. This could be due to insufficient

knowledge related to bacteria-plant interaction, as well as costly and time-consuming registration procedures (Borriss, 2011).

In recent years, many large chemical companies have become more interested in the market of biopesticides (Borriss, 2011). For example, Bayer Crop Science obtained two EPA-registered biocontrol products, Kodiak (*B. subtilis* GB03) and Yield Shield (*B. pumilus* GB34), by acquisition of Gustafson Inc. in 2004; DuPont signed an agreement to provide Marrone Bio Innovations with exclusive access to natural discoveries for development as biopesticide products in 2007; BASF obtained a license for supply and distribution of several biopesticide products in California in 2009. In addition, several large companies in different countries have collaborated with small biopesticides producers to develop their own products (<http://www.farmchemicalsinternational.com>). Examples for commercial products based on *Bacillus* formulations are listed in Table 1.2.

Plant-associated *Bacillus amyloliquefaciens*

Bacillus amyloliquefaciens was first described in 1943 as a potent producer of liquefying amylase and other extracellular enzymes of industrial importance (Fukumoto, 1943). It has been classified into the “*Bacillus subtilis*” group, which is heterogeneous and involves a group of closely related species, such as *B. subtilis*, *B. amyloliquefaciens*, *B. licheniformis*, and *B. pumilus* (Logan and Berkeley, 1984). In 1987, *B. amyloliquefaciens* was reclassified as a distinct species based on low DNA relatedness with *B. subtilis* (Priest *et al.*, 1987).

Bacillus amyloliquefaciens strain FZB42 is the most extensively studied isolate in this species, and the first Gram-positive PGPR that has been sequenced (Chen *et al.*, 2007). It is characterized as a plant growth promoting bacterium, and has been commercialized as a biofertilizer by ABiTEP GmbH corporation (online link: <http://www.abitep.de/>). Not all *B.*

amyloliquefaciens possess the ability of plant-growth promotion (Borriss *et al.*, 2011). For example, the type strain *B. amyloliquefaciens* DSM7 is a non-plant-associated isolate (Borriss *et al.*, 2011). Based on comparative analysis between the genome sequences of strains FZB42 and DSM7, differences were observed in the variable part of the genomes, although the core genome are very similar with an identity of 97.89%. Strain FZB42 dedicates 8.5 % (about 340 kb) of its total genetic capacity to antimicrobial compound synthesis, while strain DSM7 only devotes 4-5% of the whole genome for synthesis of such compounds. As a consequence, it was concluded that *B. amyloliquefaciens* FZB42 and DSM7 belong to taxonomically related but distinct units (Borriss *et al.*, 2011).

Table 1.2. Examples of commercial products based on *Bacillus* formulation

Commercial name	Active species/strain	Manufacturer
BioYield	<i>B. amyloliquefaciens</i> GB99 <i>B. subtilis</i> GB03	Novozymes Biologicals Inc., Salem, VA
Companion	<i>B. subtilis</i> GB03 <i>B. licheniformis</i> <i>B. megaterium</i>	Growth Products Ltd., White Plains, NY
EcoGuard	<i>B. licheniformis</i>	Novozymes Biologicals Inc., Salem, VA
Kodiak	<i>B. subtilis</i> GB03	Bayer Crop Science, North Carolina, NC (former Gustafson, LLC.)
Mepplus	<i>B. cereus</i> BP01	Micro-Flo Company LLC., Memphis, TN
RhizoPlus	<i>B. subtilis</i> FZB24	ABiTEP GmbH, Berlin, Germany
RhizoVital 42	<i>B. amyloliquefaciens</i> FZB42	ABiTEP GmbH, Berlin, Germany
Serenade	<i>B. subtilis</i> QST713	AgraQuest Inc., Davis, CA
Sonata	<i>B. pumilus</i> QST2808	AgraQuest Inc., Davis, CA
Subtilex	<i>B. subtilis</i> MBI600	Becker Underwood Saskatoon, Saskatchewan, Canada
Taegro	<i>B. subtilis</i> FZB24	Novozymes Biologicals Inc., Salem, VA
YieldShield	<i>B. pumilus</i> GB34	Bayer Crop Science, NC (former Gustafson, LLC.)

MECHANISMS OF PGPR IN PROMOTING PLANT GROWTH

Plant growth promotion by PGPR is a complex process. It involves exchanges of metabolites between plants and bacteria, and the interaction between the organisms is affected by many environmental factors (Bloemberg and Lugtenberg, 2001; Kim *et al.*, 2011). Several possible mechanisms have been postulated to explain how PGPRs stimulate or promote plant growth, which can be categorized as direct or indirect actions (Lugtenberg and Kamilova, 2009; Spaepen *et al.*, 2009).

Direct plant growth promotion is obtained in the absence of pathogens through either providing the plant with substances synthesized by the bacterium or facilitating the uptake of certain plant nutrients from the environment (Ahmad *et al.*, 2008; Spaepen *et al.*, 2009). This kind of action is also called “biofertilization” (Bloemberg and Lugtenberg, 2001). PGPRs directly stimulate or facilitate plant growth in several ways, including, but not limited to: 1) producing various phytohormones, such as auxins, cytokinins, and gibberellins (Santner *et al.*, 2009); 2) secreting enzymes that can modulate plant growth and development, such as reducing ethylene levels by synthesis of 1-aminocyclopropane-1- carboxylate deaminase (Naclerio *et al.*) (Penrose *et al.*, 2001); 3) enhancing asymbiotic nitrogen fixation (Adesemoye *et al.*, 2010); 4) increasing the solubilization of phosphorus and other trace element for the plant to take up (Jorquera *et al.*, 2008); and 5) synthesizing siderophores which can provide soluble iron to plants (Schwyn and Neilands, 1987).

In addition to directly stimulating plant growth, PGPRs also can help plant growth by reducing or preventing the deleterious impacts of plant pathogens. This kind of action of disease control by living organisms is also called “biological control” (Schippers *et al.*, 1987). The biological control agents can protect plants by many mechanisms, including 1) producing

antibiotics or other metabolites that inhibit pathogen growth; 2) out-competing with phytopathogens for nutrients and niches; 3) inducing plant defense against pathogen infection; 4) producing volatiles and other molecules or metabolites; and 5) predation and parasitism of plant pathogens (Lugtenberg and Kamilova, 2009). Some of above mechanisms are briefly introduced as below.

Plant hormones

Plant hormones are endogenous chemical compounds element, which can regulate the growth, development, and tissue differentiation of plants at very low concentrations (Turfitt, 1941). Many bacterial species have been found to produce phytohormones (Barea *et al.*, 1976; Arshad and Frankenberger, 1991). Several commonly reported classes of phytohormones, including auxins, cytokinins, gibberellins, ethylene, and abscisic acid are described below.

1) Auxin has been found in higher plants and in microorganisms (Wohler, 1997). Indole-3-acetic acid (IAA) is one of the most extensively studied and abundant member in the auxin family. It has been detected in several bacteria belonging to the genera *Azospirillum*, *Azotobacter*, *Enterobacter*, *Arthobacter*, *Burkholderia*, *Bacillus*, *Erwinia*, *Pantoea*, *Pseudomonas*, and *Serratia* (Teale *et al.*, 2006). About 80% of the bacteria isolated from the rhizosphere are capable of producing IAA (Patten and Glick, 1996).

Rhizobacteria secrete IAA via different pathways, such as indole-3-acetamide (Clark *et al.*, 1993; Coyne *et al.*, 2012) and indole-3-pyruvate (IPyA) intermediates (Patten and Glick, 1996). L-Tryptophan is an IAA precursor in bacteria (Patten and Glick, 1996). Root exudates of various plants contain rich supplies of natural tryptophan, which are used by rhizosphere microorganisms for synthesis and release of auxin as secondary metabolites (Dodd *et al.*, 2010). The effect of IAA on plants depends on IAA concentrations. Eliasson (1989) observed the

application of IAA stimulates root elongation at concentrations lower than 100 nM, but inhibits the growth at concentrations higher than 500 mM.

2) Cytokinins, such as zeatin and kinetin are capable of inducing division of plant cells, with the help of auxin (Van Staden and Davey, 1979). The ratio of auxins and cytokinins determines the cellular differentiation in plants: a higher auxin level favors root development, while a higher cytokinin level promotes shoot differentiation; at a 1:1 ratio of these two phytohormones, plant cell proliferation is induced (Jameson, 2000; Pertry *et al.*, 2009). Cytokinins are synthesized in root tips and developing seeds, and transported to the shoot through the xylem to regulate such factors as leaf expansion and chloroplast development (Van Staden and Davey, 1979). The spectrum of cytokinins produced by PGPRs is similar to those produced by the plant. Cytokinins produced by rhizobacteria can significantly influence the plant growth and development. Excessive amount of cytokinins can cause abnormal plant growth (Jameson, 2000).

3) More than 100 gibberellins have been isolated from microorganisms (Bottini *et al.*, 2004). These compounds are mainly involved in division and elongation of plant cells and affect almost all stages of plant growth (Pharis and King, 1985). Like auxins and cytokinins, gibberellins work in combination with other phytohormones (Bottini *et al.*, 2004). Unfortunately, the exact biosynthesis pathway for plant growth promotion by gibberellins is still obscure. It was speculated that gibberellins can increase root hair density, which acts in nutrient and water uptake in root zones (Yamaguchi, 2008).

4) The gaseous hormone ethylene plays multiple roles in plant growth such as breaking seed and bud dormancy, favoring lateral cell expansion, and inducing flower and leaf senescence and abscission (Mckeon *et al.*, 1982). High ethylene concentrations have an inhibitory effect on

plant growth (Mckeon *et al.*, 1982). Many PGPRs secrete the enzyme 1-Aminocyclopropane-1-carboxylate (Naclerio *et al.*) deaminase and regulate the amount of ethylene. This can affect the plant growth (Glick *et al.*, 1998). 1-Aminocyclopropane-1-carboxylate (ACC) is the immediate precursor of ethylene synthesis in the plant (Yoshii and Imaseki, 1982). A number of PGPRs contain ACC deaminase, which catalyzes the breakdown of ACC exuded by plant roots to ammonia and α -ketobutyrate. This process lowers the level of ACC in plants, thereby decreasing the endogenous concentration of ethylene (Glick *et al.*, 1998). Since a high level of ethylene could inhibit the root elongation after seed germination, decreased amount of ethylene in the plant results in promoting plant root and shoot growth, increasing biomass, and reducing inhibitory effects from excessive amounts of ethylene (Mckeon *et al.*, 1982; Glick *et al.*, 1998).

5) Abscisic acid (ABA) is synthesized in all plant parts, and is able to induce stomata closure, to inhibit seed germination to inhibit flowering, and plays a role in bud dormancy (Zeevaart and Creelman, 1988). The biosynthetic pathway(s) and the effect(s) of ABA-producing bacteria on plant growth are not yet fully understood (Tan *et al.*, 1997). Since ABA inhibits the synthesis of cytokinins, it can promote plant growth by interfering with the plant cytokinin pool (Dodd *et al.*, 2010).

Nitrogen fixation

In addition to symbiotic Rhizobia (Gage, 2004), some free-living rhizobacteria can enhance nitrogen nutrient uptake by plants, such as *Azospirillum*, *Herbaspirillum*, *Acetobacter*, and *Bacillus*. It is proposed that PGPRs increase the root surface area or the general root architecture, resulting in higher amounts of carbon in secreted residues released by plant roots. Increased nutrient enhances microbial activity and promotes the cycle of events that makes more N available for plants to take up (Adesemoye *et al.*, 2010).

Phosphate solubilization

Phosphate is a required nutritional element for plant growth and development (Rodriguez and Fraga, 1999). Since insoluble complexes are formed after application of phosphate fertilizer, the efficiency of phosphate use is very low (concentration of P < 5% of the total P pool) (Jiao *et al.*, 2012). Microorganisms help in making phosphorous available to plant roots, and increasing P-mobilization in soil. Many rhizosphere bacteria can solubilize insoluble mineral phosphorus by secreting active phosphatases (for organic-P release), or producing organic acid for inorganic-P release. The ability of phosphate solubilization is very common in the population of rhizobacteria, up to 40% of which have been reported to be able to solubilize P in culture (Rodriguez and Fraga, 1999). Several efficient Gram-positive strains containing this characteristic belong to the genera *Bacillus*, *Brevibacterium*, *Sarcina*, *Paenibacillus*, *Corynebacterium*, and *Micrococcus* (Rodriguez and Fraga, 1999; Jorquera *et al.*, 2008).

Minerals solubilized with siderophores

Iron is an essential components in metabolism of organisms (Schwyn and Neilands, 1987). Although iron is abundant in the soil, available forms of iron for plant use are limited since it mainly exists as ferric hydroxide with a low solubility (Schwyn and Neilands, 1987). Some PGPRs have the ability to efficiently uptake iron by producing a variety of siderophores, which are Fe^{3+} chelators able to bind this ion with high affinity (Ahmad *et al.*, 2008). The Fe^{3+} -siderophore complex is soluble and can be transported back into the cells, readily available for microbial metabolism. In addition to directly offering soluble iron for plants, PGPRs can compete for available iron with other organisms including pathogens, resulting in soluble iron being unavailable and limiting the growth of plant pathogens (Marques *et al.*, 2010).

Antibiosis

PGPRs produce a wide range of secondary metabolites, some of which inhibit the growth of organisms including plant pathogens (Schippers *et al.*, 1987). These secondary metabolites are called antibiotics, and have been used as an indicator when screening for the microorganism with antimicrobial activity *in vitro* (Bloemberg and Lugtenberg, 2001). Well-characterized antibiotic compounds (and their mechanisms) include phenazines (inhibiting electron transport), phloroglucinols (causing membrane damage and impairing the function of mitochondria), pyoluteorin and pyrrolnitrin (loss of mitochondrial activity; inhibitors of certain enzymes), hydrogen cyanide (inhibits many metalloenzymes), and cyclic lipopeptides (inserting into membranes based on surfactant properties) (Lugtenberg and Kamilova, 2009; Spaepen *et al.*, 2009).

The extensively studied antibiotic-producing PGPRs include the bacilli and pseudomonads. Production of antibiotics by *Bacillus subtilis* and related species has been recognized for over 50 years, and more than two dozen antibiotics have been identified (Stein, 2005). However, based on the analysis of whole genome sequences, the genetic capacity for synthesis of antibiotics in strain *B. subtilis* 168 is 180 kb, accounting for 4-5% of the whole genome, is less than that in *B. amyloliquefaciens* FZB42 (340 kb, 8.5 %) (Chen *et al.*, 2009).

Among these antibiotic products, peptide antibiotics represent the predominant class. Based on the different biosynthetic pathways for peptides production, the active antimicrobial compounds are either ribosomally synthesized (lantibiotics or bacteriocins) or non-ribosomally generated, including lipopeptides and polyketides (Stein, 2005). Lantibiotics are ribosomally synthesized and posttranslationally modified peptide antibiotics that contain inter-residual thioether bonds as a unique feature. They usually display a high level of target specificity against

related microorganisms, although many have a wide spectrum of antimicrobial activity. This group of antibiotics can kill target cells by forming voltage-dependent pores into the cytoplasmic membrane. Identified lantibiotics include nisin, colicin, ericin, mersacidin, and subtilin (Abriouel *et al.*, 2011).

Lipopeptides are assembled on peptide synthetases, very large protein templates, and display a modular organization to allow polymerization of monomers in an assembly-line like mechanism. Surfactins, fengycins, and iturins are the most commonly reported lipopeptides in *Bacillus* spp. They exhibit various antimicrobial effects depending on the specific target organism (Stein, 2005). Polyketide synthetases are closely related to lipopeptides since their biosynthetic pathways both use multiple Ppan carriers for binding of monomers and extending chains. In type strain *B. amyloliquefaciens* FZB42, polyketides such as bacillaene, difficidin and macrolactin have been identified (Chen *et al.*, 2007).

Colonization and competition

To possess beneficial effects for bacteria growth in the plant rhizosphere, PGPRs need to have to competitive ability to compete against other microorganisms for nutrients secreted by plant roots and niches around the roots system. Through colonization, PGPRs occupy potential binding sites for pathogens and may exclude them from the rhizosphere (Lugtenberg *et al.*, 1999). Therefore, competitive colonization ability has been considered as a crucial factor that affects PGPRs' efficacy.

Root exudates include organic acids, sugars, and amino acids (Miller *et al.*, 2009). The compositions of compounds released by plant roots help determine which group of rhizobacteria are attracted to the rhizosphere, although this also is affected by the plant species, plant cultivar and age, and environmental factors (Miller *et al.*, 2009). The sites for bacterial colonization are

toward the nutrient-rich junction areas, such as sites where lateral roots and root hairs emerge from the primary root, which was shown for *B. amyloliquefaciens* FZB42 using scanning electron microscopy. Moreover, through green fluorescent protein (GFP) labeling, biofilm-like structures on the root hair surface have been observed, suggesting biofilm formation may play an important role in rhizosphere competence (Fan *et al.*, 2011).

Induced resistance

Induced systemic resistance (ISR) represents an attractive approach to manage plant diseases since it can potentially protect a plant against a broad spectrum of plant pathogens (Kloepper *et al.*, 2004). Several bacterial components or secreted molecules of PGPR have been recognized to trigger ISR in plants, such as flagellin, lipopolysaccharides (LPS), salicylic acid, some lipopeptides, and some volatile compounds (De Vleeschauwer and Hofte, 2009). Elicitation of ISR by PGPRs was initially demonstrated using *Pseudomonas* spp. and other Gram-negative bacteria (Kloepper *et al.*, 2004). In the *Bacillus* genus, many species have been reported to have the ability to protect plant from pathogen attack through ISR. For example, *B. amyloliquefaciens*, *B. subtilis*, *B. pasteurii*, *B. cereus*, *B. pumilus*, *B. mycoides*, and *B. sphaericus* were all found to be efficient in stimulating plant ISR in some crops (Sailaja *et al.*, 1997; Enebak *et al.*, 1998; Zhang *et al.*, 2002; Choudhary and Johri, 2009). In terms of the mechanism associated with elicitation of ISR by *Bacillus* spp., research indicates that ISR is related with ultrastructural changes in plants and with cytochemical alterations as well during the process of pathogen attack (Kloepper *et al.*, 2004).

Volatiles and other factors

PGPRs can interact with plants not only by direct surface-to-surface contact, but also through volatiles at a distance. Volatiles secreted by microorganisms usually have low polarity

and high vapor pressure, which favor their diffusion in the soil, and over long distances in the atmosphere (Vespermann *et al.*, 2007). Application of volatile-producing PGPRs is a promising strategy for plant disease management, especially in post-harvest disease management (Arrebola *et al.*, 2010). To date, 770 different volatile organic compounds have been discovered in bacteria. The dominant compound groups are alcohols, alkenes, ketones, and terpenoids (Wenke *et al.* 2012).

PGPR-produced volatiles are able to affect the growth of both plants and plant pathogens, and these impacts can be positive or negative. Ryu *et al.* (2003) showed that 2,3-butanediol and its precursor acetoin, produced by *Bacillus* sp. strains GB03 and IN937, enhanced plant growth. However, in another experiment conducted by Ryu later (2005), volatiles from the same *Bacillus* species showed an inhibition effect on *Arabidopsis* growth. This inhibition depended on the distance between the plant and the test *Bacillus* strains, which suggested that the inhibitory effect may be dependent on the concentration of the secreted chemical (Ryu *et al.*, 2005).

Among the hundreds of identified volatiles produced by bacteria, some may be toxic to plants, such as HCN (Blom *et al.*, 2011); some volatiles such as dimethyl disulfide, ammonia and 3-phenylpropionic acid, have a negative impact on both plants and pathogens (Wenke *et al.*, 2012). In contrast, some volatiles are able to trigger induced systemic resistance in plants (Ryu *et al.*, 2004; Ryu *et al.*, 2005). Sang (2011) reported application of strain GST09 (*Flavobacterium* sp.) and ISE14 (*Chryseobacterium indologenes*) effectively stimulated pepper fruit ripening and yield, and 2,4-di-*tert*-butylphenol may be related to this phenomenon.

**CHAPTER 2: CHARACTERIZING A NOVEL STRAIN OF *BACILLUS*
AMYLOLIQUEFACIENS BAC03 FOR POTENTIAL BIOLOGICAL CONTROL
APPLICATION**

This chapter has been previously published:

Meng, Q.X., Jiang, H.H., Hanson, L.E., Hao, J.J. (2012) Characterizing a novel strain of *Bacillus amyloliquefaciens* BAC03 for potential biological control application. *Journal of Applied Microbiology*, 113 (5): 1165-1175.

ABSTRACT

Bacterial strain BAC03, isolated from potato common scab suppressive soil, was identified as *Bacillus amyloliquefaciens* by analyzing sequences of fragments of the *recA*, *recN*, *cheA*, and *gyrA* genes. BAC03 displayed antagonistic activity against *Streptomyces* spp. on agar plates using a co-culture method. In greenhouse assays, BAC03 applied in potting mix significantly reduced common scab ($P < 0.05$), and potentially increased the growth of potato plants ($P < 0.05$). An antimicrobial substance extracted from BAC03 by ammonium sulfate precipitation was identified as an LCI peptide using liquid chromatography-mass spectrometry. The antimicrobial activity of either a BAC03 liquid culture or the ammonium sulfate precipitate fraction was stable under a wide range of temperatures, and pH levels, as well as following incubation with several chemicals, but was removed by all proteinases tested. To conclude, *Bacillus amyloliquefaciens* strain BAC03 displayed strong antimicrobial activity, suppression of potato common scab, and may potentially enhance plant growth. LCI peptide is associated with some of the antimicrobial activity. BAC03 has the potential to be developed as a commercial biological control agent for potato common scab management.

INTRODUCTION

Biological control has the potential to play an important role in managing plant diseases (Liu *et al.*, 1995; Guetsky *et al.*, 2002; Singhai *et al.*, 2011). The efficacy of most reported biological control agents varies by strain, therefore more effective strains of beneficial microorganisms are always of interest (Lugtenberg and Kamilova, 2009). Biological control agents have shown strong activities for inhibition of plant pathogens (Compant *et al.*, 2005; Haas and Defago, 2005), promotion of plant growth (Spaepen *et al.*, 2009), competitive colonization (Fan *et al.*, 2011), and induction of plant defense systems against pathogens (Kloepper *et al.*, 2004). Some microorganisms have at least one of these characteristics, but most studied *Bacillus* spp. possess multiple modes of action (Kloepper *et al.*, 2004; Ongena and Jacques, 2008; Arguelles-Arias *et al.*, 2009). In addition, the formation of endospores makes the *Bacillus* genus highly resistant to adverse environmental conditions (Nicholson *et al.*, 2000), which favors the commercialization of biological control products in transportation and shelf life (Fravel, 2005).

Antimicrobial activity is the most easily observed sign of antagonism in biological control agents and has been extensively studied (Naclerio *et al.*, 1993; Yoshida *et al.*, 2001; Hu *et al.*, 2010). Various antimicrobial substances produced by *Bacillus* spp. have been identified, such as iturins (Gueldner *et al.*, 1988; Hiradate *et al.*, 2002), fengycins (Toure *et al.*, 2004; Hu *et al.*, 2007) and surfactins (Vitullo *et al.*, 2012), which belong to non-ribosomally synthesized peptides (Ongena *et al.*, 2007). In addition to these metabolites, ribosomally synthesized antimicrobial compounds were also detected (Stein, 2005).

Potato common scab (*Streptomyces* spp.) is an important disease worldwide, and can cause significant reduction in the economic value of potatoes (Loria *et al.*, 1997; Loria *et al.*, 2006). The pathogens produce a phytotoxin, thaxtomin A, which is the only known pathogenicity

determinant and induces disease symptoms (Bignell *et al.*, 2010a). The disease is difficult to manage as the pathogens survive saprophytically in and on plant debris or organic matter in soil, and have a ubiquitous distribution (Loria *et al.*, 2006). Many approaches have been used to manage common scab, including resistant cultivars (Douches *et al.*, 2009), crop rotation (Larkin, 2008), using green manures or organic amendments (Larkin and Griffin, 2007), and chemical treatments (Tegg *et al.*, 2012). However, resistant germplasm is very limited, and the rest of the practices are partially effective (Dees and Wanner, 2012). Hence, additional methods for the control of common scab are needed.

Biological control using microorganisms can be a potential strategy for sustainable common scab management. The most commonly studied bacterial genera for the control of potato common scab include *Pseudomonas* spp. (Singhai *et al.*, 2011; St-Onge *et al.*, 2011), and *Streptomyces* spp. (Liu *et al.*, 1995; Eckwall and Schottel, 1997; Beausejour *et al.*, 2003; Hiltunen *et al.*, 2009). *Pseudomonas* sp. LBUM 223 has been shown to inhibit the growth of *S. scabies*, because the *Pseudomonas* produces the antibiotic compound phenazine-1-carboxylic acid (PCA) that represses gene expression for thaxtomin A (St-Onge *et al.*, 2011). *Pseudomonas mosselii* promotes potato growth and also induces systemic disease resistance in the plant (Singhai *et al.*, 2011). *Streptomyces melanosporofaciens* strain EF-76 produces geldanamycin, which reduces the severity of potato common scab under both controlled experiments and field conditions (Beausejour *et al.*, 2003). *Streptomyces diastatochromogenes* strain PonSSII does not affect the growth of most organisms tested, but is inhibitory against *Streptomyces scabies* (Eckwall and Schottel, 1997).

Some studies have been reported using *Bacillus* spp. to manage potato common scab, including *Bacillus* sp. sunhua (Han *et al.*, 2005) and *B. subtilis* (Schmiedeknecht *et al.*, 1998).

As combined organisms with different modes of action have been shown to enhance biological control activity (Guetsky *et al.*, 2002), adding a robust and effective biological control agent to the available options provides greatly enhanced opportunities for disease management.

Unfortunately, the bacteria mentioned in the above studies are still at a research stage; therefore, additional studies are needed.

A bacterial strain BAC03, belonging to the genus *Bacillus*, was isolated from a potato field that has shown suppressiveness to potato common scab in Michigan (Meng *et al.*, 2012b). It inhibits a broad spectrum of microorganisms *in vitro*, including *Streptomyces* spp. that cause potato common scab (Meng *et al.*, 2011). The biological characteristics and the potential application of this strain for disease control, especially for potato common scab management are of my interest. The objectives of this study were to identify bacterial strain BAC03 to species, evaluate its antimicrobial activity, determine one or more antimicrobial substances produced by this strain, and examine the potential for common scab management.

MATERIALS AND METHODS

Microorganisms and identification of BAC03

A bacterial strain, designated BAC03, was isolated from soil of a Michigan potato field that has been shown to be suppressive to potato common scab (Meng *et al.*, 2012b). *Bacillus amyloliquefaciens* FZB42 was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH, USA). All other microorganisms used in this study and their sources are listed in Table 2.1.

In order to identify BAC03, sequence analysis was conducted using selected genes. BAC03 cultured in tryptic soy broth (TSB, EMB Chemical Inc., Gibbstown, NJ, USA) for 24 h was used for DNA extraction. The FastDNA Spin Kit (MP Biomedicals, Solon, OH, USA) was

used according to manufacturer's instructions. Polymerase chain reaction (PCR) was conducted to amplify fragments of the 16S rDNA, *recA* and *recN* (DNA repair and recombination proteins), *cheA* (ahistidine kinase), and *gyrA* (gyrase subunit A) genes with primers listed in Table 2.2. Primers for genes *cheA* and *gyrA* were designed based on the consensus sequence of these two genes retrieved from several *B. amyloliquefaciens* strains in National Center for Biotechnology Information (NCBI) database, and validated with DNAMAN software (Lynnon Corporation, Quebec, Canada). Each PCR reaction had a total volume of 25 μ l, containing 5 U of Taq DNA polymerase, 1X Taq polymerase PCR buffer (Promega, Madison, WI, USA), 200 mM dNTP mixture, 0.2 mM of each primer, and 1 μ l (2 to 25 ng) of template DNA. Thermocycler (Bio-Rad Scientific Inc, Hercules, CA, USA) settings were as follows: an initial denaturation for 5 min at 94°C, followed by 36 cycles of 40 s at 94°C, 40 s at 58°C, and 1.5 min at 72°C; and extension for 7 min at 72°C. After PCR product visualization by electrophoresis on 1.2% (w/v) agarose gel stained with GelGreen (Biotium, Inc., Hayward, CA, USA), the PCR products were purified with a PCR purification kit (Denville Scientific Inc., Metuchen, NJ, USA), and sequenced at the Michigan State University Genomic Technology Support Facility (East Lansing, MI, USA). The sequences were analyzed using the BLAST algorithm against the GenBank database. This experiment was conducted twice, and with three replicates each time.

Table 2.1. Inhibition activities of *Bacillus amyloliquefaciens* strain BAC03 against *Streptomyces* spp.

Pathogen	* Source (isolated from)	** Inhibition zone diameter (mm)
<i>Streptomyces acidiscabies</i> 49003	ATCC (tuber)	8.6 ± 0.6
<i>Streptomyces aureofaciens</i> 5404	NRRL (soil)	7.8 ± 1.0
<i>Streptomyces scabies</i> 1231	NRRL (tuber)	10.8 ± 0.8
<i>Streptomyces scabies</i> 49173	ATCC (tuber)	25.3 ± 1.8
<i>Streptomyces</i> sp. DS3024	Hao lab (soil)	11.6 ± 1.1
<i>Streptomyces stelliscabiei</i> Her21	Hao lab (tuber)	14.5 ± 1.2
† Control		0

* ATCC: American Type Culture Collection (<http://www.atcc.org/>), Manassas, VA, USA. NRRL: USDA-ARS Agriculture Collection (<http://nrml.ncaur.usda.gov/>), Peoria, IL, USA. Yeast malt extract was used for the bacterial growth.

** The inhibitory activity was tested on yeast malt extract medium, with 15 µl of BAC03 suspension (10^7 CFU ml⁻¹) applied on a sterile filter paper disk. Each mean value is the average of 8 measurements. † Control was set with a filter paper with TSB only.

Table 2.2. Primers for polymerase chain reactions used in this study

Gene	Primer	Sequence (5'-3')	Reference
16s rDNA	63-F	CAGGCCTAACACATGCAAGTC	(Dennis <i>et al.</i> , 2003)
	1387-R	GGGCGGWGTGTACAAGGC	
<i>recA</i>	recA-F	TGAGTGATCGTCAGGCAGCCTTAG	(Arguelles-Arias <i>et al.</i> , 2009)
	recA-R	TTCTTCATAAGAATACCACGAACCGC	
<i>recN</i>	recN-F	CTTTTGCGATCAGAAGGTGCGTATCCG	(Arguelles-Arias <i>et al.</i> , 2009)
	recN-R	GCCATTATAGAGGAAGTACG ATTTC	
<i>cheA</i>	cheA-F	GTTTGAAGCGCTTGATCATCTAGAA	This work
	cheA-R	GGTTCATTGAAGAATCCAGTCTTTC	
<i>gyrA</i>	gyrA-F	CGTACGCAATGAATGATTTAGGCATG	This work
	gyrA-R	ATTATGAAAGACGTCTGCAGGGCCGTT	
<i>lci</i>	lci-F	CGCGGATCCATGAAATTCAAAAAAGTGTT	(Hu <i>et al.</i> , 2010)
	lci-R	GCGTCGACTTATTTATCTACACTTTCAT	
<i>txtA</i>	txtA-F	TGCTCAACTCCGTGATCCAGTA	(St-Onge <i>et al.</i> , 2011)
	txtA-R	GGGACACCTCGCGCAGTA	
<i>txtC</i>	txtC-F	ACCATCTCGCTGTCCTTGGT	(St-Onge <i>et al.</i> , 2011)
	txtC-R	CGTGGACGACGGAGAACTTC	

Antimicrobial activity of BAC03 against *Streptomyces* spp.

Antimicrobial activity of BAC03 was determined against *Streptomyces* spp. using a co-plate assay (Yoshida *et al.*, 2001). One-hundred microliter spore suspensions (10^5 CFU ml⁻¹, determined by dilution plating) of various *Streptomyces* spp. isolates were deposited across the plate with a sterile cell spreader on yeast malt extract agar (YME, EMB Chemical Inc., Gibbstown, NJ, USA) plates. Fifteen microliters of a 10^7 CFU ml⁻¹ of BAC03, cultured for 24 h in TSB, were placed as a drop on a sterile filter paper disk (5 mm diam., Whatman #1, Piscataway, NJ, USA), which was placed on the agar medium with 2 disks per plate, at 2 cm distance from each other. After the plates were incubated at 28°C in darkness for 3 days, the diameter of the inhibition zone (if any) was measured with a ruler. Analysis of each isolate was replicated four times, and this trial was conducted twice.

Biological control activity assay

A pot assay was conducted in a greenhouse to examine the biological control activity of BAC03. *Streptomyces scabies* (ATCC49173) was cultured in oatmeal broth (Loria *et al.*, 1995) at 28°C for 4 to 5 days in a incubator shaker at 180 rpm, and the concentration of *Streptomyces* in the liquid culture was determined by plating on YME. Potato tuber pieces ('Snowden') with at least one eye were surface disinfested with 1% NaClO for 5 min, and then rinsed with sterile distilled water three times. After air-drying, the tubers were planted in potting mix (ASB Greenworld Inc., New Brunswick, VA, USA) and grown in a growth chamber at 25°C until seedling emergence. Each potato seedlings was transferred to a 3.78 L plastic pot with potting mix, infested with *S. scabies* by pouring the inoculum on top of the potting mix at a final concentration of 10^6 colony forming units (CFU) cm⁻³. For BAC03 application in soil, a liquid

culture of BAC03 was added to the potting mix as a drench to give a final concentration of 10^5 or 10^6 CFU cm⁻³. Treatment with BAC03 was done twice; once when seedlings were transplanted and a second drench 20 days later. The treatments included 1) control with *S. scabies* inoculum plus TSB; 2) control of non-infested potting mix mixed with TSB; 3) potting mix inoculated with BAC03 at 10^5 CFU cm⁻³, and infested by *S. scabies*; and 4) potting mix inoculated with BAC03 at 10^6 CFU cm⁻³, and infested by *S. scabies*. Plants were watered every 2 to 3 days and fertilizer (Miracle-Gro all purpose plant food, the Scotts Company, Marysville, OH, USA) was applied as needed. There were four replications (pots) for each treatment. Six weeks after transplanting, the height of the plant from the soil line to the apex of potato plant was measured with a ruler. Potato tubers were harvested 10 weeks after transplanting. Tubers were examined for lesions and given a severity rating using the 0 to 5 scale of Hao *et al.* (2009) where 0 = no symptoms, 1 = 1 to 10% surface area with superficial or raised lesions, 2 = 11 to 25% surface area with superficial or raised lesions, 3 = 26 to 50% surface area with superficial or raised lesions, 4 = more than 50% surface area with superficial or raised lesions or 6 to 25% pitted lesion area, and 5 => 50% surface area with superficial or raised lesions or > 25% pitted area. Potato yield was determined by measuring the weight of all potato tubers from each pot. This trial was done twice.

Precipitation of potential antimicrobial substances

Two extraction methods were used to separate different types of secondary metabolites. For all extractions, BAC03 was grown in 200 ml of TSB at 28°C on a shaker incubator at 180 rpm for 48 h. The culture was centrifuged at 12,000 g for 20 min at 4°C and the supernatant was collected.

For potential proteinaceous secondary metabolites (ribosomally synthesized metabolites) (Entian and Klein, 1993), BAC03 culture supernatant was extracted using ammonium sulfate precipitation (Sutyak *et al.*, 2008) as described below. Eighty percent ammonium sulfate was added to the supernatant, and incubated at 4°C overnight. The mixture was centrifuged at 12,000 g for 30 min at 4°C, and the supernatant was discarded. The precipitate was dissolved in 10 mM sodium phosphate buffer (pH 6.0), applied to a Sephadex G-50 column (Pure Biotech LLC, Middlesex, NJ, USA), and centrifuged at 2000 g for 3 min. The eluted fluid was passed through a 0.22 µm filter membrane (Millipore, Billerica, MA, USA), and the antimicrobial activity of this substance against *S. scabies* was tested by the agar diffusion assay as described above. This extract was designated as ammonium sulfate precipitated material (ASP) for the rest of the study.

Extraction of lipopeptides (non-ribosomally synthesized peptide) was carried out according to the combination of acid precipitation and solvent extraction described before (Vater *et al.*, 2002) with slight modifications. The pH of the culture filtrate was adjusted to 2.0 by adding 6 N HCl followed by precipitation at 4°C overnight. The pellet derived from centrifugation (12,000 g for 30 min at 4°C) following precipitation was dissolved in 100% methanol. The mixture was passed through a 0.22 µm filter membrane (Millipore, Billerica, MA, USA). The antimicrobial activity of the extract against *S. scabies* was tested with the agar diffusion assay described above. This material was designated acid precipitate material for the rest of the study.

The concentration of protein in the ASP was determined by colorimetric absorbance at 595 nm using a Bradford Protein assay kit (Thermo Fisher Scientific Inc., Rockford, IL, USA, source of protein is bovine serum albumin) with a spectrophotometer (NanoDrop Technologies

Inc., Wilmington, DE, USA) based on a standard curve using bovine serum albumin. All these experiments were conducted three times.

Antimicrobial activity of ASP against *Streptomyces scabies*

To quantitatively test the effect of the ASP on *S. scabies*, a spore suspension of *S. scabies* (10^5 CFU ml⁻¹) was mixed with BAC03 ASP at concentrations equivalent to 0, 15, 30, 45, 60, 75, and 90 µg of protein ml⁻¹, as determined by the Bradford assay for one hour. Then the mixture was serially diluted and spread on YME plate, and incubated at 28°C in the dark. Any inhibitory effect was evaluated by counting *S. scabies* colonies on the plate after 3-day incubation in comparison with a control of plating the spore suspension alone.

To determine whether the extract was bactericidal or bacteriostatic, 10^5 CFU ml⁻¹ of a *S. scabies* spore suspension was incubated with ASP at a final concentration of 30, 60, and 90 µg ml⁻¹. After 0, 1, 3 and 5-day incubation at 28°C in the dark, the mixture was filtered through a sterile 0.22 µm membrane, and the *S. scabies* collected from the filter surface was diluted followed by spreading on YME to determine the concentration of viable *S. scabies*. Four replications were set for each treatment, and these two trials were conducted twice.

Effects of temperature, pH, enzymes, and chemical solvents on antimicrobial activity of liquid culture and ASP of BAC03

Both BAC03 liquid culture and its derived ASP were examined for their reaction to various factors, including temperatures, pH levels, enzymes, and chemicals. To test the effect of temperature, one microliter of BAC03 spore solution or 100 µl of ASP was incubated in a 1.5 ml micro tube, which was placed in a heating block (Denville Scientific, Inc., Metuchen, NJ, USA) at temperatures of 40, 60, 80, and 100°C for 30 min, or in an autoclave (Consolidated sterilizer

systems Inc., Boston, MA, USA) at 121°C for 15 min. To test the effect of pH levels, the pH was adjusted from 1.0 to 14.0 by whole pH units using sterile 1 N HCl or 1 N NaOH. Material was incubated at these pH levels overnight at 4°C. Prior to assessing activity, the pH was readjusted to pH 7.0. Several enzymes were tested for their effect on activity by incubating the BAC03 culture or ASP with 10 mg ml⁻¹ of the following enzymes for 2 h at the optimal temperature for each enzyme according to the manufacturer's instruction. Enzymes were trypsin (MP Biomedicals, Solon, OH, USA, 25°C, pH 7.6), proteinase K (Sigma-Aldrich, Inc. St Louis, MO, 37°C, pH 7.5), pepsin (Sigma-Aldrich, Inc., 37°C, pH 2.0), α -chymotrypsin (Sigma-Aldrich, Inc., 25°C, pH 7.8), and catalase (MP Biomedicals, 25°C, pH 7.0). Effect of various chemical solvents (Table 2.3) was tested by incubating the substance for 5 h at 25°C with 10% (vol/vol) organic solvents. Antimicrobial activities of the treated culture and ASP were tested against *S. scabies* using the disc diffusion assay as described above. Enzyme or solvent only was tested at the same time as a negative control. Three replicates were set for each treatment, and all the experiments were conducted twice.

Scanning electron microscope analysis

To examine the effect of ASP on *S. scabies*, scanning electron microscopy was used to observe the morphology of mycelia on media. Ten microliters of ASP was dropped onto a *S. scabies* 2-day-old colony. Sodium phosphate buffer was used as a control. The plates were incubated at 28°C in the dark. Five days later when the control colonies became white with mycelia, both cultures were coated with metallic osmium and processed according to the methods of Fan *et al.* (2011) by the Center for Advanced Mycology at Michigan State University and examined with a scanning electron microscope JEOL JSM 6400 (Japan Electron Optics Laboratories, Tokyo, Japan) at high voltage (10 kV). This trial was carried out twice.

Molecular mass and chemical structure of ASP

ASP was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) (Scholz *et al.*, 2011). Following electrophoresis, the gel was vertically cut into two parts. One part of the gel, containing the sample and molecular weight standards, was stained with Coomassie Blue protein stain. The other part, containing the same sample, was tested for antimicrobial activity using the method of Naclerio *et al.* (1993). Briefly, the gel was treated with 20% isopropanol-10 mM Tris-HCl (pH 7.5) for 3 h, rinsed for 1.5 h in 10 mM Tris-HCl (pH 7.5), and washed twice with distilled water for a total of 1 h. The gel was placed in a Petri dish with 1% water agar, and overlaid with 0.8% YME mixed with a bacterial suspension of *S. scabies* (10^7 CFU ml⁻¹). The plate was incubated at 28°C for 48 h, then dyed with thiazolyl blue tetrazolium bromide (MTT, Sigma-Aldrich Co. LLC., St. Louis, MO, USA) to stain living organisms, and observed for any unstained zone(s).

The active band associated with the antimicrobial activity above (if any) was excised, eluted from the gel, and digested (Shevchenko *et al.*, 1996). Liquid chromatography-mass spectrometry (LC/MS/MS) was conducted at the Michigan State University Proteomics Facility. The resulting MS/MS spectra were converted to peak lists using BioWorks Browser v3.3.1 (Thermo Fisher Scientific Inc, Pittsburgh, PA, USA) and searched against all known gram-positive bacterial protein sequences downloaded from NCBI. The Mascot output was analyzed using Scaffold v3.08 to probabilistically validate protein identifications using the ProteinProphet2 computer algorithm. Assignments validated above the Scaffold 95% confidence filter were considered valid. This experiment was conducted two times.

PCR detection

Based on the identified protein, the corresponding predicted nucleotide sequence was amplified using PCR with specific primers (Table 2.2) reported by Hu *et al.* (2010), and genomic DNA of BAC03 as the template. The DNA extraction, PCR amplification and sequence determination were conducted as described above. The sequence of the amplification was compared to the NCBI Embank database using the BLAST algorithm.

Statistical analysis

Data were analyzed using SAS software (Version 9.2, SAS Inc., Cary, NC, USA). Procedure GLM was used for analysis of variance, and Fisher's least significance difference (LSD) multiple comparisons were performed for mean separation where ANOVA showed differences. Procedure REG was used for linear regression. If there was no interaction between repeated trials ($P > 0.05$), data were combined from all trials.

RESULTS

Identification of BAC03

The 16s rRNA sequence of BAC03 had a similarity of 99% with both *B. subtilis* (accession number: JN700079.1) and *B. amyloliquefaciens* (accession number: JQ245705.1). Sequences of fragments from *recA*, *recN*, *cheA*, and *gyrA* genes showed the highest similarity between strain BAC03 and *B. amyloliquefaciens*. Specifically, the similarity with *B. amyloliquefaciens* was 98% for *recA* (accession number: JN048426.1), and 99% for *recN* (accession number: CP000560.1), *cheA* (accession number: FN652798.1), and *gyrA* (accession number: AB612173.1).

Antimicrobial activity of BAC03 against *Streptomyces* spp.

BAC03 displayed antagonistic activities against *Streptomyces* spp. in plate tests. Inhibition zone diameters ranged between 0.78 and 2.53 cm (Table 2.1), with the largest inhibition zone (2.53 cm) against *S. scabies* strain 49173.

Biological control activity assay

In the greenhouse, the severity of potato common scab was significantly reduced ($P < 0.05$) by *B. amyloliquefaciens* BAC03 at concentrations of both 10^5 and 10^6 CFU cm⁻³ potting mix (Fig. 2.1 A). In addition, plant height and potato tuber weight was significantly increased ($P < 0.05$, Fig. 2.1 B) in association with BAC03 application at both concentrations in the presence of *S. scabies* compared to the controls. Enhanced growth was greater ($P < 0.05$) at 10^6 than at 10^5 CFU cm⁻³ potting mix of BAC03 (Fig. 2.1).

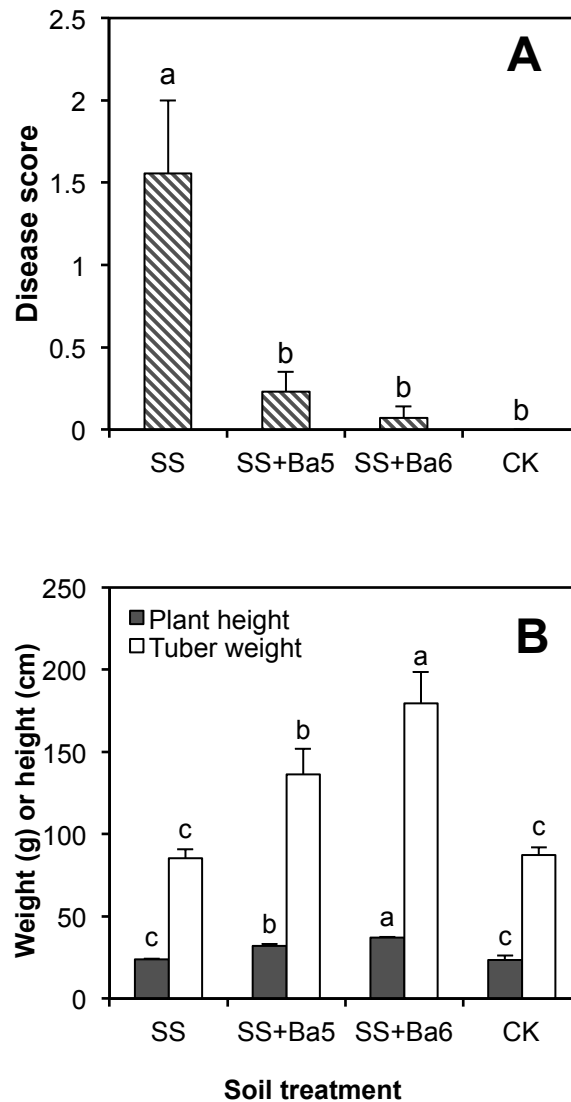


Figure 2.1. Effect of *Bacillus amyloliquefaciens* BAC03 on the severity of common scab (A) and growth of potato (B) in the presence of *Streptomyces scabies*. Potato tubers were planted in potting mix amended with *S. scabies* at a final concentration of 10^6 colony forming unit (CFU) cm^{-3} plus tryptic soy broth (SS) or *S. scabies* with BAC03 at 10^5 CFU cm^{-3} (SS + Ba5), or with BAC03 at 10^6 CFU cm^{-3} (SS + Ba6). Tryptic soy broth alone was used as a control (CK). Plant height was determined 6 weeks after transplanting by measuring the plant from the soil line to the apex of potato plant with a ruler. Potato yield was determined by measuring the weight of all potato tubers from each pot. In disease rating (A), each bar is the average of 20 to 24 observations. In examination of potato growth, each bar is the average of 8 observations. Error bars represent standard error of the means of observations. Mean values of responses in each treatment were compared using Fisher's LSD multiple range comparisons at $\alpha = 0.05$. Values of the bars with the same letters were not significantly different.

Characterization of an antimicrobial substance from *B. amyloliquefaciens*

Based on the results of the agar diffusion assay, the AP fraction showed low activity (< 0.5 cm) and was not examined further. The material from the ASP fraction produced larger inhibition zones (> 2 cm) than the AP fraction produced, and thus further study concentrated on this material. The ASP derived from the bacterium *B. amyloliquefaciens* BAC03 significantly reduced ($P < 0.05$) the number of *S. scabies* colonies by the co-culturing method, and the effect was positively correlated with the ASP concentration (Fig. 2.2 A). The inhibition was near 100% when the ASP concentration was $75 \mu\text{g ml}^{-1}$. In an assay for bactericidal effect, a reduction in the number of *S. scabies* colonies following removal from the ASP was observed after co-incubation for different times, and the effect was increased with increasing exposure time (Fig. 2.2 B).

The antimicrobial activity was not affected by catalase, or any of the organic solvents tested (Table 2.3), but activity was completely removed by treatments with proteinase K, trypsin, pepsin, or chymotrypsin. The activity was relatively heat stable, and maintained the same level of activity when exposed to temperatures up to 60°C , but activity was totally lost following autoclaving. The culture broth and ASP were also active over a wide pH range, but the growth inhibition was reduced in extremely acid ($\text{pH} < 3$) or alkaline ($\text{pH} > 12$) conditions (Table 2.3).

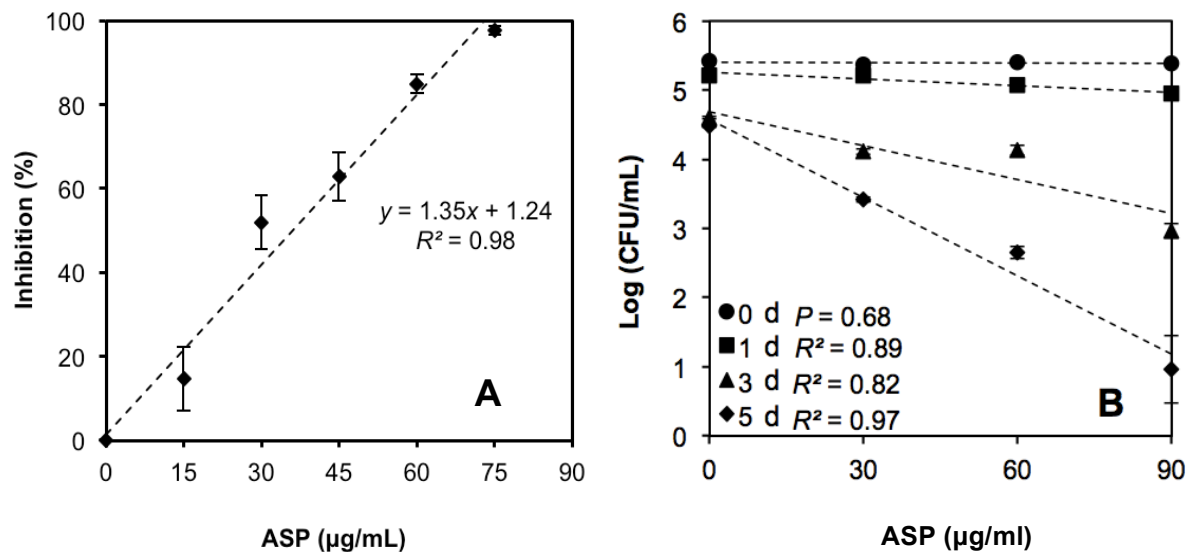


Figure 2.2. Antimicrobial activity of the ammonium sulfate precipitation fraction (ASP) from *Bacillus amyloliquefaciens* BAC03 tested against *Streptomyces scabies* liquid culture. (A) A spore suspension of *S. scabies* (10^5 CFU ml⁻¹) was mixed with BAC03 ASP at concentrations equivalent to 0, 15, 30, 45, 60, 75, and 90 µg of protein ml⁻¹ as determined by the Bradford assay. The mixture was spread on yeast malt extract agar plates and incubated at 28°C in the dark. Inhibition of *S. scabies* was calculated as $[100 \times (\text{population in control} - \text{population in the treated}) / \text{population in control}]$. In the regression equation, y represents inhibition and x represents ASP concentration. (B) Bactericidal activity of BAC03 ASP. *Streptomyces scabies* was recovered after incubation with ASP for 0, 1, 3, and 5 days, followed by rinsing off ASP through a 0.22 µm membrane with sterile distilled water three times. Correlation coefficients (R^2) between ASP concentration (x axis) and *S. scabies* population (y axis) are shown for each day of evaluation, with $P < 0.05$ for regressions of days 1, 3, and 5. Each point is an average of 8 observations. Bars on each value show standard errors.

Scanning electron microscope analysis

Colonies of the ASP non-treated culture turned white or whitish grey after 7 days of growth (5 days after treatment), while there was no any color change observed in ASP treated *S. scabies* colonies. The morphology of mycelia observed under the SEM displayed typical spiral spore chains (Schaad *et al.* 2001) on non-treated plates (Fig. 2.3). However, there was no change observed of the morphology of mycelia on ASP-treated culture (Fig. 2.3).

Molecular mass and chemical structure determination of ASP and PCR testing

The ASP fractioned by SDS-PAGE displayed a clear band with a molecular mass of approximately 10 kDa after MTT staining, indicating no bacterial growth (Fig. 2.4). LC/MS/MS results indicated that the most probable compound from this fraction was an LCI peptide. A PCR product was obtained using a pair of primers specific to an *lci* gene (Hu *et al.*, 2010). The sequence similarity of the amplified putative *lci* was 99% between strain BAC03 and *B. amyloliquefaciens* FZB42 (accession number: CP000560.1) or C-31 (accession number: FJ904931.1). The DNA sequence and putative translated peptide sequences were compared to those of reported *B. amyloliquefaciens* strains. All of the strains had a putative peptide of 94 amino acid. The sequence of the LCI peptide in BAC03 was 100% identical with strain C31, but had one amino acid different from FZB42 (Hu *et al.* 2010).

Table 2.3. Effects of temperature, pH level, enzyme treatment, and chemical solvents on the inhibitory activity of *Bacillus amyloliquefaciens* as measured by an agar disc diffusion assay against the growth of *Streptomyces scabies*

Treatment	Inhibitory activity of BAC03 (% ± standard deviation)*	
	Liquid culture**	ASP †
Non-treated	100	100
Chemicals		
Acetone	100	100
Ethyl acetate	100	100
Ethyl alcohol	100	100
Chloroform	100	100
Methyl alcohol	100	100
Enzymes		
Proteinase K	5 ± 3	0
Trypsin	7 ± 2	0
Pepsin	15 ± 4	0
Chymotrypsin	8 ± 4	0
Catalase	100	100
Temperature (°C) / incubation time		
40 / 30	100	100
60 / 30	100	100
80 / 30	89 ± 3	61 ± 4
100 / 30	26 ± 6	10 ± 3
121 / 15	0	0
pH		
1	76 ± 3	55 ± 6
3	100	100
5	100	100
10	100	100
12	100	100
14	0	0

* Inhibitory activity was measured by an agar disc diffusion assay against the growth of *Streptomyces scabies*, and calculated as [(diameter of inhibition zone on treated plate)/ (diameter of inhibition zone of control)]*100%. Each mean value is the average of 6 measurements.

** Strain BAC03 was cultured in tryptic soy broth for 24 h (10^7 CFU ml⁻¹) on a shaker at 180 rpm.

† Crude antimicrobial substance was extracted by ammonium sulfate precipitation from the culture supernatant, and filtered through a Sephadex G-50 column and 0.22 µm filter membrane.

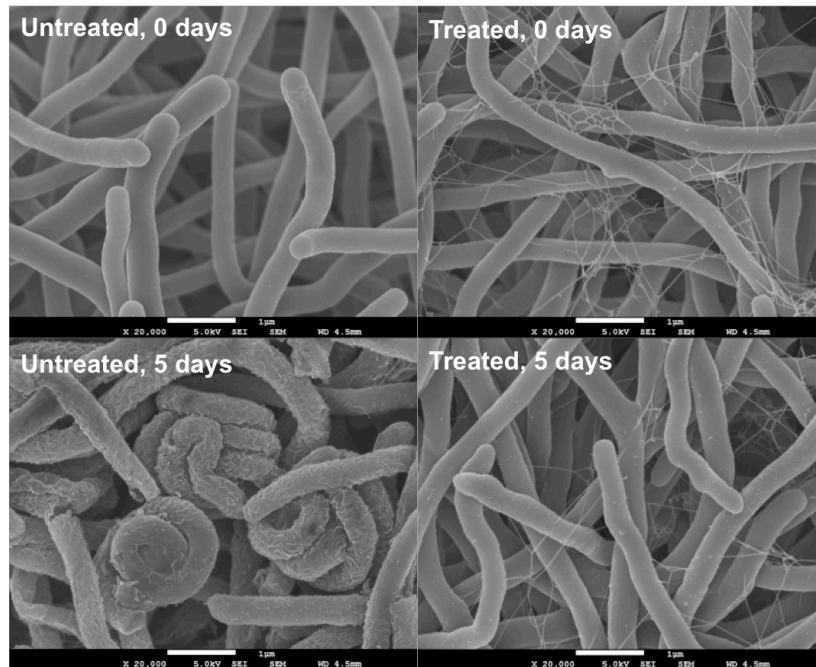


Figure 2.3. Scanning electron micrographs of mycelial morphology of *Streptomyces scabies* grown on yeast malt extract agar. Ten microliters of sodium phosphate buffer (untreated), or ammonium sulfate precipitation (ASP) (treated) was dropped onto a *S. scabies* 2-day-old colony. Five days later, the morphology of mycelia was observed under SEM. The cultures were processed following the method of Fan *et al.* (2011) and coated with metallic osmium in preparation for SEM.

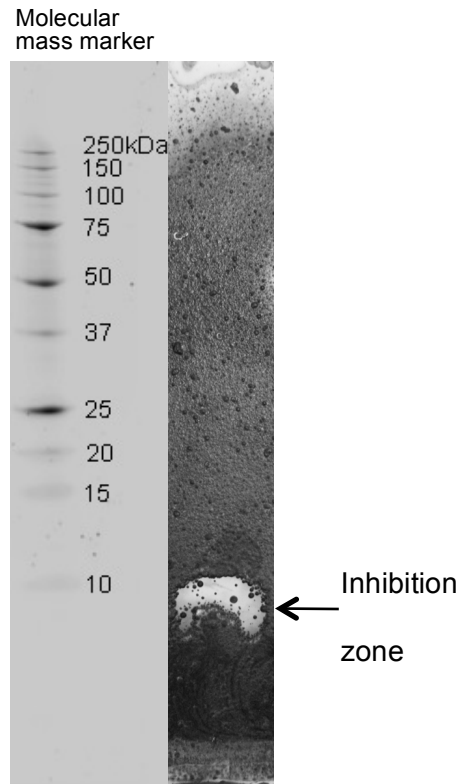


Figure 2.4. Active fraction of ammonium sulfate precipitate (ASP) from *Bacillus amyloliquefaciens* BAC03 by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After fractionation on SDS-PAGE, the gel was overlaid with melt yeast malt extract agar containing spores of *Streptomyces scabies* (10^7 CFU ml⁻¹), and incubated at 28°C for 2 days. Response was observed by staining the bacterial cells with thiazolyl blue tetrazolium bromide. A clear zone indicates an inhibition zone. The atomic mass of the active compound(s) was estimated by comparison to the position of the markers (in kDa).

DISCUSSION

Bacillus amyloliquefaciens is a closely related species to *B. subtilis* (Arguelles-Arias *et al.*, 2009), which has been demonstrated or commercialized to be used as biological control agent for decades (Gueldner *et al.*, 1988; Sailaja *et al.*, 1997; McSpadden Gardener and Fravel, 2002a; Joshi and Gardener, 2006). By analyzing multiple gene fragments such as *recA*, *recN*, *cheA*, and *gyrA* used in this study, strain BAC03 was confirmed as *B. amyloliquefaciens*. *Bacillus amyloliquefaciens* has been recognized as a good plant growth promoter and root colonizer (Fan *et al.*, 2011). In addition, the genetic capacity for synthesis of secondary metabolites in *B. amyloliquefaciens* (340kb) is larger than that in *B. subtilis* (180 kb) (Chen *et al.*, 2009). Based on results from this study, it is expected to develop BAC03 into a commercial product, similar to *B. amyloliquefaciens* FZB42 (Chen *et al.*, 2009), the product of which has not been registered in the US though (ABiTEP GmbH, Berlin, Germany).

The strain BAC03 displayed a strong antagonism against *Streptomyces* spp. as shown in this study, as well as against a range of plant pathogens (unpublished), which may indicate a potential for use against multiple plant diseases. The inhibitory effect on *Streptomyces* spp. was shown *in vitro*, and a significant reduction in the severity of potato common scab was found in this study. This promising result should be further investigated for the feasibility of field application of BAC03 at a large scale. In addition to disease suppression, a higher tuber weight and plant height was observed in the presence of the pathogen with BAC03. However, in order to prove the capability of direct plant-growth-promoting in strain BAC03, a further study in the absence of pathogens need to be conducted.

A large part of the *in vitro* antagonism of BAC03 was associated with a proteinaceous fraction showing high similarity to an LCI peptide. A similar antimicrobial substance was first

reported in *B. subtilis* strain A014, which had only 46 amino acid residues (Liu et al., 1990) compared to the 94 amino acid residues predicted for BAC03. The one identified in the current study has been found in *B. amyloliquefaciens* C31 (Hu et al., 2010). These products have shown strong antimicrobial activities against various microorganisms, which may be a factor for disease suppression in BAC03 (Hu et al., 2010). Furthermore, this compound has a bactericidal effect, as shown by the reduction in colony numbers in the current study.

The culture broth of BAC03 was more tolerant to adverse conditions than the extracted ASP fraction from the bacterium. This may be because the living cells can protect the compounds from degradation and continue to produce the chemicals (Nicholson et al., 2000). It also is possible that other compounds are present in the culture broth. In the current study, the acid precipitate fraction showed a small level of inhibition against *Streptomyces* spp. in assays on agar plates. These may be providing some of the activity in the crude culture broth. *Bacillus* spp. can have more than one antibiotic (Ongena and Jacques, 2008), either ribosomally or non-ribosomally synthesized (Stein, 2005). For example, *B. amyloliquefaciens* FZB42 has two ribosomally synthesized antimicrobial substances, plantazolicin (Scholz et al., 2011) and amylocyclicin (Butcher and Helmann, 2006). In addition, non-ribosomally synthesized metabolites have also shown inhibitory activity against microorganisms and have drawn more attention (Chen et al., 2009). A genetic study showed that 8.5% of the entire genomic capacity of *B. amyloliquefaciens* FZB42 is devoted to the non-ribosomal synthesis of secondary metabolites, which includes polyketides (bacillaene, difficidin, and macrolactin), lipopeptides (surfactin, fengycin, and bacillomycin D), and siderophores (bacillibactin and the product of the *nrs* cluster) (Scholz et al., 2011). In order to exert more potential of BAC03, it is necessary to further investigate whether BAC03 has these or similar antibiotics or new antibiotics.

In conclusion, the results presented here indicate the potential to use BAC03 as a biological control agent for common scab management. LCI peptide is potentially correlated with antimicrobial activities. However, further investigation is needed on modes of action, spectrum of effects on other pathogens, effects on plant growth promotion, and the biological control efficacy of strain BAC03 in the field.

**CHAPTER 3: EFFECTS OF APPLICATION METHODS WITH *BACILLUS*
AMYLOLIQUEFACIENS BAC03 ON THE CONTROL OF SCAB CAUSED BY
*STREPTOMYCES SCABIES***

ABSTRACT

Bacillus amyloliquefaciens strain BAC03 is a novel bacterial strain that previously had been studied for biological control of common scab (*Streptomyces scabies*). In order to optimize its efficacy, various application strategies were investigated for their effect on scab in radish and potato. Assessments included application using a foliar spray, a seed treatment, or applications to potting mix. Tests were conducted with different timing, frequency, and concentrations of BAC03 in potting mix that was infested with *S. scabies*. Results showed that foliar application and seed treatment with BAC03 did not affect the disease severity in either radish or potato. BAC03 applied 5 days before planting completely suppressed ($P < 0.05$) radish scab, but the later BAC03 was applied the less effective it was ($P < 0.05$). BAC03 at 10^5 CFU cm⁻³ potting mix or higher concentrations (up to 5×10^6 CFU cm⁻³) was effective ($P < 0.05$) at reducing severity of radish scab. Increasing the frequency of BAC03 application did not increase efficacy for disease reduction. In addition, BAC03 increased ($P < 0.05$) the biomass of radish roots and leaves in the presence of the pathogen. In population dynamics, as determined by qPCR, the amount of *S. scabies* when BAC03 was applied before radish planting was significantly lower ($P < 0.5$) than that in other treatments with BAC03 application at later stages.

INTRODUCTION

Streptomyces spp. are a group of gram-positive filamentous bacteria (Loria *et al.*, 1997). Among the more than 900 species in this group a small number of species are pathogenic to plants and cause scab symptoms on plants, including potato, radish, beet, carrot, turnip, and other tap-root crops (Goyer and Beaulieu, 1997; Bignell *et al.*, 2010a). The members in this group share some common characteristics: they form filamentous hyphae, have a saprophytic life style, contain high G+C contents in their genomic DNA, secrete many enzymes, and produce desiccation-resistant spores. All these characteristics are favorable for *Streptomyces* spp. to become competitive soil inhabitants (Loria *et al.*, 2006), and result in the management of scab caused by pathogenic *Streptomyces* spp. being difficult to control (Dees and Wanner, 2012). In the case of potato common scab, the pathogen infection usually starts an initiation of the tuber or into roots by penetrating through natural openings or wounds (Stevenson *et al.*, 2001). Secretion of thaxtomin A by pathogenic *Streptomyces* spp. facilitates their continuous penetration and nutrient acquisition via inhibition of cellulose synthesis (Fry and Loria, 2002). Scab symptoms become enlarged as the tuber or root expansion (Stevenson *et al.*, 2001).

Streptomyces scabies was the first species described as a pathogen causing scab and is a predominant plant pathogenic *Streptomyces* species associated with scab around the world (Thaxter, 1892; Loria *et al.*, 2008; Bignell *et al.*, 2010a). Management of potato common scab has been considered a tough job for both growers and researchers (Hiltunen *et al.*, 2009; Dees and Wanner, 2012). There are many tools used for managing scab, such as using resistant cultivars (Douches *et al.*, 2009), maintaining high soil moisture (Lapwood *et al.*, 1973), reducing soil pH (Lacey and Wilson, 2001), applying chemical treatments (Davis *et al.*, 1976), crop rotation (Larkin *et al.*, 2011), and organic soil amendments (Lazarovits *et al.*, 1999; Larkin and

Tavantzis, 2013). However, none of above approaches has been satisfactory for scab control because some of them have side effects (Wharton *et al.*, 2011), or the resources, such as for resistant cultivars, are limited (Wanner and Haynes, 2009), or the effect of the management on disease control is not consistent among locations or seasons (Larkin, 2008).

Biological control using beneficial microorganisms offers a promising addition to traditional management tools (Lugtenberg and Kamilova, 2009). In the management of potato common scab, different microorganisms have been studied, such as *Pseudomonas* spp. (Singhai *et al.*, 2011; St-Onge *et al.*, 2011), non-pathogenic *Streptomyces* spp. (Liu *et al.*, 1995; Beausejour *et al.*, 2003; Hiltunen *et al.*, 2009), and *Bacillus* spp. (Han *et al.*, 2005). The genus *Bacillus* consists of many plant beneficial bacteria (Kloepper *et al.*, 2004), which can favor plant growth by using several tools, such as: secreting antimicrobial substances (Ongena and Jacques, 2008), promoting plant growth by providing more growth-required nutrition (Spaepen *et al.*, 2009), repelling other unfavorable microorganisms by competing for space and/or nutrients (Fan *et al.*, 2011), or inducing plant disease defense systems (Kloepper *et al.*, 2004). Another important characteristic of *Bacillus* species is that, compared to other beneficial microorganisms, the endospores of the *Bacillus* group are more resistant to adverse environmental conditions and can help the bacteria survive in some conditions during storage and transportation (Abriouel *et al.*, 2011).

Bacillus amyloliquefaciens strain BAC03 (Patent No.: US 7,615,366 B2) was isolated from a soil naturally suppressive to potato common scab (Meng *et al.*, 2012b). This bacterium has shown antimicrobial activity against *Streptomyces* spp. *in vitro* (Meng *et al.*, 2012a) and a consistent efficacy in potato common scab suppression in the field in two seasons and two locations (Meng *et al.*, 2013). However, a number of factors can affect the efficacy of biological

control. The interactions taking place in the rhizosphere among biocontrol agents, pathogens, plants, as well as other biological and environmental factors are complex and can affect the biocontrol efficacy (Bloemberg and Lugtenberg, 2001; Kim *et al.*, 2011). In order to test the efficacy of BAC03, and provide information for large-scale application, the effect of different strategies for BAC03 application on disease suppression of potato common scab should be determined. The objectives of this study were to analyze the effects of various application strategies on the efficacy of BAC03 in controlling disease caused by *S. scabies* and study the rhizosphere population dynamics of both BAC03 and *S. scabies*. This study may offer useful information to aid in application strategy determination for reducing scab.

MATERIALS AND METHODS

Bacterial cultures

Bacillus amyloliquefaciens FZB42, the active ingredient in commercial biocontrol product RhizoVital42 (ABiTEP GmbH Inc., Berlin, Germany), was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH, USA). *Bacillus subtilis* QST713 was obtained from the commercial product Serenade (Bayer CropScience Inc., Monheim, Germany) by culturing the product (powder) in tryptic soy broth (TSB; EMB Chemical Inc., Gibbstown, NJ, USA). All *Bacillus* strains were cultured on tryptic soy agar (TSA; EMB Chemical Inc.). *Streptomyces scabies* 49173 was obtained from the American Type Culture Collection (Manassas, VA, USA.), and cultured on yeast malt extract agar (YME; EMB Chemical Inc.).

Inoculum preparation and planting

For *Bacillus* spp. inoculum, the bacterial cultures were grown in TSB at 28°C on an incubator shaker (Thermo Fisher Scientific Inc., Rockford, IL, USA) at 180 rpm for 48 h in the dark. The concentration of bacterial cells in the liquid culture was determined by dilution plating on TSA. For preparation of *Streptomyces scabies* inoculum, strain 49173 was streaked on YME and incubated in the dark at 28°C for two weeks. A spore suspension was prepared by adding 10 ml of sterile distilled water to each culture plate and scraping the colonies using a sterile inoculation loop. The concentration of the spore suspension was determined by dilution plating on YME (Hao *et al.*, 2009).

Potato tuber pieces (cv. 'Snowden') with at least one eye were surface disinfested with 1% NaClO for five minutes and rinsed three times with sterile distilled water. The tubers were planted in 3.78-L plastic pots containing soil-less potting mix (ASB Greenworld Inc., New Brunswick, VA, USA) in a greenhouse (Michigan State University greenhouse facility, East Lansing, MI). Growth conditions in the greenhouse were around 18 to 22°C with a 14-h photoperiod supplemented by light at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. Radish ('Cherry Belle', Burpee Inc. Warminster, PA, USA) seeds were pre-germinated on sterile moist filter paper (No.1, Whatman, Pittsburgh, PA) in a Petri dish by incubating overnight at 25°C. After germination, seedlings were transplanted into potting mix, with 2 seedlings per pot, 0.5 cm below the potting mix surface in a 1-L pot, and incubated in a growth chamber (24°C and 14 h light).

BAC03 application by using seed treatment and foliar spray

The study was conducted on both potato and radish. Potato tuber pieces were soaked in a *Bacillus* liquid culture (10^6 CFU ml⁻¹) in TSB for 20 min and then air-dried for 24 h at room temperature before planting. To test foliar spray application, 20 ml of BAC03 liquid culture (10^6

CFU ml⁻¹) was sprayed with a pump sprayer onto potato leaves starting when the plant shoot height reached 15 cm above the surface of the potting mix. The BAC03 was applied weekly for four weeks. Potting mix covered with a plastic film to avoid contamination from the foliar spray. For pathogen inoculation, spore suspensions of *S. scabies* were poured from a beaker on the surface of the potting mix (10⁶ CFU cm⁻³ potting mix) two weeks after plant germination, and this was repeated two weeks later. A negative control for *Bacillus* application used sterile TSB to replace the BAC03 culture. Five replicates were used for each treatment. Potato tubers were harvested four month after planting and lesion severity on the tuber was rated for lesions and given a severity rating using the 0–5 scale (Hao *et al.*, 2009). This trial was conducted twice.

For radish seed treatment, radish seeds were soaked in a BAC03 liquid culture (10⁶ CFU ml⁻¹) for 20 min before pre-germination. For the foliar spray test, 10 ml of BAC03 culture broth (10⁶ CFU ml⁻¹) was sprayed onto radish leaves as above 10 days after planting, and repeated every three days for a total of four times. The potting mix in the pots was covered with a plastic film to avoid contamination from the foliar spray. A spore suspension of *S. scabies* was applied as a drench on the potting mix (10⁶ CFU cm⁻³ potting mix) five days before planting.

Inoculation with TSB only was used as a negative control. Five replicates were used for each treatment. Radishes were harvested six weeks after planting and lesion severity was rated according to the method of Wanner (2004). The trial was carried out twice.

BAC03 application at different stages

In order to determine the optimal timing of BAC03 application for scab suppression, treatments at different radish growth stages were tested, including 5 and 2 days before planting

(DBP), as well as 10, 20, and 30 days after planting (DAP). A liquid culture of BAC03 was added to the potting mix as a drench to give a final concentration of 10^5 CFU cm⁻³ potting mix. *Streptomyces scabies* was directly applied as a drench on the potting mix (10^6 CFU cm⁻³ potting mix) two DBP. Treatments with *S. scabies* combined with TSB or TSB-only were used for controls. There were 4 replicates for each treatment. Disease severity was determined as above (Wanner, 2004). The weight of leaves and roots were evaluated separately at harvest (6 weeks after planting). The trial was carried out twice.

BAC03 application with different concentrations

A series of BAC03 concentrations, which included 1×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 , and 5×10^6 CFU cm⁻³ potting mix, were tested for radish scab suppression. *Streptomyces scabies* (10^6 CFU cm⁻³ potting mix) was inoculated at two DBP. A *Bacillus* liquid culture was diluted and applied as a drench into the potting mix 10 DAP to get the designated concentrations. Treatments with *S. scabies* combined with TSB or TSB-only were used for controls. Four replicates were included for each treatment. The disease rating and biomass assessment were conducted as described above. This experiment was conducted twice.

BAC03 application with different frequencies

Streptomyces scabies (10^6 CFU cm⁻³ potting mix) was inoculated in potting mix at two DBP. BAC03 was applied either once (at 10 DAP), or the same total volume of inoculum was divided and applied for a total of two (at 8 and 12 DAP), three (at 7, 10, and 13 DAP), or four (at 7, 9, 11, and 13 DAP) times. Treatments with *S. scabies* combined with TSB or TSB-only were used for controls. Four replicates were applied for each treatment. The methods for planting,

disease rating, and biomass measurement were as described above. This experiment was conducted twice.

Comparison of BAC03 with *B. subtilis* QST713 and *B. amyloliquefacisn* FZB42

BAC03, QST713 and FZB42 were evaluated for scab reduction in radish. *Bacillus* were applied as liquid cultures at 10 DAP to a final concentration of 10^5 CFU cm⁻³ potting mix. Four replicates were used for each treatment. The methods for planting, disease rating, and biomass measurement were as described above. This experiment was conducted twice.

Population dynamics of *Streptomyces scabies* 49173 and *Bacillus amyloliquefaciens* BAC03 in the radish rhizosphere

Radish planting and preparation of *S. scabies* inoculum were conducted as above. Spore suspension of *S. scabies* was mixed into potting mix by directly applying the spore suspension as a drench into the mix to get a final concentration of 10^6 CFU cm⁻³ potting mix (treatment SS). Five days after *S. scabies* application, radish was planted. *Bacillus amyloliquefaciens* BAC03 liquid cultures were prepared as described above. BAC03 was inoculated at either 5 DBP (treatment B1+SS), or 5 DAP (treatment B2+SS). Controls were BAC03 only (treatment B), *Streptomyces scabies* and tryptic soy broth (treatment SS), and tryptic soy broth only (treatment TSB). Nine replicates were used for each treatment.

The radish rhizosphere soil was sampled at 10, 20, and 30 DAP. For sampling, radish plants were carefully dug out from the potting mix in order not to disrupt the root integrity. Two plants from each pot were vigorously shaken by hand for one minute. Then the roots were placed in a 1.5 ml microcentrifuge tube containing one-milliliter of sterile phosphate buffered saline solution ($1 \times$, NaCl 8 g L⁻¹, KCL 0.2 g L⁻¹, Na₂HPO₄ 1.44 g L⁻¹, KH₂PO₄ 0.27 g L⁻¹, pH 7.4),

and vortexed ($3000 \times \text{rpm}$) for two minutes. The roots were removed and the tube was centrifuged at $20,000 \times g$ for 10 min. The supernatant was poured out. One tenth of a gram of precipitate was used for DNA extraction. Total DNA was extracted using a FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) following the manufacturer's instructions.

To compare the level of rhizosphere population of both *Streptomyces scabies* and *Bacillus amyloliquefaciens* BAC03, the amount of organisms were estimated by measuring their DNA in the rhizosphere soil with qPCR. To quantify the *S. scabies* population the *txtA* gene was amplified with the primer set *txtA*-F (TGCTCAACTCCGTGATCCAGTA) and *txtA*-R (GGGACACCTCGCGCAGTA) (St-Onge *et al.*, 2011). To quantify the population of *B. amyloliquefaciens* BAC03 the *lci* gene was amplified with a set of primers designed for the *lci*-F (TGCCTTACTGATGTCTGCCG) and *lci*-R (CGGCATCTGCTTTCGTTGG). Reactions were prepared in triplicate in 96-well optical plates using a mix consisting of 10 μl of ABI SYBR Green PCR master mix (2 X) (Applied Biosystems, Carlsbad, CA, USA), 2 μl of 1/10 diluted DNA, 0.5 μl of each primer (10 μM), and 7 μl of sterile double distilled water. Reactions containing sterile water served as negative controls for each PCR. External standards for quantification consisted of five concentrations of serially diluted *S. scabies* and *B. amyloliquefaciens* genomic DNA (1, 0.1, 0.01, 0.001, 0.0001 $\text{ng } \mu\text{l}^{-1}$), and were included in triplicate in each PCR run to calculate quantities of *S. scabies* and *B. amyloliquefaciens* in the potting mix samples. The PCR was carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems, Carlsbad, CA, USA) using the conditions described by the manufacturer with 45 amplification cycles. To confirm single products, the melting curve and gel electrophoresis were used (St-Onge *et al.* 2011). This experiment was conducted twice.

Statistical analysis

Data were analyzed using SAS software (version 9.2, SAS Inc., Cary, NC, USA). For plant weight, procedure GLM was used for the analysis of variance, and Fisher's least significant difference (LSD) multiple comparisons were performed for mean separation of yield where ANOVA showed significant differences. Non-parametric data analysis was used for disease analysis, and Kruskal-Wallis test was performed to compare mean values. If there was no interaction between repeated trials ($P > 0.05$), data were combined from all trials.

RESULTS

Foliar spray and seed treatment

Foliar spray with BAC03 did not affect the severity of scab either in potato or radish compared to that of broth-treated plants. In the potato trial, disease scores were around 0.9 for the BAC03-treated, and 0.8 for the non-treated plants. In the radish trial, disease scores were 0.7 for BAC03-sprayed, and 0.8 for non-treated plants. The disease was not significantly reduced when radish and potato seeds were treated with BAC03 and grown in soil infested with *S. scabies*.

BAC03 application at different stages

Treatments with a soil drench of BAC03 applied at different stages of radish growth all reduced scab compared to the non-treated control (Fig. 3.1 A). The scab disease was not detectable in the treatment with BAC03 applied 5 DBP (Fig. 3.1 A). Biomass assessment showed that treatment with BAC03 applied 10 DAP gave the highest weight of radish leaves (Fig. 3.1 B).

BAC03 application at different concentrations

Treatments with BAC03 concentrations from 1×10^5 to 5×10^6 CFU cm⁻³ potting mix all significantly reduced scab in radish compared to the inoculated control (Fig. 3.2 A). Radish plants treated with 1×10^5 , 5×10^5 , 1×10^6 , and 5×10^6 CFU cm⁻³ potting mix had a higher weight of leaves than other treatments and controls (Fig. 3.2 B); radish plants treated with 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 , and 5×10^6 CFU cm⁻³ potting mix had a higher root weight than other treatments and controls (Fig. 3.2 B).

BAC03 application with different frequencies

There were no significant differences in disease severity among treatments with BAC03 at different application frequencies (Fig. 3.3 A). However, BAC03 applied more than one time had a higher weight of radish leaves than that with one time application and the controls (Fig. 3.3 B). In the weight assessment of radish roots, all treatments with BAC03 application increased the weight of radish compared to the controls treated with *S. scabies* and TSB (Fig. 3.3 B).

Comparison of BAC03 with *B. subtilis* QST713 and *B. amyloliquefaciens* FZB42

There was no significant difference in disease suppression among the bacterial strains (Fig. 3.4 A). However, treatment with BAC03 gave a higher weight of radish leaves and roots than treatments with FZB42 and QST713 (Fig. 3.4 B).

Population dynamics of *Streptomyces scabies* and *Bacillus amyloliquefaciens* BAC03 in the radish rhizosphere

The amounts of genomic DNA of both *S. scabies* and *B. amyloliquefaciens* in the rhizosphere soil of radish treated with BAC03 were determined by a qPCR assay. In the detection of *S. scabies*, the bacterial population displayed various dynamic responses to different

treatments (Fig. 3.5 A). Without BAC03 addition (treatment SS), the levels of *S. scabies* were around $1.4 \mu\text{g g}^{-1}$ potting mix at 10 and 20 DAP, and about $1 \mu\text{g g}^{-1}$ potting mix at 30 DAP (Fig. 3.5 A). With the addition of BAC03 before radish planting (treatment B1+SS), the level of *S. scabies* population was about $0.34 \mu\text{g g}^{-1}$ potting mix at 10 DAP, and $0.19 \mu\text{g g}^{-1}$ potting mix detected at 30 DAP (Fig. 3.5 A). When BAC03 was applied after radish planting (treatment B2+SS), *S. scabies* population were determined at $0.94 \mu\text{g g}^{-1}$ potting mix at 10 DAP and $0.45 \mu\text{g g}^{-1}$ potting mix at 30 DAP. No *S. scabies* was detected in the controls (treatment B and TSB) (Fig. 3.5 A).

In the estimation of *B. amyloliquefaciens* population in the rhizosphere by detecting *lci* gene, no DNA was amplified from the treatments SS and TSB (Fig. 3.5 B). When BAC03 was applied after radish planting, the levels were determined $1.44 \mu\text{g g}^{-1}$ at 10 days to $0.74 \mu\text{g g}^{-1}$ at 30 DAP (Fig. 3.5 B, B2+SS). In other two treatments with BAC03 applied before radish planting (B1+SS) and with BAC03 application only (B), the levels of *B. amyloliquefaciens* population were lower than that detected in the treatment of B2+SS (Fig. 3.5 B).

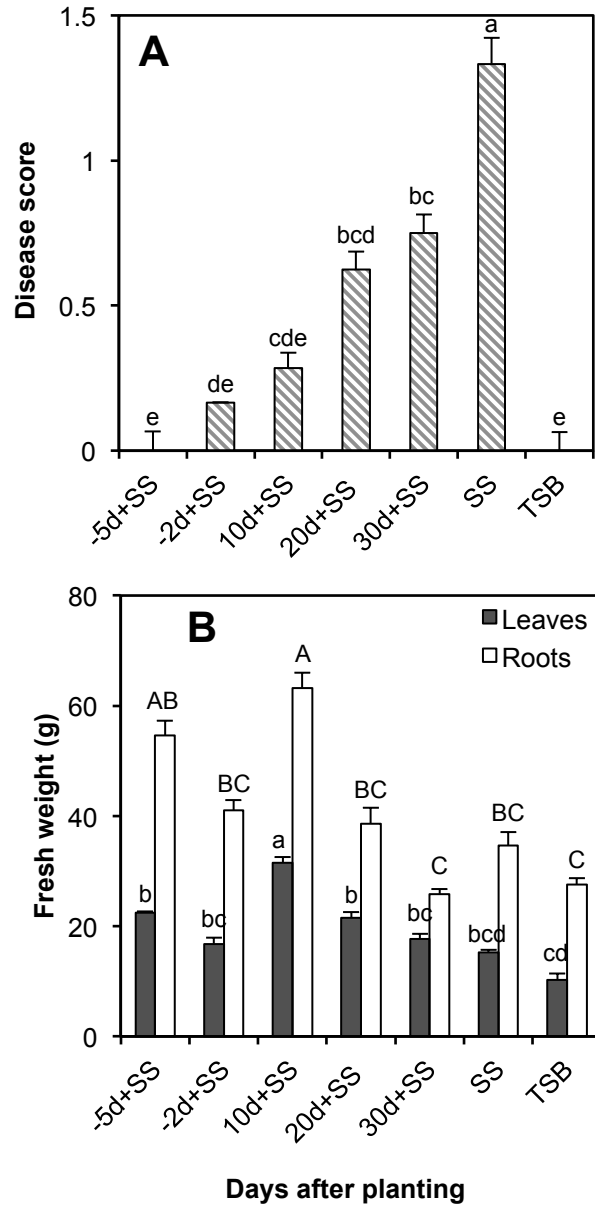


Figure 3.1. Effect of application timing of *Bacillus amyloliquefaciens* BAC03 as a soil drench on scab and fresh weight of radish. *Streptomyces scabies* (10^6 CFU cm^{-3} potting mix) was inoculated 2 days before planting. BAC03 culture was drenched into the potting mix to get a final concentration of 10^5 CFU cm^{-3} potting mix at different stages of radish growth, including 5 and 2 days (d) before, as well as 10, 20, and 30 days (d) after planting. Treatment with tryptic soy broth (TSB) only or (*S. scabies* + TSB) (SS) were used as controls. Mean values are the average of two trials, with four replicates each. Bars with the same letter indicate no significantly difference ($P < 0.05$). Mean values were separated by lower-case (for radish leaves) or capital (for radish roots) letters at significance level $\alpha = 0.05$. Tubers were examined for lesions and given a severity rating using the 0–5 scale of Wanner et al. (2004).

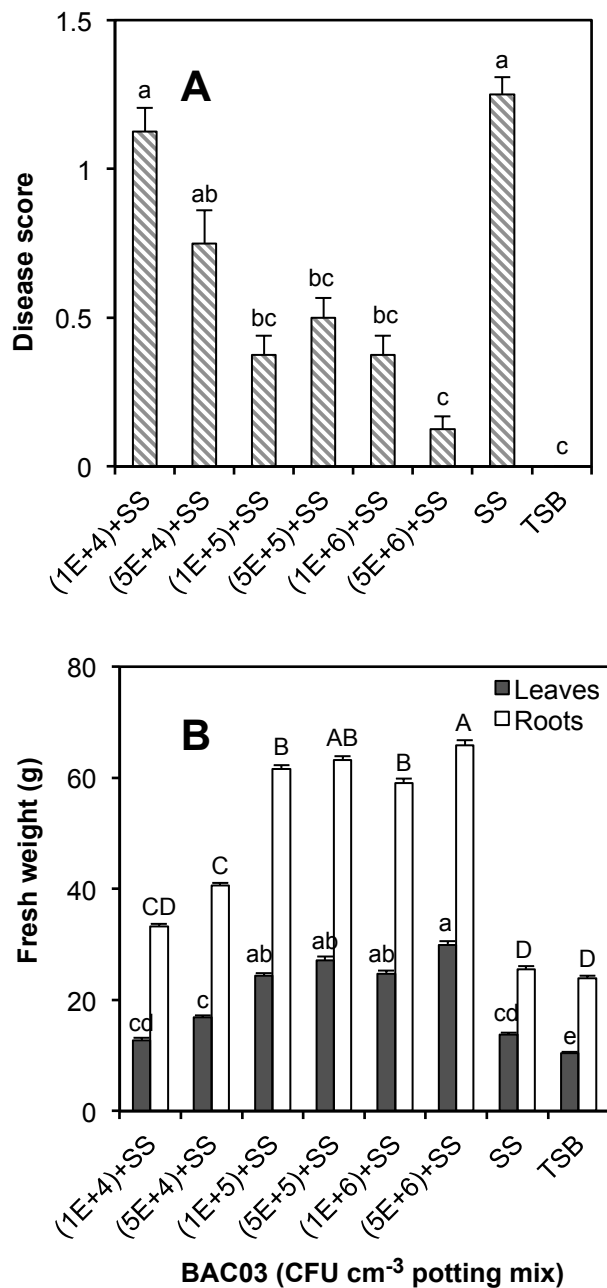


Figure 3.2. Effect of *Bacillus amyloliquefaciens* BAC03 applied with different concentrations as a soil drench on scab and fresh weight of radish. *Streptomyces scabies* (10^6 CFU cm^{-3} potting mix) was inoculated 2 days before planting. BAC03 liquid culture was drenched into the potting mix to get final concentrations of 10^4 , 5×10^4 , 10^5 , 5×10^5 , 10^6 , 5×10^6 CFU cm^{-3} potting mix, at 10 days after planting. Treatment with tryptic soy broth (TSB) only or (*S. scabies* + TSB) (SS) were used as controls. Mean values are the average of two trials, with four replicates each. Bars with the same letter indicate no significantly difference ($P < 0.05$). Mean values were separated by lower-case (for radish leaves) or capital (for radish roots) letters at significance level $\alpha = 0.05$. Tubers were examined for lesions and given a severity rating using the 0–5 scale of Wanner et al. (2004).

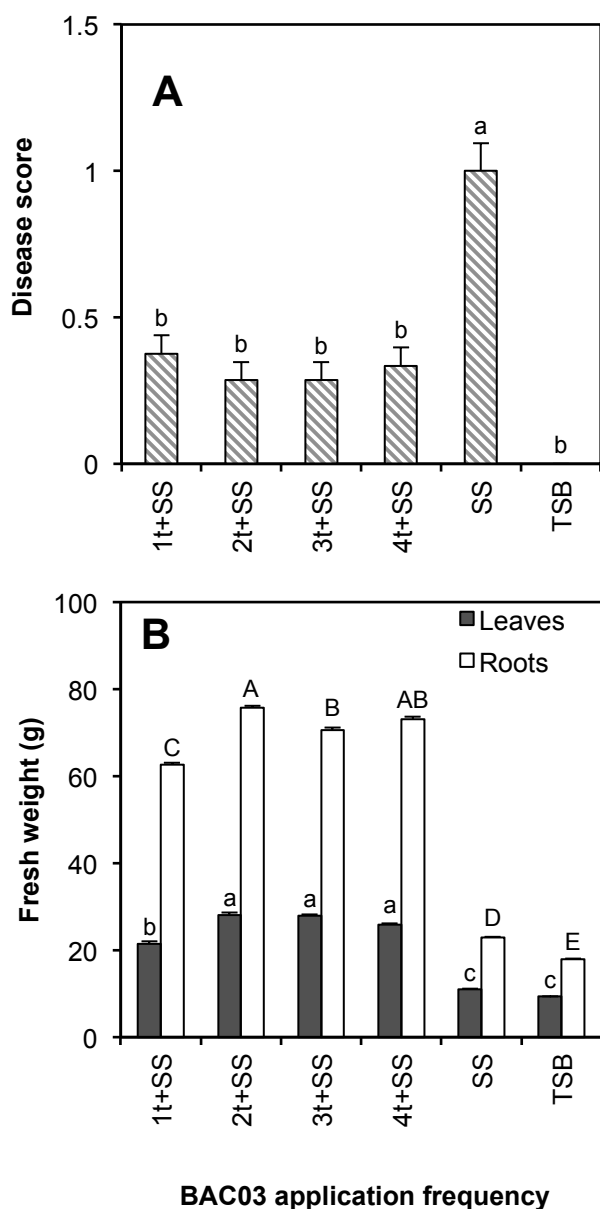


Figure 3.3. Effect of application frequency of *Bacillus amyloliquefaciens* BAC03 as a soil drench on scab and fresh weight of radish. *Streptomyces scabies* (10^6 CFU cm^{-3} potting mix) was inoculated 2 days before planting. BAC03 was applied either once (1t) at 10 days after planting (DAP), or the same total volume of inoculum was divided and applied for a total of two times (2t) at 8 and 12 DAP, three times (3t) at 7, 10, and 13 DAP, or four times (4t) at 7, 9, 11, and 13 DAP. Treatment with tryptic soy broth (TSB) only or (*S. scabies* + TSB) (SS) were used as controls. Mean values are the average of two trials, with four replicates each. Bars with the same letter indicate no significantly difference ($P < 0.05$). Mean values were separated by lower-case (for radish leaves) or capital (for radish) letters at significance level $\alpha = 0.05$. Tubers were examined for lesions and given a severity rating using the 0–5 scale of Wanner et al. (2004).

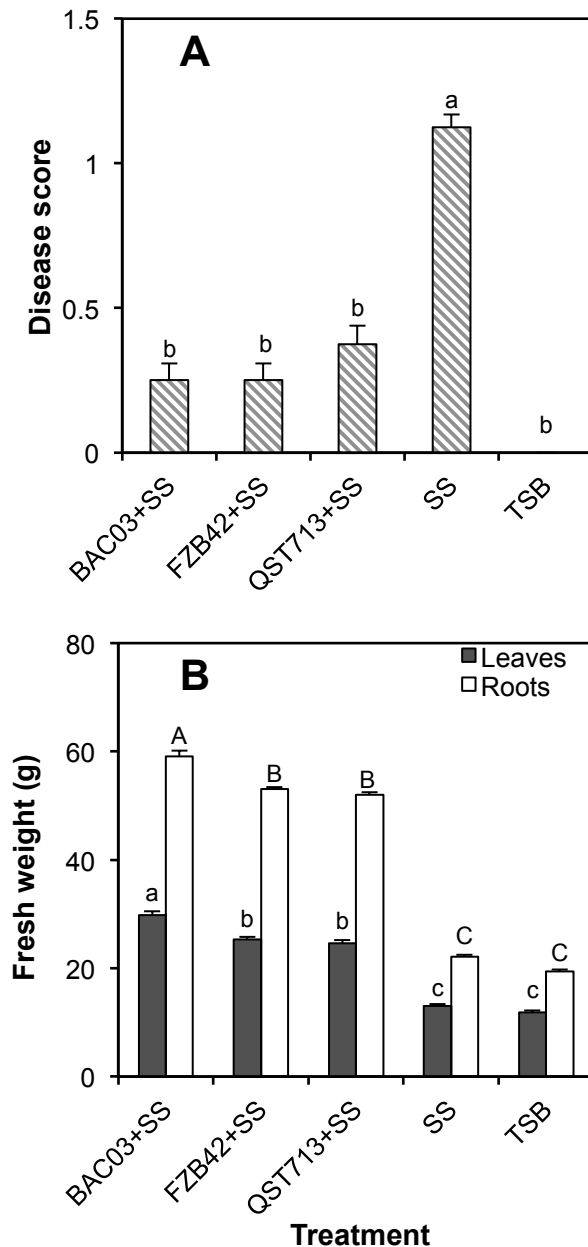


Figure 3.4. Efficacy comparison of *Bacillus amyloliquefaciens* BAC03, *B. subtilis* QST713, and *B. amyloliquefaciens* FZB42 as a soil drench on scab and fresh weight of radish. *Streptomyces scabies* (10^6 CFU cm^{-3} potting mix) was inoculated 2 days before planting. Liquid cultures of BAC03, QST713, and FZB42 were applied at 10 days after radish planting to get a final concentration of 10^5 CFU cm^{-3} potting mix. Treatment with tryptic soy broth (TSB) only or (*S. scabies* + TSB) (SS) were used as controls. Mean values are the average of two trials, with four replicates each. Bars with the same letter indicate no significantly difference ($P < 0.05$). Mean values were separated by lower-case (for radish leaves) or capital (for radish roots) letters at significance level $\alpha = 0.05$. Tubers were examined for lesions and given a severity rating using the 0–5 scale of Wanner et al. (2004).

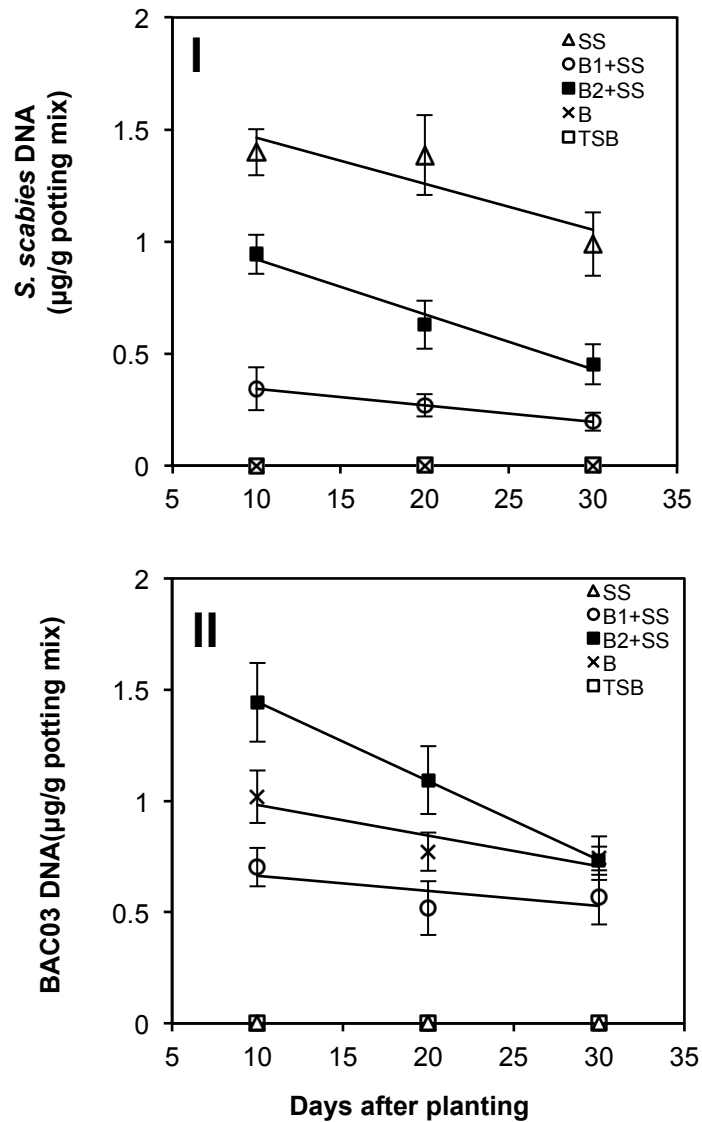


Figure 3.5. Population dynamics (converted from amount of total DNA in the sample) of *Streptomyces scabies* (panel I) and *Bacillus amyloliquefaciens* BAC03 (panel II) in radish rhizosphere potting mix analyzed by quantitative polymerase chain reaction. BAC03 was applied either five days before planting (DBP, treatment B1+SS), or five days after planting (DAP) of radish (B2+SS). Controls were BAC03 only (B) applied at 5 DBP, *S. scabies* only (SS) applied at 5 DBP, and tryptic soy broth (TSB) applied at both 5 DBP and 5 DAP. Values of treatments TSB and B overlap due to the same measurements (all zeros). Error bars represent standard error of the means of eighteen replicates.

DISCUSSION

Results of this study indicated that the efficacy of *Bacillus amyloliquefaciens* BAC03 for scab control was affected by the application method. Foliar application and seed treatment with BAC03 did not significantly reduce scab severity, while application of BAC03 as a soil drench before planting resulted in reducing radish scab, and this inhibition was reduced with later BAC03 applications. Application of BAC03 at concentrations higher than 10^5 CFU cm⁻³ as a drench to the potting mix gave significant reduction of radish scab. BAC03 performed equally well compared with two commercial biocontrol products based on *Bacillus* isolates (*B. subtilis* QST713 and *B. amyloliquefaciens* FZB42).

Understanding how the biocontrol agent interact with plants and pathogens could help to design effective application strategies (Perez-Garcia *et al.*, 2011). Since different biocontrol agents can use different mechanisms (Ongena and Jacques, 2008; Kim *et al.*, 2011), the strategy for their application can be species- or strain-specific (Ryu *et al.*, 2003; Blom *et al.*, 2011). For example, some *Bacillus* spp. are effective at reducing diseases when applied plant seed treatments (Zhang *et al.*, 2009; Wharton *et al.*, 2012; Martins *et al.*, 2013). However, in this study *Bacillus* strain BAC03 did not show any effect in scab suppression by using seed treatment or as a foliar spray. Therefore, it is not recommended to use this isolate seed treatment or foliar spray for BAC03 application in scab control.

A concentration of 10^5 CFU cm⁻³ potting mix was the threshold for significantly reducing scab of the levels tested in this study (Fig. 3.2, A). Based on the results of previous studies, the concentrations of biological control agents in the *Bacillus* spp. used in disease suppression are around 10^7 to 10^8 CFU cm⁻³ or g⁻¹ soil (Han *et al.*, 2005; Zhang *et al.*, 2009).

Therefore, the effective concentration for scab inhibition concluded from this study is relatively low compared to other reports and would be useful in reducing the application dose in commercial application (Spaepen *et al.*, 2009). Application of BAC03 at various frequencies did not affect scab suppression if the total amount were the same (Fig. 3.3, A). A critical period for scab development is during radish root expansion, especially at the beginning of development (Bignell *et al.*, 2010a). Therefore, scab could be effectively reduced as long as the addition of the biocontrol agent covers this critical disease development stage.

Antagonism and rhizosphere colonization are two major tools used by biocontrol agents (Bloemberg and Lugtenberg, 2001; Ongena and Jacques, 2008; Fan *et al.*, 2011). In a previous study (Meng *et al.*, 2012a, chapter 2), BAC03 was shown to produce LCI peptide, which is associated with some antimicrobial activity *in vitro* against *S. scabies*. Moreover, biocontrol agents in the *Bacillus* group are well documented to produce antimicrobial substance, such as iturins, fengycins, and surfactines (Stein, 2005). Colonization is another critical trait for exerting their beneficial effect in the rhizosphere (Fan *et al.*, 2011). Good colonizers are able to compete with plant pathogens for nutrients secreted by the root and for sites that can be occupied on the root (Lugtenberg and Kamilova, 2009). When both BAC03 and *S. scabies* were applied before planting, no disease was observed at harvest (Fig. 3.1, A). One possible reason for this result could be that BAC03 inhibited and repelled the pathogens, resulting in minimizing the negative influence of *S. scabies* on radish.

Results of population dynamics in the radish rhizosphere soil detected by qPCR showed various dynamic changes of BAC03 and *S. scabies* levels in different treatments. BAC03 applied before planting reduced the population of the pathogen in the plant rhizosphere soil (Fig. 3.1, A), indicating an antagonistic activity play an important role during this period. The effects of

BAC03 applied after radish planting was not as good as that with application before planting (Fig. 3.1, A). One possible reason could be that the period was shorter for BAC03 to interact with the pathogen in the treatment with later application compared to the treatment with earlier BAC03 application. BAC03 levels dropped quickly right after its application (Fig. 3.5, B). This needs to be considered in disease management. Moreover, even though the concentration of *S. scabies* applied as a drench was ten times higher than that of BAC03, the detected concentrations of both bacteria were at a similar level. This indicates BAC03 had an advantage in survival and competition compared with *S. scabies*.

The biomass of radish leaves and roots were increased when inoculated with *S. scabies* and *B. amyloliquefaciens* BAC03. Since the pathogen was inoculated, it cannot be concluded that BAC03 has ability of plant growth promotion. However, the results from this study could serve as an indication that BAC03 potentially has some ability for plant growth promotion. To better exert this potential of strain BAC03 for plant health, the possibility of growth promotion in BAC03 needs to be further studied (see chapter 4).

The optimal application stage of BAC03 for radish growth promotion may be different from that for scab disease suppression (Fig. 3.1, A and B). It could be because the mechanisms used by BAC03 in these two processes were different. In disease suppression, when BAC03 was applied early, the biocontrol agent could have a opportunity to interact with the pathogen and more possibility to colonize the plant rhizosphere roots, especially during the early stage of rhizosphere roots development. However, since growth enhancement may be through nutrient offered by BAC03, the stage of radish root expansion could be the most critical period for growth promotion. For the same total volume of BAC03, multiple applications resulted in a higher biomass of radish (Fig. 3.3, B). This relationship between rate and response might relate

to the survival of the *Bacillus* strains, since the viable period of BAC03 could be prolonged by dividing the application into several times.

In conclusion, *B. amyloliquefaciens* BAC03 displayed an effect in a scab disease suppression in radish, and exerted a potential with specific application strategies, which could provide practical information for large-scale application.

CHAPTER 4: CHARACTERIZATION OF *BACILLUS AMYLOLIQUEFACIENS* STRAIN BAC03 FOR PLANT GROWTH PROMOTION

ABSTRACT

Plant growth promoting rhizobacteria (PGPR) can play an important role in plant health and growth. *Bacillus amyloliquefaciens* strain BAC03 has been shown to be an antagonist against *Streptomyces scabies* in previous studies (chapter 2 and 3), but its efficacy on plant growth promotion needs to be investigated. In this study, BAC03 was tested for potential growth promotion on nine selected plants types, including beet, carrot, cucumber, pepper, potato, radish, squash, tomato, and turnip, at the concentration of 10^5 colony forming unit cm^{-3} potting mix under greenhouse conditions. Results showed that BAC03 increased the growth at various levels of some tested plants in different plant parts. Application of BAC03 at 10 days after planting was associated in radish with the highest biomass gain compared to applications at other stages. Multiple applications of BAC03 giving the same total amount of inoculum resulted in higher weights of radish roots and leaves compared to the controls. In looking at potential mechanisms, BAC03 produced indole-3-acetic acid and ammonia, as well as showing a 1-aminocyclopropane-1-carboxylate deaminase activity, some of which could be potentially related to plant growth promotion. Volatiles released from BAC03 might be responsible for the negative effect on seed germination and seedling growth, the impact on the growth of *S. scabies* as well. Acetoin and 2,3-butanediol were detected as major components of the volatile by using gas chromatography–mass spectrometry analysis.

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are microorganisms that can be present within or in the vicinity of the plant rhizosphere (Kloepper *et al.*, 1980). They have been documented to improve the plant growth by providing promotive substance or facilitating plants uptake of nutrients from the environment (Lugtenberg and Kamilova, 2009). A large array of bacteria, such as strains in *Enterobacter*, *Arthobacter*, *Burkholderia*, *Pseudomonas*, *Bacillus*, *Azospirillum*, *Klebsiella*, *Serratia*, and *Paenibacillus*, have been reported to have the ability to enhance plant growth (Spaepen *et al.*, 2009). In addition to the trait of direct plant growth enhancement, some PGPRs may increase plant growth indirectly by preventing deleterious effect of plant pathogenic microorganisms (Kim *et al.*, 2011) and increasing plant tolerance to environmental stresses, such as flooding (Grichko and Glick, 2001), salt stress (Mayak *et al.*, 2004a), water deprivation (Mayak *et al.*, 2004b), and excess of heavy metals (Zhuang *et al.*, 2007).

PGPRs can directly enhance plant growth through a broad range of mechanisms, including: 1) producing or changing the concentration of various phytohormones, such as auxins, cytokinins, and gibberellins (Santner *et al.*, 2009); 2) secreting enzymes that can modulate plant growth and development, such as reducing ethylene level by synthesis of 1-aminocyclopropane-1-carboxylate deaminase (Yang and Hoffman, 1984; Penrose *et al.*, 2001); 3) supplying plant with nutrients, such as enhancing asymbiotic nitrogen fixation (Doberein and Campelo, 1971) 4) increasing the solubilization of phosphorus and other trace element for plant uptake (Gyaneshwar *et al.*, 2002); and 5) synthesizing siderophores which can provide soluble iron to plants (Scher and Baker, 1982).

Enhancement of plant growth by some isolates of root-colonizing *Bacillus* spp. is well documented (Chanway *et al.*, 1988; Turner and Backman, 1991; Ryu *et al.*, 2003). For example, *Bacillus amyloliquefaciens* strain FZB42 was demonstrated to produce indole-3-acetic acid (IAA), which is related to plant growth promotion activity validated by mutation (Idris *et al.*, 2007). *Bacillus subtilis* and *B. megaterium* are beneficial to plant growth, and production of cytokinin by some *Bacillus* isolates may relate to promotion (Arhipova *et al.*, 2007). Plant growth promotion has been associated with production of either gibberellin or jasmonic acid (Forchetti *et al.*, 2007). Moreover, volatile organic compounds from some *B. subtilis* have been shown to trigger growth promotion in *Arabidopsis* by regulating auxin homeostasis (Zhang *et al.*, 2007). Further research on this group will help to accelerate the development and application of new products that improve crop quality and yields.

Bacillus amyloliquefaciens strain BAC03 has antimicrobial (Meng *et al.*, 2012a) and biological control activities in greenhouse and field conditions (Meng *et al.*, 2013). It also displayed potential growth promotion ability under the stress of plant pathogen exposure (Meng *et al.*, 2012a). To utilize this bacterium for enhancing plant growth to get a better result, it is necessary to evaluate BAC03 for plant growth activity without phytopathogens and determine the optimal strategies of BAC03 application for plant growth promotion. The aims of this work were to 1) assess plant growth responses to BAC03 treatment; 2) test different strategies for BAC03 application in radish growth promotion; 3) test the effect of BAC03 on seed germination and seedling growth of a variety of plants; 4) detect substance(s) synthesized by BAC03 that may be associated with plant growth promotion; and 5) characterize volatiles released by BAC03 that may impact plant growth.

MATERIALS AND METHODS

Bacterial cultures

Bacillus amyloliquefaciens FZB42, the active ingredient in commercial biocontrol product RhizoVital42 (ABiTEP GmbH Inc., Berlin, Germany), was obtained from the *Bacillus* Genetic Stock Center (Columbus, OH, USA). *Bacillus subtilis* QST713 was obtained from the commercial product Serenade (Bayer CropScience Inc., Monheim, Germany) by culturing the product (powder) in tryptic soy broth (TSB; EMB Chemical Inc., Gibbstown, NJ, USA). All *Bacillus* strains were cultured on tryptic soy agar (TSA; EMB Chemical Inc.).

Plant growth promotion assay in different plants

To examine the effect of BAC03 on plant growth, nine different types of plant were used. Seeds of radish (cv. 'Cherry Belle', Burpee Inc. Warminster, PA, USA), beet (cv. 'Burpee's Red Ball'), carrot (cv. 'Touchon'), cucumber (cv. 'Bush Champion'), pepper (cv. 'Bush Belle Hybrid'), squash (cv. 'Black Beauty'), tomato (cv. 'Summer Girl Hybrid'), and turnip (cv. 'Purple Top') were pre-germinated on sterile moist filter paper (No.1, Whatman, Pittsburgh, PA) in a Petri dish (VWR International, LLC, Radnor, PA, USA), incubated at 25°C. The germinated seeds were placed in a 1-L pot containing potting mix (ASB Greenworld Inc., New Brunswick, VA, USA), and placed in a growth chamber (24°C, 14 h light, and 90% relative humidity), with 2 seedlings per pot.

To prepare the inoculum of BAC03, a bacterium was grown in Tryptic soy broth (TSB; EMB Chemical Inc., Gibbstown, NJ) at 28°C on an incubator shaker (Thermo Fisher Scientific Inc, Rockford, IL, USA) at 180 rpm for 48 h. Liquid culture of BAC03 was applied as a drench 15 days after planting (DAP), to a final concentration of 10^5 CFU cm⁻³ potting mix (ASB Greenworld Inc.). This final concentration of BAC03 was used, unless mentioned otherwise.

Sterile TSB was used to treat the potting mix as a negative control. Four replicates were used for each treatment. Radish, squash, cucumber, and turnip were harvested six weeks after planting, while beet, tomato, pepper, and carrot were harvested two months after planting. Height, number of flowers, and/or fresh and dry weight of both leaves and roots, were measured after harvesting, depending on the plant type (Table 4.1). This experiment was conducted twice.

For potato, tuber pieces (cv. 'Snowden') with at least one eye were incubated in the potting mix in a growth chamber (25°C, 14 h light, and 90% relative humidity) until sprouting, then transferred in 3.78 L plastic pots containing potting mix in the greenhouse (18 to 22°C with a 14-h photoperiod supplemented by light at 200 $\mu\text{mol m}^{-2} \text{s}^{-1}$). A liquid culture of BAC03 (10^7 CFU ml^{-1}) was added to the potting mix as a drench 10 days after planting (DAP), to give a final concentration of 10^5 CFU cm^{-3} potting mix. A negative control of non-infested potting mix mixed with TSB was used. Six weeks after transplanting, the height of the plant from the soil line to the apex of potato was measured with a ruler. Plants were harvested three months after transplanting. Tuber yield was determined by measuring the weight of potato tubers from each pot.

BAC03 application at different stages

BAC03 was applied at five different time during radish growth, including five days before planting (DBP), 1, 10, 20, and 30 DAP with the same concentration of 10^5 CFU cm^{-3} potting mix. Treatment with TSB only was used for a control. There were four replicates (pots) for each treatment. Fresh and dry weight of leaves and roots were determined at harvest, six weeks after planting. This trial was conducted twice.

BAC03 application with different concentrations

BAC03 concentrations tested included 1×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 , and 5×10^6 CFU cm⁻³ potting mix. Diluted *Bacillus amyloliquefaciens* BAC03 liquid culture was applied as a drench into the potting mix 10 DAP of radish. Treatment with TSB only was used for a control. Four replicates were included for each treatment. The fresh and dry weight of leaves and roots were measured after harvest, which was at 6 weeks after planting. The method for a radish planting was the same as described above. This experiment was conducted twice.

BAC03 application with different frequencies

BAC03 liquid culture was applied as a drench between 7 to 13 DAP to get a concentration of 10^5 CFU cm⁻³ potting mix. BAC03 was applied either once (at 10 DAP), or the same volume of inoculum was divided and applied two (at 8 and 12 DAP), three (at 7, 10, and 13 DAP), and four (at 7, 9, 11, and 13 DAP) times. The same volume of TSB was used as a control. Four replicates were used for each treatment. The methods for planting and biomass measurement were the same as before. This experiment was conducted twice.

Comparisons of BAC03 with *Bacillus subtilis* QST713 and *B. amyloliquefaciens* FZB42

Liquid cultures of each of the three bacteria were applied 10 DAP of radish to get a final concentration of 10^5 CFU cm⁻³ potting mix. Four replicates were used for each treatment. The methods for planting and measurement were the same as mentioned above. This experiment was conducted twice.

Effects of BAC03 on seed germination and seedling growth

To examine the effect of BAC03 on seed germination, seeds of eight types of plants were put in the potting mix (100 cm^{-3}) in Petri dishes. Beet, carrot, cucumber, pepper, radish, squash,

tomato, and turnip were used. Diluted BAC03 culture was mixed into the potting mix to make final concentrations of 10^5 , 10^6 , and 10^7 CFU cm^{-3} potting mix. Ten seeds were set per plate, and three plates were used as replicates for each plant type. The length of each seedling (from apex to the base of seedlings, no involvement of the length of seed) was measured one or two weeks after planting. This assay was conducted twice.

Seeds of beet, carrot, cucumber, pepper, radish, squash, tomato, and turnip were pre-germinated on moist filter paper in a Petri dish incubated at 25°C. The germinated seeds were placed into potting mix (100 cm^{-3}) in a Petri dish, which was infested with BAC03 at concentrations of 10^5 , 10^6 , and 10^7 CFU cm^{-3} potting mix. Ten seeds were used per plate, and three plates used as replicates per plant. The length of each seedling was measured 10 DAP as above. This assay was conducted twice.

Detection of substance(s) that have been associated with plant growth promotion

1-Aminocyclopropane-1-carboxylate (ACC) is an immediate precursor of ethylene in higher plants (Naclerio *et al.*). ACC-deaminase hydrolyses ACC into ammonia and α -ketobutyrate (Onofre-Lemus *et al.*, 2009). To determine the amount of α -ketobutyrate, cell pellets of the BAC03 culture were suspended in 5 ml of 0.1 M Tris-HCl (pH 7.6), and centrifuged at $20,000 \times g$ for 5 min. The pellets were re-suspended in 2 ml of 0.1 M Tris-HCl (pH 8.5). Thirty microliters of toluene were added to the cell suspension vortexed for 30 s. Twenty microliters of 0.5 M ACC (Sigma-Aldrich Co., St Louis, MO, USA) were added to 200 μl of the resulting suspension and incubated at 30°C for 15 min. One milliliter of 0.56 M HCl was added into the suspension which was centrifuged for 5 min at $20,000 \times g$. Two milliliters of the resulting supernatant were vortexed together with one milliliter of 0.56 M HCl. Two

milliliters of 2,4-dinitrophenylhydrazine reagent (0.2%, 2,4-dinitrophenylhydrazine in 2 M HCL, Sigma-Aldrich Co.) was added to each and vortexed, followed by incubation at 30°C for 30 min. Two milliliters of 2 M NaOH were added and the absorbance of the mixture was read at 540 nm (UNICAM HELIOS spectrophotometer, Thermo Scientific Inc., Waltham, USA). The concentration of α -ketobutyrate was determined by comparing the absorbance at 540 nm of a sample to a standard curve of α -ketobutyrate (Sigma-Aldrich Co., St Louis, MO, USA) ranging between 0.1 and 1.0 μ M. A stock solution of 100 μ M α -ketobutyrate (Sigma-Aldrich Co.) was prepared in 0.1 M Tris-HCl pH 8.5 and stored at 4°C. The stock solution was diluted with the same buffer to make a 10 μ M solution prior to use (Honma and Shimomura, 1978).

Indole-3-acetic acid (IAA) production was tested according to the modified method as previously described by Wohler (1997). Briefly, bacterial cultures were grown for 48 h in TSB at 28°C on an incubator shaker, followed by centrifugation at $7,000 \times g$ for 10 min. The pellet was incubated at 37°C for 24 h in 3 ml of sodium phosphate buffer (pH7.5) amended with glucose (1%) and tryptophan (1%, Sigma-Aldrich Co.). After incubation, the mixture was amended with two milliliters of 5% trichloroacetic acid (Sigma-Aldrich Co.) and one milliliter of 0.5M CaCl₂. The solution was filtered through filter paper (No.2, Whatman, Pittsburgh, PA). Two milliliters of Salkowski reagent (2 ml 0.5 M FeCl₃ and 98 ml 35% perchloric acid, Sigma-Aldrich Co.) was added into three milliliters of the filtrate. This mixture was incubated for 30 min at 25°C in the dark. Optical density was taken at 530 nm with a spectrophotometer (Thermo Scientific Inc., Waltham, MA, USA). Concentration of IAA produced by cultures was estimated by comparison with a measured with standard graph of IAA in the range of 10-100 μ g ml⁻¹.

Bacterial isolates were tested for the production of ammonia in peptone water. A loop of cultures of test isolates were inoculated into 10 ml of peptone water (peptone 10g L⁻¹, sodium

chloride 5 L^{-1} , pH 7.5) (Cappuccino and Sherman, 1992) and incubated for 48 to 72 h at 28°C . Five hundred microliters of Nessler's reagent (Sigma-Aldrich Co.) was added to each tube and the reaction color was observed visually. Development of color from yellow to brown was considered as a positive reaction for ammonia production (Ahmad *et al.*, 2008).

Chrome azurol S agar plates were prepared based on the description by Schwyn and Neilands (1987), and inoculated with test organisms ($10 \mu\text{l}$ of 10^6 CFU ml^{-1}), followed by incubation at 28°C for 48 to 72 h. Development of a yellow-orange halo around the colony was considered as a positive reaction for siderophore production (Schwyn and Neilands, 1987).

Test for potential volatiles on seed germination and seedling growth

The bioassay was done in closed Petri dishes (9 cm diameter, VWR Inc., Radnor, PA, US) in the presence of a BAC03 culture and plant seeds or seedlings (Fig. 4.1). BAC03 was cultured on TSA plates (plate on the top) by spreading $50 \mu\text{l}$ of liquid culture with a sterile cell spreader. Liquid culture involved different concentrations, including 10^5 , 10^6 , and 10^7 CFU ml^{-1} . Radish seeds without any prior treatment or germinated radish seedlings (pre-germinated in a Petri dish with moist filter paper for 24 h at 25°C) were placed in another Petri dish with moist filter paper (Fig. 4.1, plate at the bottom). With the lid removed, these plates containing seeds or seedlings (Fig. 4.1, plate at the bottom) were covered with TSA plates containing BAC03 culture (Fig. 4.1, plate on the top). The double dishes (Fig. 4.1, plates on the top and at the bottom) were sealed with a double layers of paraffin film (Para film M, Chicago, IL, USA). Ten seeds or seedlings were tested for each plate, and three replicate plates were used for each treatment. *Bacillus subtilis* QST713 and *B. amyloliquefacisn* FZB42 were included in this experiment. TSA

plates without bacterial culture were sealed with radish seeds or seedlings as a negative control. This experiment was conducted twice.

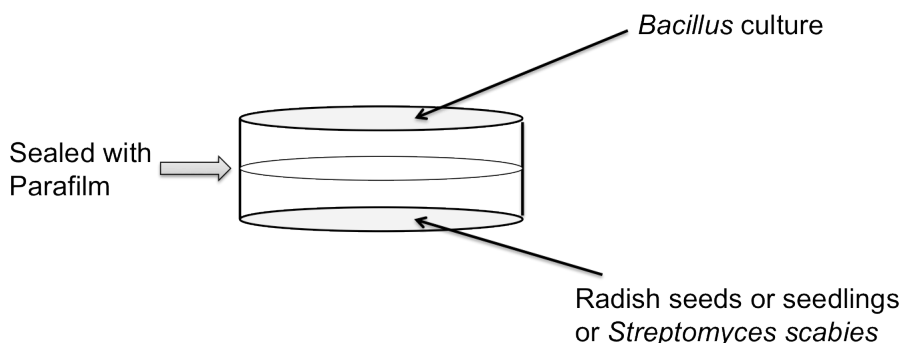


Figure 4.1. Unattached culture method used for the test of effect of potential volatiles

Effect of potential volatiles on the growth of *Streptomyces scabies*

Effects of volatiles released from BAC03 were tested on both colony growth and sporulation of *S. scabies*. Fifty microliters of a spore suspension of *S. scabies* (ATCC49173) was spread on yeast malt extract agar (YME; EMD Chemical Inc., Gibbstown, NJ, US) plates (Fig. 4.1). BAC03 was cultured by spreading 50 μl of liquid culture (10^7 CFU ml^{-1}) with a sterile cell spreader on TSA (EMD Chemical Inc.). After the lid was removed, two culture plates were placed face-to-face and sealed with a double layer of paraffin film (Fig. 4.1), and incubated at 28°C. Numbers of *S. scabies* colonies were counted after three, four, and five days of incubation. To test for effects of BAC03 potential volatiles on *S. scabies* sporulation, 50 μl of a spore suspension (10^4 CFU ml^{-1}) of *S. scabies* was spread on YME plates as above. Three days later, plates with visible *S. scabies* colonies were sealed with plates inoculated with BAC03 as above. The numbers of colonies that turned white or whitish grey were observed after five, seven, and nine days of incubation. *Bacillus subtilis* QST713 and *B. amyloliquefacisn* FZB42 were used as

comparative bacteria. TSA plates without *Bacillus* were covered and sealed with YME plates incubated with *S. scabies* as a negative control. This experiment was carried out twice.

Gas chromatography–mass spectrometry (GC-MS) analysis of the volatile organic compounds (VOCs) for BAC03

Ten microliters of BAC03 liquid culture (10^7 CFU ml⁻¹, prepared with the method above) was transferred to 15 ml of TSB in a 40-ml glass vial with a screw cap with a silicon septa (Cole-Parmer Inc., Vernon Hills, IL, USA). Prior to sampling, the vials were stored at 40°C for one hour to equilibrate the solid-phase and the phase of VOCs in the vial. The vials were incubated on a shaker at 180 rpm and 30°C for 48 h. GC-MS was conducted at the Michigan State University Mass Spectrometry and Metabolomics Core facility (East Lansing, MI, USA). A 50/30 µm solid-phase microextraction fiber (Supelco, Bellefonte, PA) was inserted into the vial for extraction of VOCs at 40°C for 40 min. The fiber was removed from the vial and inserted into a gas chromatograph / time-of-flight mass spectrometer (Waters GCT Premier, Milford, MA, USA). The GC column was a DB-WAX (30 m length x 250 µm inner diameter x 0.250 µm film thickness; Agilent J&W Columns, Santa Clara, CA, USA). The inlet conditions consisted of a temperature of 225°C, a split injection with a 10:1 ratio, and a purge rate of 50 mL min⁻¹ at a time of 1 minute. Ultra-high purity helium was used as the carrier gas at a constant flow rate of 1.4 mL min⁻¹. The oven temperature program was as follows: 40°C for 1.5 min, increased at 10°C min⁻¹ to 220°C. The mass spectrometer parameters included ionization by electron ionization (EI), a m/z range of 10-800, a scan time of 0.2/s, and an inter-scan time of 0.1 s. The data acquisition time was 19.5 min per sample.

Identification of the VOCs was done by comparing the mass spectra and retention times of the individual VOC with those for the standard compounds deposited in the database of the National Institute of Standards and Technology (NIST) by using the Agilent Chemstation Probability Based Match algorithm (Probability Based Matching, Agilent. Technologies, Santa Clara, CA). Background-subtracted total ion chromatograms (TICs) of the samples were overlain and visually inspected to determine the presence of compounds those appear to be different between samples.

Statistical analysis

Data were analyzed using SAS software (version 9.2, SAS Inc., Cary, NC, USA). Procedure GLM was used for the analysis of variance, and Fisher's least significant difference (LSD) multiple comparisons were performed for mean separation where ANOVA showed significant differences. If there was no interaction between repeated trials ($P > 0.05$), data were combined from all trials.

RESULTS

Plant growth promotion assay in different plants

Plants from nine different plant types showed that plant growth promotion was observed in all measurement categories for all plant species, including plant height, number of flowers, and biomass of plant part (Table 4.1). However, the efficacies for plant growth enhancement varied depending on the plant type, from less than 100% to above 200% of the control (Table 4.1).

Strategy of BAC03 application for radish growth promotion

In the test of fresh weight of radish, treatments with BAC03 applied at -5, 1, 10, and 20 DAP had higher weight statistically in both root and leaf biomass, but no growth promotion effect was observed when BAC03 was applied at 30 DAP (Fig. 4.2, I). For dry weight, treatments with BAC03 applied at -5, 10, and 20 DAP had higher weight results in both radish root and leaf than control (Fig. 4.2, II).

BAC03 at 5×10^4 CFU cm⁻³ potting mix and higher concentrations significantly increased fresh weight of radish leaves and roots compared to the control (Fig. 4.3, I). BAC03 gave a higher dry weight than the TSB control (Fig. 4.3, II). Treatments with BAC03 at 5×10^4 CFU cm⁻³ potting mix and higher concentrations gave a higher dry weight of radish root than other treatments (Fig. 4.3, II).

For both fresh and dry weight measurements, BAC03 applied more than one time increased weight in radish roots and leaves compared to the treatment with a single application of BAC03 (Fig. 4.4, I and II).

In the test of radish roots biomass (fresh and dry weight), the effect of BAC03 was not significantly different from that of FZB42, a higher weight compared to the treatment of QST713 in both fresh and dry weight measurement (Fig. 4.4, I and II). There was no significant difference in the weight of radish leaves treated with the three isolates (Fig. 4.4, I and II).

Effects of BAC03 on seed germination and seedling emergence

BAC03 at all three concentrations inhibited seed germination of eight different types of plants at various level. The inhibition rate for carrot, cucumber, radish, squash, tomato, and turnip were higher than 90% (Fig. 4.6 A). For seedling growth, BAC03 inhibited seedling growth

of most plants at all concentrations, with the exception of pepper treated with BAC03 at 10^5 CFU cm⁻³ and squash treated with BAC03 at 10^5 and 10^6 CFU cm⁻³ potting mix (Fig. 4.6 B).

Detection of substances potentially related to plant growth promotion

Results of plant growth promotion substances and activity were displayed in the table 4.2. All three *Bacillus* isolates produced IAA and NH₃, as well as showing ACC-deaminase activity while no siderophore production was detected. In the assessment of IAA production and ACC-deaminase activity, *B. amyloliquefaciens* strains BAC03 and FZB42 had higher concentrations than *B. subtilis* QST713 (Table 4.2).

Volatiles released from BAC03

Volatiles from three *Bacillus* strains displayed inhibition on both seed germination and seedling growth, regardless of bacterial concentrations. The inhibition rate on seed germination (around 80 to 90%) was higher than those on seedling growth (around 40 to 50%) (Fig. 4.7 A and B).

Volatiles released from *Bacillus* strains inhibited colony growth (Fig. 4.8 A) and sporulation (Fig. 4.8 B) of *S. scabies*. However, the inhibition effect decreased over time (Fig. 4.7 A and B).

Major VOCs released from *B. amyloliquefaciens* BAC03, *B. amyloliquefaciens* FZB42, and *B. subtilis* QST713 were the same, including putative 3-hydroxy-2-butanone (acetoin) and 2,3-butanediol (Table 4.3). In addition, a substance, with a retention time of 9.05 min was detected but was not identified based on the NIST 2005 database (Table 4.3).

Table 4.1. Plant growth promotion assay in different plants using *Bacillus amyloliquefaciens* BAC03 at concentration of 10^5 CFU cm^{-3} potting mix

Plant	Height (cm)		Weight of leaves (g)		Dry weight of leaves (g)		No. of flowers	Weight of root or tubers (g)		Dry weight of root (g)	
Radish	16.8	^a 50%	17.0	233%	5.8	152%	^z ND	59.5	294%	12.6	385%
Turnip	18.8	41%	27.1	105%	7.6	90%	ND	19.0	428%	3.0	400%
Beet	16.5	83%	15.6	291%	3.2	146%	ND	10.6	715%	2.7	800%
Carrot	34.6	70%	14.0	311%	5.0	233%	ND	29.6	212%	4.8	243%
Potato	38.1	64%	102.6	97%	34.7	50%	4.2	172.1	88%		ND
Cucumber	34.4	115%	50.1	175%	17.7	190%	31		ND		ND
Squash	26.6	48%	49.9	187%	12.8	190%	31		ND		ND
Pepper	28.6	52%	15.1	268%	3.6	200%	3.3		ND		ND
Tomato	45.7	29%	47.7	107%	18.0	128%	3.3		ND		ND

^a Plant growth promotion efficacy was calculated as [(Treatment – control) / control] * 100%.

^z ND: not detected.

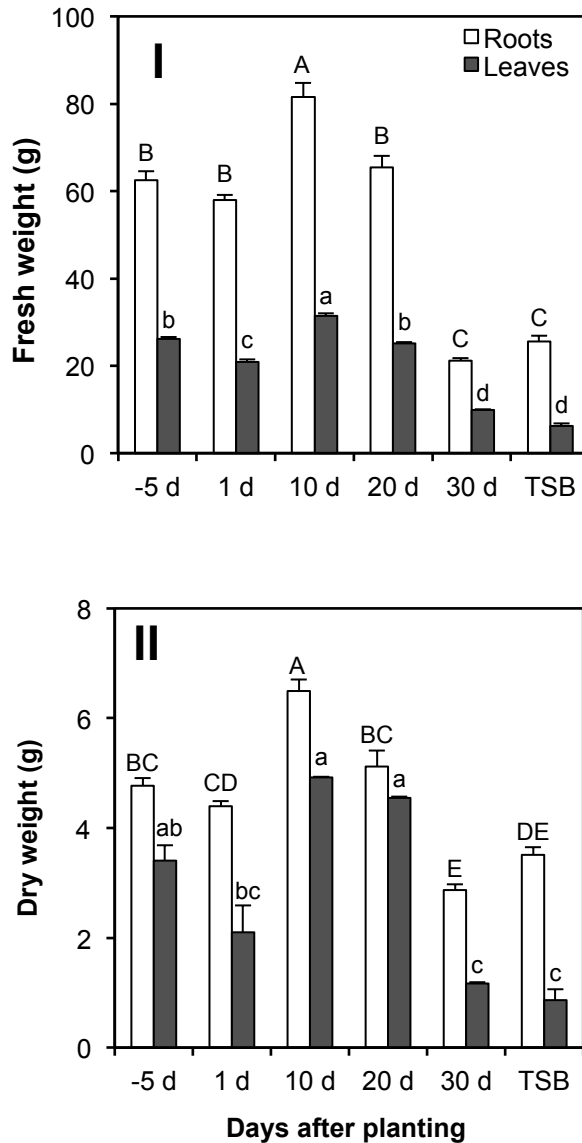


Figure 4.2. Effects of different application timings of *Bacillus amyloliquefaciens* BAC03 on radish growth in fresh (I) and dry (II) weight of roots (white bars) or leaves (black bars). BAC03 was applied at 5 days before planting (-5 d), or 1, 10, 20, and 30 days after radish planting (1d, 10d, 20d, and 30d) at a concentration of 10^5 CFU cm^{-3} potting mix. Treatment with tryptic soy broth (TSB) only was used for a control. Mean values are the average of two trials, with four replicates each. Error bars represent standard error of the means of observations. Multiple comparisons were conducted for weight measurement. Means with the same letters for each tissue type (roots or leaves) are not significantly different on the basis of least significant difference ($P < 0.05$).

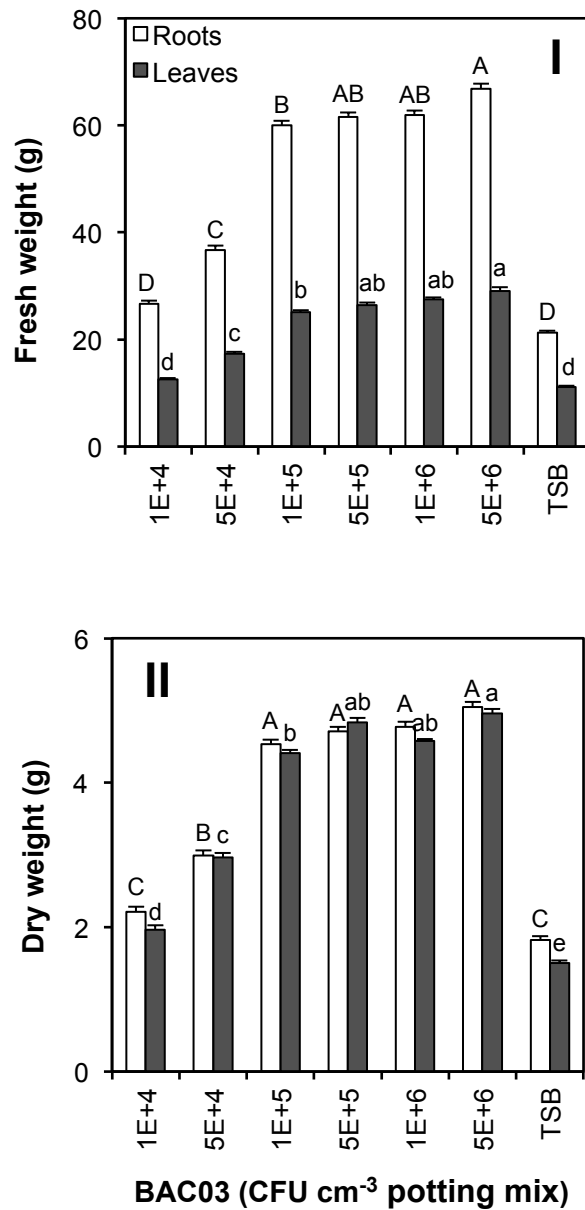


Figure 4.3. Effects of different *Bacillus amyloliquefaciens* BAC03 initial concentrations on radish growth in fresh (I) and dry (II) weight of roots (white bars) or leaves (black bars). BAC03 concentrations tested included 1×10^4 , 5×10^4 , 1×10^5 , 5×10^5 , 1×10^6 , 5×10^6 CFU cm⁻³ potting mix. Diluted *Bacillus amyloliquefaciens* BAC03 liquid culture was drenched into the potting mix 10 DAP of radish. Tryptic soy broth (TSB) was used for a control. The values were the average of two trials with four replicates. Error bars represent standard error of the means of observations. Multiple comparisons were conducted for weight measurement. Means with the same letters for each tissue type (roots or leaves) are not significantly different on the basis of least significant difference ($P < 0.05$).

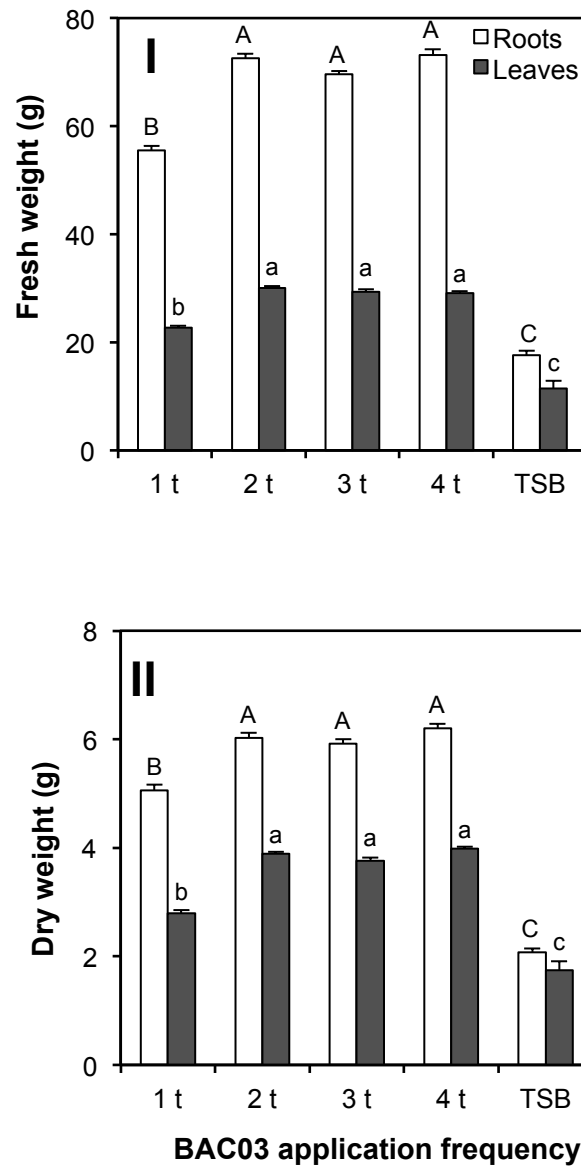


Figure 4.4. Effects of different application frequencies of *Bacillus amyloliquefaciens* BAC03 (10^5 CFU cm^{-3} potting mix) on radish growth in fresh (I) and dry (II) weight of roots (white bars) or leaves (black bars). BAC03 was applied either for once (1 t), or the same volume of BAC03 was divided into 2, 3, or 4 (2 t, 3 t, and 4 t). Tryptic soy broth (TSB) was used for a control. The values shown are the average of two trials with four replicates. Error bars represent standard error of the means of observations. Multiple comparisons were conducted for weight measurement. Means with the same letters in each measurement category (roots or leaves) are not significantly different on the basis of least significant difference ($P < 0.05$).

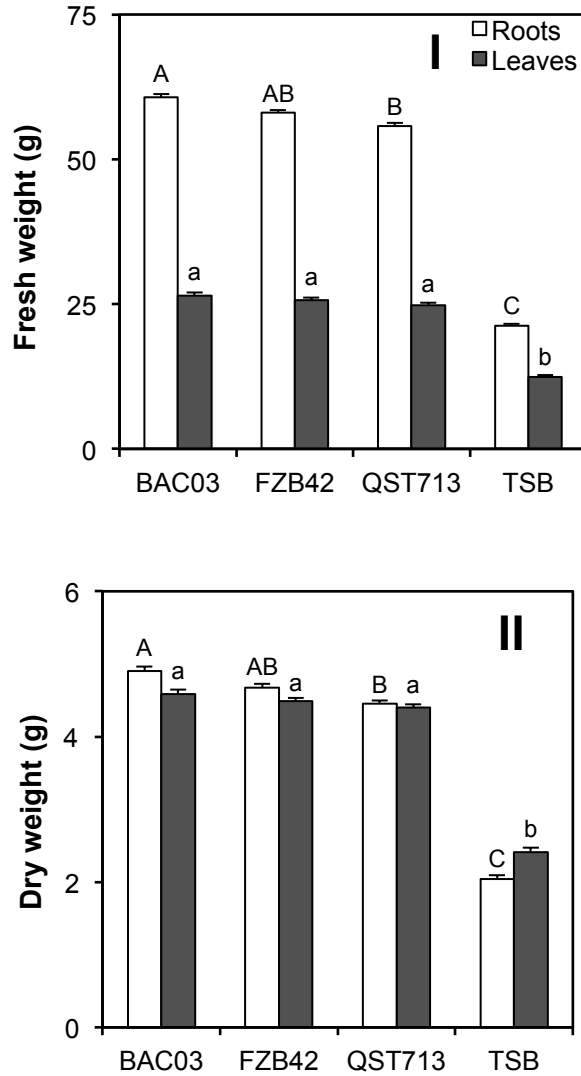


Figure 4.5. Comparison of *Bacillus amyloliquefaciens* BAC03, *B. subtilis* QST713, and *B. amyloliquefaciens* FZB42, at 10^5 CFU cm^{-3} potting mix, on radish growth as fresh (I) and dry (II) weight of roots (white bars) or leaves (black bars). The values were the average of two trials with four replicates. Error bars represent standard error of the means of observations. Multiple comparisons were conducted for weight measurement. Means with the same letters in each measurement category (roots or leaves) are not significantly different on the basis of least significant difference ($P < 0.05$).

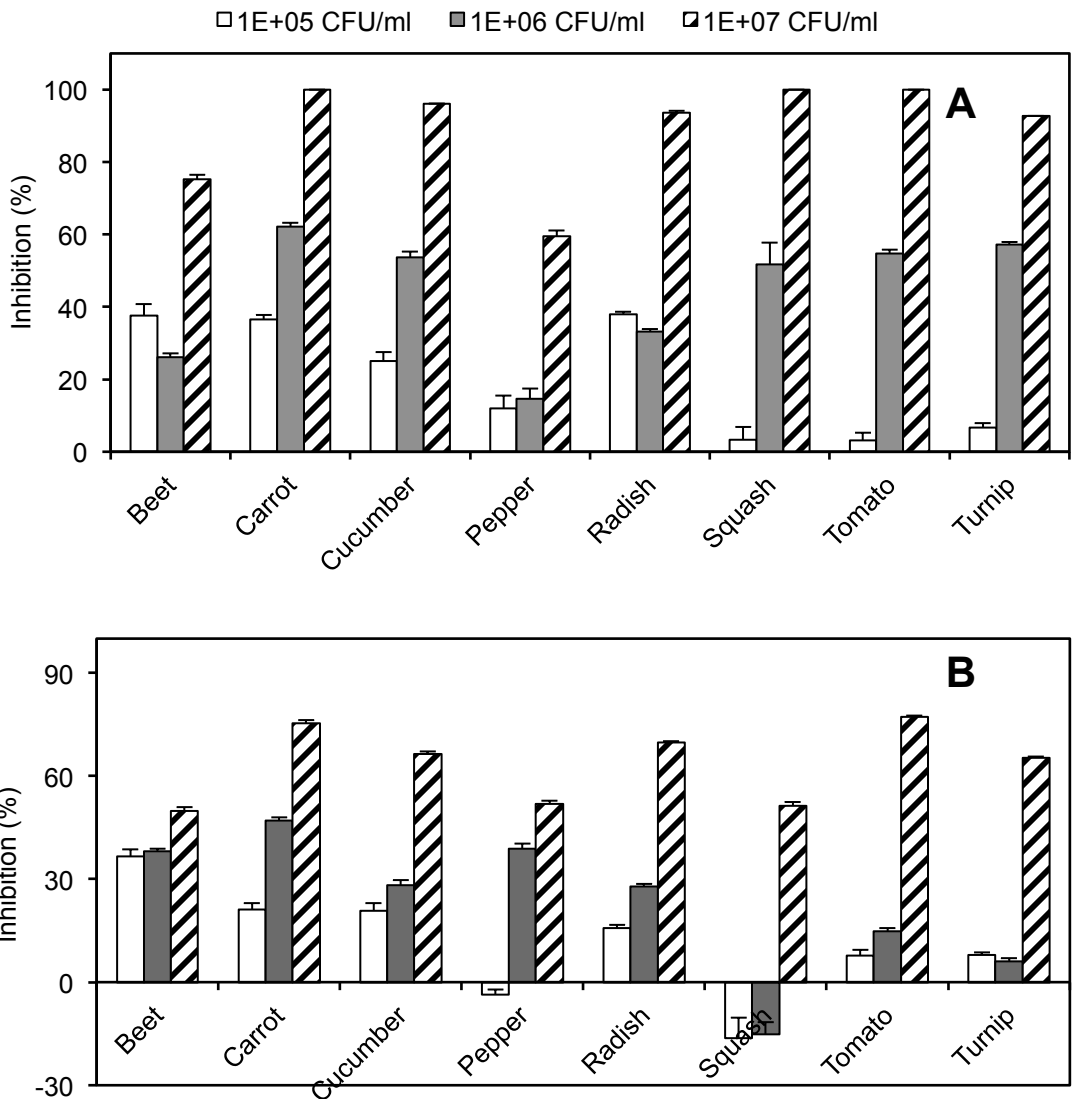


Figure 4.6. Effects of *Bacillus amyloliquefaciens* BAC03 on seed germination (A) and seedling growth (B) of select plants at three concentrations (10^5 , 10^6 , and 10^7 CFU ml⁻¹ potting mix). Non-treated seeds (A) or pre-germinated seedlings (B) of different plants were placed into potting mix (100 cm^{-3}) in a Petri dish, which was inoculated with BAC03 at the above concentrations. Ten seeds were used per plate, and three plates used as replicates per plant. The length of each seedling (from apex to the base of seedlings, no involvement of the length of seed) was measured one or two weeks after planting. This assay was conducted twice. Inhibition was calculated as the length of [(control – treatment) / treatment] $\times 100\%$. Values were averaged from two trials, with thirty plant seeds each trial. Bars for mean values are standard errors.

Table 4.2. Substances potentially related to plant growth promotion detected from *Bacillus amyloliquefaciens* strains BAC03 and FZB42, and *B. subtilis* strain QST713

	BAC03	FZB42	QST713
^a ACC-deaminase activity ($\mu\text{mol } \alpha\text{-ketobutyrate g}^{-1}$)	^x 0.26 ± 0.08 a	0.28 ± 0.05 a	0.10 ± 0.03 b
IAA production ($\mu\text{g ml}^{-1}$)	21.3 ± 0.60 a	19.5 ± 0.80 a	15.2 ± 0.40 b
NH ₃ production	^y D	D	D
Siderophore production	^z ND	ND	ND

^a ACC: 1-Aminocyclopropane-1-carboxylate; IAA: Indole-3-acetic acid.

^x Values are expressed as means \pm standard errors ($n = 8$). Means with different letters in each row are significantly different from each other on the basis of least significant difference ($P < 0.05$).

^y D: color detected; development of color from yellow to brown was considered as a positive reaction for ammonia production (Ahmad *et al.*, 2008) ($n = 8$).

^z ND: not detected; development of a yellow-orange halo around the colony was considered as a positive reaction for siderophore production (Schwyn and Neilands, 1987).

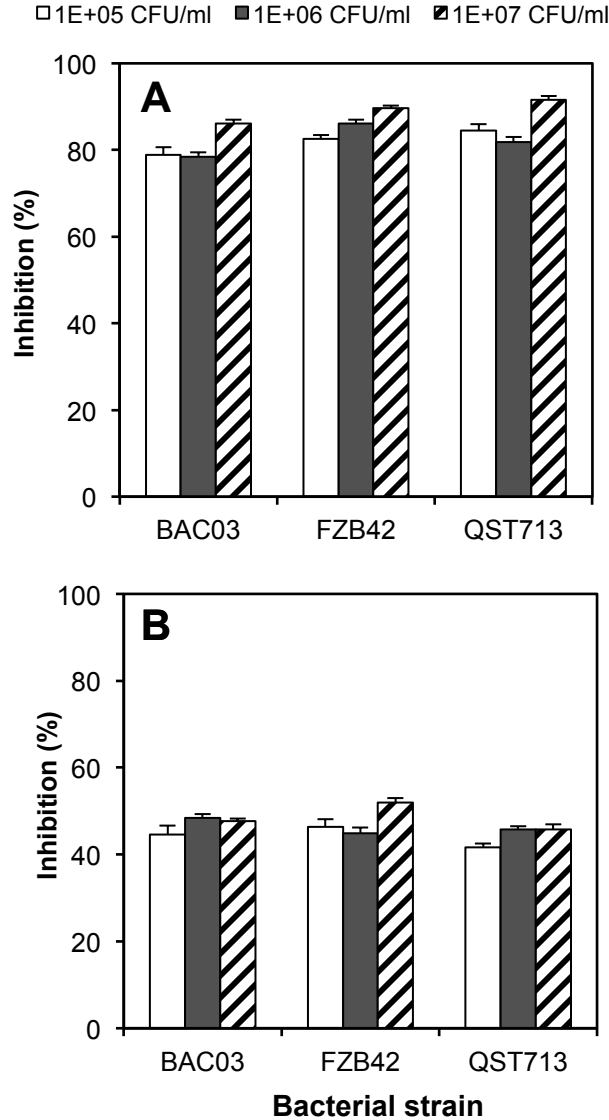


Figure 4.7. Effect of volatiles released by *Bacillus amyloliquefaciens* strains BAC03 and FZB42, and *B. subtilis* strain QST713 on radish seed germination (A) and seedling growth (B). The bioassay was done in closed Petri dishes in the presence of *Bacillus* culture (inverted plate, 50 μ l of liquid culture at 10^5 , 10^6 , and 10^7 CFU ml⁻¹) and plant seeds or seedlings (plate at the bottom). The double dishes (plates on the top and the bottom) were sealed with double layers of paraffin film. Ten seeds or seedlings were tested for each plate, and three replicate plates were used for each treatment. TSA plates without bacterial culture were sealed with radish seeds or seedlings as a negative control. This experiment was conducted twice. Inhibition rate was calculated as (control – treatment) / treatment \times 100%. Values were averaged from two trials, with thirty replications each. Bars over mean values are standard errors.

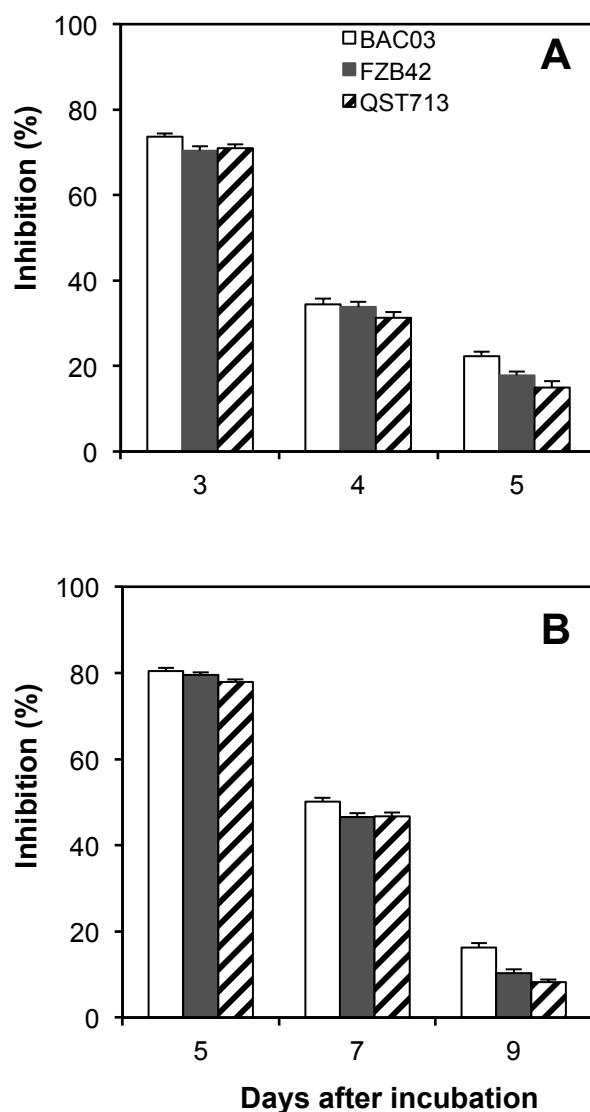


Figure 4.8. Effect of volatiles from *Bacillus amyloliquefaciens* strains BAC03 and FZB42, and *B. subtilis* strain QST713 on colony growth (A) and sporulation (B) of *Streptomyces scabies*, tested on yeast malt extract agar plates. The bioassay was done in closed Petri dishes in the presence of *Bacillus* culture (50 μ l of liquid culture at 10^7 CFU ml $^{-1}$) and *S. scabies* culture (50 μ l of a spore suspension at 10^4 CFU ml $^{-1}$). The double dishes (plates on the top and at the bottom) were sealed with double layers of paraffin film and incubated at 28°C. Numbers of *S. scabies* colonies after three, four, and five days of incubation were counted (A). To test the effect on sporulation of *S. scabies* (B), 3-days-old cultures were used and the numbers of colonies that turned white or whitish grey were counted after five, seven, and nine days of incubation. TSA plates without *Bacillus* were covered and sealed with YME plates cultured with *S. scabies* as a negative control. This experiment was carried out twice. Inhibition rate was calculated as [(control – treatment) / treatment] \times 100%. Values were averaged from two trials. Bars over are standard errors.

Table 4.3. Volatile organic compounds released from *Bacillus amyloliquefaciens* strains BAC03 and FZB42, and *B. subtilis* strain QST713 detected by using gas chromatography-mass spectrometry

Retention time (min)	^y Putative identification	Relative peak areas (%)			
		BAC03	FZB42	QST713	Blank control
7.81	3-hydroxy-2-butanone (acetoin)	94.7	97.3	100	ND
9.05	Unknown	1.7	1.4	3.7	ND
11.00	Benzaldehyde	^z ND	ND	ND	10.7
11.17	2,3-butanediol	2.2	7.8	6.7	ND

^y Compounds were identified by comparison of retention time and mass spectral data against National Institute of Standards and Technology (NIST) library.

^z ND: not detected.

DISCUSSION

The present work has addressed the performance of a potential PGPR strain *B. amyloliquefaciens* BAC03 on plant growth. The ability to cause growth promotion by BAC03 was tested in nine different types of plants with different measurements. Ten days post planting was an optimal timing for BAC03 application to gain a good efficacy for radish growth promotion. The root system quickly develops at this stage, which nurses more bacteria such as BAC03 to colonize by providing them with nutrient exudates. Result showed that multiple applications of BAC03 was better than a single application in enhancing radish growth. This might be due to a combination of survival of the bacterium and prolonged period of maintaining the bacterial population at a high level by multiple applications.

BAC03 inhibited seed germination and seedling growth, as shown in eight different types of plants. The negative effect of BAC03 on plant early growth could be associated with volatiles released from this strain (Blom *et al.*, 2011), as shown in the data in this study. It has been known that volatiles can be a very powerful tool used by many PGPRs to combat with other microorganisms including plant pathogens (Vespermann *et al.*, 2007; Arrebola *et al.*, 2010). Since the compounds are not target specific, plant growth can be affected as well (Wenke *et al.*, 2012). Such effects can be either promotion or inhibition for plant growth (Ryu *et al.*, 2003; Ryu *et al.*, 2005; Blom *et al.*, 2011). For example, in an assessment of an effect of volatile-mediated impact of *Bacillus* spp. on *Arabidopsis* seedlings, plant growth was promoted and the length of seedlings were increased six-fold (Ryu *et al.*, 2003). However, with the isolates in the same species, *Arabidopsis* seedlings were killed in another study (Ryu *et al.*, 2005). Concentration of secreted chemicals or different composition of the volatiles released from various isolates could be responsible for these contrasting effects (Ryu *et al.*, 2005). According to this speculation,

volatiles released from BAC03 could not be deleterious to the plants at lower concentrations. The negative effect observed on plant seedlings in this study could be because the concentration of released chemicals were relatively high towards small seedlings, while the concentration decreased over time, and to be beneficial to promote growth when the plant approaches maturity.

Among the volatiles released by PGPRs, 2,3-butanediol and its precursor acetoin are the most common organic volatiles; those are considered as candidates to be responsible for the effects of plant growth promotion (Han *et al.*, 2006; Blom *et al.*, 2011). Although acetoin and 2,3-butanediol were detected from the volatiles released by BAC03 in this study, it may not be concluded that they are responsible for the negative effect on plant seedlings. Volatiles released by bacteria consist of organic compounds and permanent gases. Although GC-MS with SPME used in this study is a powerful method for detecting volatile organic compounds (Huang *et al.*, 2011), it does not detect permanent gases, such as hydrogen cyanide (Alstrom and Burns, 1989; Astrom, 1991). Blom (2011) reported that HCN accounts for a significant extent of the deleterious effects when growing *Arabidopsis thaliana* in the presence of certain bacterial volatiles. Permanent gases that might be released by strain BAC03 and their function need to be considered in future study.

BAC03 produces IAA, NH₃, and has ACC deaminase activity. These compounds are all potentially associated with plant growth promotion in other studies (Loper and Schroth, 1986; Santner *et al.*, 2009; Teale *et al.*, 2006; Grichko and Glick, 2001). IAA is the most common phytohormone detected in PGPRs, and can play a very important role in plant growth promotion (Loper and Schroth, 1986; Santner *et al.*, 2009). Development of plant growth is sensitive to concentrations of phytohormones (Teale *et al.*, 2006). In addition to phytohormone production by PGPR, plants can be induced by certain PGPRs to secrete IAA (Marques *et al.*, 2010). The

enzyme ACC deaminase, secreted by some PGPRs, is able to catalyze ACC, the immediate precursor of ethylene, resulting in the concentration of ethylene being decreased. Since high levels of ethylene inhibits the root elongation after seed germination, reduced amount of ethylene in the plant can result in promoting plant root and shoot growth (Penrose and Glick, 2003). Moreover, plants that are treated with ACC deaminase-producing bacteria are more resistant to a variety of stressful conditions (Grichko and Glick, 2001; Onofre-Lemus *et al.*, 2009). Production of ammonia was reported positively related to nitrogen accumulation and elongation of the plant roots, as well as biomass production (Marques *et al.*, 2010).

In conclusion, *B. amyloliquefaciens* strain BAC03 has activities of plant growth promotion through the secretion of several substances. It can be a good candidate for a plant growth promoter used in agricultural production.

**CHAPTER 5: MANAGING COMMON SCAB CAUSED BY *STREPTOMYCES* SPP.
USING *BACILLUS AMYLOLIQUEFACIENS* BAC03 AND OTHER BIOMATERIALS**

This chapter has been published:

Meng, Q.X., Hanson, L.E., Douches, D. and Hao, J.J. (2013) Managing common scab caused by *Streptomyces scabies* using *Bacillus amyloliquefaciens* BAC03 and other biomaterials. *Biological control*, 67, 373-79.

ABSTRACT

Streptomyces spp. cause scab in plants like potato and radish. To effectively control this disease, biologically based materials were examined for their efficacies. In greenhouse or growth chamber tests potting mix was infested with *Streptomyces scabies* (10^6 CFU cm⁻³) followed by applying different products. *Bacillus amyloliquefaciens* strain BAC03 (10^5 CFU cm⁻³) reduced disease severity ($P < 0.05$) and potentially enhanced growth of radish ($P < 0.05$); chestnut tissues at 50% volume, oregano and clove essential oils ($0.1 \mu\text{g cm}^{-3}$), and ground horseradish ($> 0.18 \text{ g m}^{-3}$) significantly reduced scab in potato and/or radish ($P < 0.05$). In two Michigan fields in 2011 and 2012, chestnut tissues (1.15 kg m^{-2}), oregano essential oil (1.5 ml m^{-2}), and ground horseradish (0.38 g m^{-2}) were incorporated into the soil two weeks before planting. BAC03 (10^6 CFU ml⁻¹, 1 L m^{-2}) was applied as a soil drench four times at 2-week intervals starting one month post planting. BAC03 significantly ($P < 0.05$) reduced disease severity of potato scab in two seasons. It also increased potato tuber weight in 2011 ($P < 0.05$) at two locations. The rest of the treatments caused significant disease reduction, but were less effective than BAC03, and the efficacy varied depending on location and year.

INTRODUCTION

Streptomyces scabies is the major species of pathogenic *Streptomyces* spp., which are distributed worldwide and cause potato common scab (Loria *et al.*, 1997; Wanner, 2006), and scab disease in radish and other crops (Goyer and Beaulieu, 1997). The economic losses in most potato production areas are significant due to lack of effective management strategies (Wanner and Haynes, 2009; Dees and Wanner, 2012). Since most of the necrotic lesions are formed on the potato tuber surface, cosmetic damage contributes more than yield reduction to economic losses (Loria *et al.*, 1997). The pathogen can survive as a saprophyte for long periods in the soil, and different species of the pathogen having distinct genetic backgrounds, making disease management difficult (Loria *et al.*, 2006; Wanner, 2007; Hao *et al.*, 2009; Hiltunen *et al.*, 2009; Jiang *et al.*, 2012).

Many approaches have been used to manage common scab, but few are practical or effective (Larkin and Griffin, 2007; Larkin, 2008; Hiltunen *et al.*, 2009). Using cultivars resistant to common scab is the most effective method of disease management, but availability of resistant germplasm is limited (Douches *et al.*, 2009b; Wanner and Haynes, 2009). Lower soil pH and increasing soil moisture can reduce the disease severity (Lapwood *et al.*, 1970; Davis *et al.*, 1976; Lacey and Wilson, 2001). However, contradictory results exist by using these two strategies (Lapwood *et al.*, 1973; Larkin *et al.*, 2011). Use of pentachloronitrobenzene is partially effective (Davis *et al.*, 1976), but it can cause environmental concerns due to its long half-life in soil (Wharton *et al.*, 2011). Therefore, additional or alternative strategies for managing common scab are needed.

Among cultural practices, organic soil amendments, green manures, and crop rotation have been shown to reduce common scab (Abbasi *et al.*, 2006; Sakuma *et al.*, 2011; Singhai *et*

al., 2011). Addition of biological materials has provided promising results for disease suppression by promoting activity and diversity of resident microbial organisms, increasing plant-beneficial organisms, and promoting antagonists toward pathogens (Larkin, 2008). Many biological materials have been studied for potato common scab management, such as fish emulsion (Abbasi *et al.*, 2006); soymeal, meat and bone meal (Lazarovits *et al.*, 1999); lopsided oat green manure (Sakuma *et al.*, 2011); and mustard green manure (Larkin and Griffin, 2007). In addition, several groups of plant growth promoting rhizobacteria (PGPR) displayed promising activities for biological control of common scab, such as *Pseudomonas* spp. (Al-Mughrabi, 2010; Singhai *et al.*, 2011), *Bacillus* spp. (Schmiedeknecht *et al.*, 1998; Han *et al.*, 2005), and non-pathogenic *Streptomyces* spp. (Liu *et al.*, 1995; Hiltunen *et al.*, 2009).

To seek effective biological control options, various materials have been examined in laboratory and greenhouse trials (Bi *et al.*, 2012; Hao *et al.*, 2012; Meng *et al.*, 2012a). Extracts of chestnut tissue displayed a strong antimicrobial activity against many plant pathogens, which probably is associated with antimicrobial compounds such as flavonol glycoside and several terpenoid substances (Hao *et al.*, 2012). Clove and oregano essential oil has shown a high level of suppressive activity against *Phytophthora capsici* (Bi *et al.*, 2012). Ground horseradish tissue inhibited the growth of several *Streptomyces* spp. *in vitro* (Meng, unpublished). *Bacillus amyloliquefaciens* strain BAC03 (Patent No.: US 7,615,366 B2) was isolated from a soil naturally suppressive to potato common scab (Meng *et al.*, 2012b), and has shown biological control activity (Meng *et al.*, 2012a).

In this study, several materials were tested for their ability to manage common scab under controlled and field conditions. The objectives of this study were to evaluate five biologically derived materials, including chestnut tissue, clove and oregano essential oils, horseradish, and *B.*

amyloliquefaciens strain BAC03, for their ability to suppress scab either in potato or radish under greenhouse and field conditions.

MATERIALS AND METHODS

Preparation of bacterial inoculum

Streptomyces scabies ATCC49173 (from the American Type Culture Collection, and isolated from potato tuber) was cultured in oatmeal broth at 28°C for 4 to 5 days in an incubator shaker at 180 rpm (Loria *et al.*, 1995). The concentration of *S. scabies* in the liquid culture was determined by dilution plating on yeast malt extract (YME; EMB Chemical Inc., Gibbstown, NJ, USA) agar. *Bacillus amyloliquefaciens* strain BAC03 was cultured in tryptic soy broth (TSB; EMB Chemical Inc.) at 28°C for 48 h at 180 rpm. The concentration was determined by dilution plating on tryptic soy agar (TSA; EMB Chemical Inc.) plates.

***Bacillus amyloliquefaciens* BAC03 on scab and plant growth of radish.**

Radish ('Cherry Belle', Burpee Inc. Warminster, PA, USA) seeds were pre-germinated in a Petri dish with moist filter paper overnight at 25°C. After germination, seedlings were transplanted in a 1 L pot containing potting mix (ASB Greenworld Inc., New Brunswick, VA, USA) in a greenhouse (Michigan State University greenhouse facility, East Lansing, MI). Growth conditions in the greenhouse were around 18 to 22°C with a 14-h photoperiod supplemented by light at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$. *Bacillus amyloliquefaciens* strain BAC03 liquid culture was applied as a drench into the potting mix to a final concentration of 10^5 CFU cm^{-3} one week post seedling emergence. A liquid culture of *S. scabies* strain ATCC49173 was added to the potting mix by drenching to give a final concentration of 10^6 CFU cm^{-3} one week post

seedling emergence. There were four replicates for each treatment. Radishes were harvested and weighed 6 weeks post planting. Disease severity was determined by measuring the lesion severity using the 0 to 5 scale of Wanner (2004), where 0 = no disease and 5 = the whole root is covered by scab lesions. This study was conducted two times.

Effect of chestnut tissues on potato common scab in growth chamber

Chestnut tissues (shell/pellicle complex) were obtained from chestnut peeling process using a commercial brulage peeling line (Boema S. P. A., Neive, Italy), located at the Michigan State University Roger's Reserve, Jackson, MI. Potato seed tubers (cv. 'Snowden') were surface-disinfested with 1% NaClO for 5 min and rinsed with sterile distilled water three times. Potato seed tubers were air dried at room temperature and planted in 3.78 L pots containing mixtures of potting mix and chestnut tissues at 0:1, 1:1, and 1:0 (chestnut tissues:potting soil = vol:vol), with four replicates each. Growth chamber settings were 16 h of light and 8 h of dark, with light intensity of $200 \mu\text{mol m}^{-2} \text{s}^{-1}$, and constant temperature of 18°C. One week after seedling emergence, a liquid culture of *S. scabies* strain ATCC49173 was applied as a drench to the potting mix to give a final concentration of 10^6 CFU cm^{-3} potting mix. Potato tubers were harvested four months post planting, examined for lesions, and given a severity rating using a 0 to 5 scale (Hao *et al.*, 2009), where 0 = no symptoms, 1 = 1 to 10% surface area with superficial or raised lesions, 2 = 11 to 25% surface area with superficial or raised lesions, 3 = 26 to 50% surface area with superficial or raised lesions, 4 = more than 50% surface area with superficial or raised lesions or 6 to 25% pitted lesion area and 5 => 50% surface area with superficial or raised lesions or > 25% pitted area. The average disease score was calculated as $\Sigma (\text{score} \times \text{number of tubers with that score}) / \text{total number of tubers evaluated}$. The experiment was conducted twice.

Effect of oregano and clove essential oils on scab in potato and radish.

Commercial oregano essential oil (OE; Aura Cacia Inc., Urbana, IA, USA) and clove essential oil (CE; Aura Cacia Inc., Urbana, IA) were dissolved in dimethyl sulfoxide (DMSO; Krackeler Scientific Inc., Albany, NY, USA) as stock solutions (0.8 mg ml^{-1}) and stored at 4°C in the dark. Potato seed tubers (cv. 'Snowden') and germinated radish seedlings (cv. 'Cherry Belle'), prepared as described above, were sown in potting mix, immediately followed by drenching with clove or oregano essential oils, which resulted in final concentrations in potting mix of approximately $0.1 \text{ } \mu\text{g cm}^{-3}$. DMSO was used as a control. One week after seedling emergence a liquid culture of *S. scabies* strain ATCC49173 was applied as a drench to the potting mix to give a final concentration of 10^6 CFU cm^{-3} potting mix. Disease was rated six weeks after radish planting and four months after potato planting, with the criteria mentioned above (Wanner, 2004; Hao *et al.*, 2009). The experiments were conducted twice.

Effect of ground horseradish on potato common scab

Horseradish root (J.R. Kelly Inc., Collinsville, IL) purchased from fresh market was ground using a blender (Waring Laboratory Inc., Torrington, CT). The ground horseradish tissue was mixed into potting mix at 0, 0.18, 0.33, 0.91, and 1.8 g m^{-3} (dry weight) with four replicates each. One week after seedling emergence, a liquid culture of *S. scabies* strain ATCC49173 was added as described above. Disease ratings were conducted 4 months post planting at harvest (Hao *et al.*, 2009). The experiment was conducted twice.

Field trials

Field trials were conducted at the Montcalm Research Center (N43°21.148', W85°10.510', designated as MRC) at Lakeview, MI, and Michigan State University Soil Science

Research Center (N42°42.942', W84°27.953', designated as MSU) at East Lansing, MI in 2011 and 2012. The field trial was divided into four blocks (replications), with 12 rows (0.6 by 7.5 m per row) in each block. Six treatments were involved in the trial and two-row plots were arranged for each treatment in each block. The plots were arranged in a randomized complete block design. Twenty-two seed tubers were planted in each row with 30 cm spacing. Potatoes were planted on the 3rd and 27th of June in 2011, and the 22nd May and 5th of June in 2012. Scab-free (by visual evaluation for lesions) potato (cv. 'Snowden') seed tubers were planted along the furrow. Pesticides and fertilizers were applied following standard procedures used in commercial production.

Oregano essential oil (Aura Cacia Inc., Urbana, IA) was dissolved in DMSO and added to water to obtain a final concentration of 0.35% (vol/vol) for OE and 1.15% (vol/vol) for DMSO. Horseradish was cut into small pieces and ground using a blender as above. The final concentration was adjusted to 0.17 kg L⁻¹ (dry weight/water). Chestnut tissue (1.15 kg m⁻², dry weight), ground horseradish (38 g m⁻², dry weight), and oregano essential oil (1.5 ml m⁻²) were incorporated into the soil with a rototiller (Earthquake Inc., Cumberland, WI, USA) two weeks before planting. Soil was covered with a plastic sheet (Husky Inc., Grand Prairie, TX, USA) for two weeks, followed by planting potato seed tubers. A diluted BAC03 culture suspension [10^6 colony forming unit (CFU) ml⁻¹] in TSB was applied four times post planting at two-week intervals starting one month post planting. The BAC03 liquid culture was manually applied to each potato plant by slowly drenching in areas close to plant roots (0.25 m in diameter around the main stem, which resulted in a concentration of 1 L m⁻² at 10^6 CFU ml⁻¹). For a control

treatment, the same volume of TSB was applied as a drench using the same method.

Potatoes were harvested on the 19th of September and the 10th of October in 2011, and the 28th of September and the 22nd of October in 2012. Fifty tubers were randomly selected from each two-row plot for evaluation. Disease lesions on potato tubers were rated using the scale mentioned earlier (Hao *et al.*, 2009). For yield assessment, all potatoes in a two-row (3 m row⁻¹) plot were manually harvested and weighed.

Statistical analysis

Data were analyzed using SAS software (version 9.2, SAS Inc., Cary, NC, USA). Procedure GLM was used for the analysis of variance, and Fisher's least significant difference (LSD) multiple comparisons were performed for mean separation of yield where ANOVA showed significant differences. Non-parametric data analysis was used for disease analysis, and Kruskal-Wallis test was performed to compare mean values. Regression was performed for the relationship between horseradish concentration and disease score. Effect of horseradish on scab severity was analyzed using Spearman's rank correlation. If there was no interaction between repeated trials ($P > 0.05$), data were combined from all trials.

RESULTS

Effect of biomaterials tested in greenhouse or growth chamber

Bacillus amyloliquefaciens BAC03 significantly ($P < 0.05$) reduced the severity of radish scab. The average disease scores were 0.3 and 1 for BAC03 treatment and control, respectively (Table 5.1). In addition, application of *S. scabies* increased the fresh weight of radish leaves (14%) and roots (10%), but this increase was much less than application of *S. scabies* with BAC03 (126% and 166% for leaves and roots respectively, Table 5.1).

Disease severity on potato tubers was significantly reduced when chestnut tissues were incorporated in potting soil, and negatively correlated ($R^2 = 0.96$) with the amount of chestnut material applied (Table 5.2). This effect was observed in both trials.

In radish, oregano and clove essential oils significantly reduced disease severity ($P < 0.05$), and no disease was observed in potato under the oregano essential oil treatment (Fig. 5.1). Based on the results of greenhouse trials, oregano essential oil was selected for further study in the field.

Horseradish reduced common scab in the greenhouse. As the concentration of ground horseradish increased in potting soil, the severity of common scab decreased, and no disease was observed in the treatment with 1.8 g m^{-3} of horseradish (Fig. 5.2).

Table 5.1. Effect of *Bacillus amyloliquefaciens* BAC03 on scab (*Streptomyces scabies*) and plant fresh weight of radish in greenhouse

Treatment	Disease score	Fresh weight (g plant ⁻¹)	
		Leaf	Root
Non-treated	0 c **	5.7 ± 0.4 c	10.4 ± 0.4 c
<i>Streptomyces scabies</i> (10 ⁶ CFU cm ⁻³) *	1.0 ± 0.2 a	6.5 ± 0.4 b	11.4 ± 0.5 b
<i>Streptomyces scabies</i> (10 ⁶ CFU cm ⁻³) and BAC03 (10 ⁵ CFU cm ⁻³)	0.3 ± 0.2 b	12.9 ± 0.9 a	27.7 ± 0.8 a

* One week post seedling emergence, a liquid culture of *Streptomyces scabies* strain ATCC49173 was added to the potting mix by drenching to give a final concentration of 10⁶ CFU cm⁻³. Growth conditions in the greenhouse were around 18 to 22°C with a 14-h photoperiod supplemented by light at 200 μmol m⁻² s⁻¹.

** Values are means (average of 16 measurements) ± standard deviations. Multiple comparisons were performed using Fisher's least significant difference test at a significance level $\alpha = 0.05$. Values followed by same letters in a column are not significantly different. Disease severity was determined by estimating the lesion severity using the 0 to 5 scale of Wanner (2004), where 0 = no disease and 5 = the whole root is covered by scab lesions. This study was conducted two times.

Table 5.2. Effect of chestnut tissues on potato common scab in greenhouse

Ratio of chestnut tissue in potting mix (%)	Average disease score	
	24 May 2010	10 July 2010
0*	1.3 ± 0.1 a**	2.4 ± 0.3 a
50	0.7 ± 0.1 b	1.0 ± 0.2 b
100	0.3 ± 0.1 c	0.3 ± 0.1 c

* One week post seedling emergence, a liquid culture of *Streptomyces scabies* strain ATCC49173 was added to the mixture of potting mix and chestnut tissues by drenching to give a final concentration of 10^6 CFU cm^{-3} . Growth conditions in the greenhouse were around 18 to 22°C with a 14-h photoperiod supplemented by light at $200 \mu\text{mol m}^{-2} \text{s}^{-1}$.

** Values are means (average of 20 measurements) ± standard deviations. Multiple comparisons were conducted in each trial using Fisher's least significant difference test. Means with the same letters were not significantly different at $\alpha = 0.05$. Severity of common scab was evaluated using a 0 to 5 scale (Hao *et al.*, 2009), where 0 = no disease and 5 = 50% surface area of the tuber covered by scab lesions.

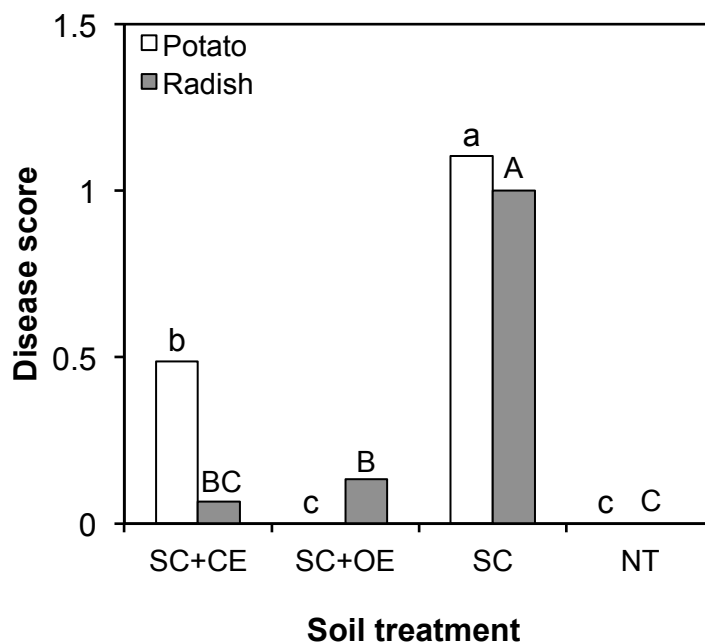


Figure 5.1. Effect of oregano essential oil (OE, $0.1 \mu\text{g cm}^{-3}$ soil) and clove essential oil (CE, $0.1 \mu\text{g cm}^{-3}$ soil) on scab in potato (open bars) and radish (filled bars). One week post seedling emergence potting mix was infested with *Streptomyces scabies* (SC, 10^6CFU cm^{-3}) except non-treated control (NT). Mean values were separated by lower-case (for potato) or capital (for radish) letters at significance level $\alpha = 0.05$. Each bar is the average of 15 to 25 of observations.

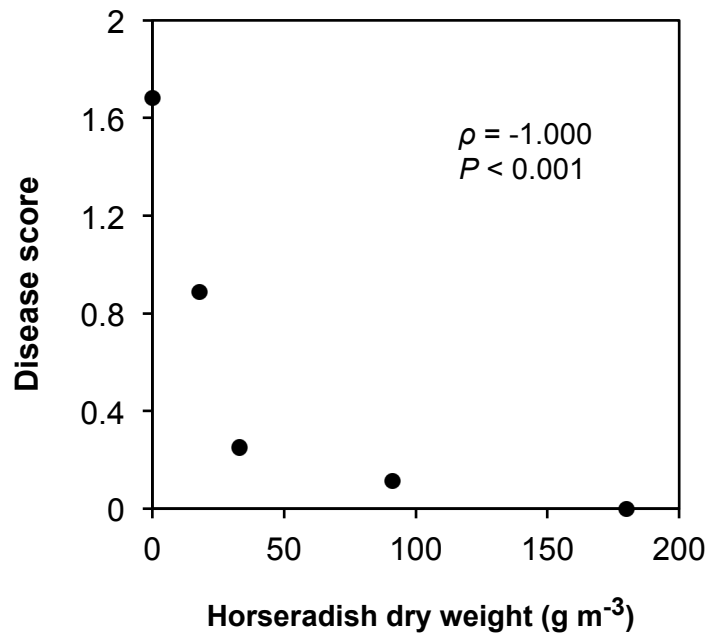


Figure 5.2. Effect of ground horseradish on potato common scab tested in the greenhouse. Ground horseradish was incorporated in potting mix at various concentrations (g m^{-3}). For regression analysis, the concentration was transformed to $[1/(\text{concentration} + 0.13)]$. A liquid culture of *Streptomyces scabies* strain ATCC49173 was added to the potting mix by drenching to give a final concentration of 10^6 CFU cm^{-3} potting mix one week post seedling emergence. Data represents two combined trials. Severity of common scab was evaluated using a 0 to 5 scale (Hao *et al.*, 2009), where 0 = no disease and 5 = 50% surface area of the tuber covered by scab lesions.

Field trials

There were interactions between year, location, and treatment ($P < 0.05$), therefore, the data were analyzed individually for each year and location. In the 2011 field trial at MRC, BAC03, oregano essential oil, and horseradish significantly reduced scab severity compared to the controls, with BAC03 (average disease score 0.6) and horseradish (0.8) having the greatest efficacy ($P < 0.05$). The disease severity under chestnut-tissue treatment was 1.1, lower than that in the TSB control, but not significantly different from the non-treated control ($P < 0.05$) (Fig. 5.3 A). In the MSU trial, BAC03 had significant reduction in common scab severity, with a disease score of 0.4 ($P < 0.05$). The effects of chestnut tissue, oregano essential oil, and horseradish were not significantly different from non-treated (Fig. 5.3 B). In the 2012 trial at both MRC and MSU, BAC03 was the only treatment that significantly reduced the disease severity of potato common scab, although it was not significantly different from the essential oil treatments at the MSU location (Fig. 5.3 C and D).

In the 2011 trial at MRC, *Bacillus amyloliquefaciens* strain BAC03 was the only treatment that displayed a yield promotion (with weight of 2.7 kg m^{-2}), compared to the TSB control (1.8 kg m^{-2}), although the ground horseradish also did so versus the untreated control (Table 5.3). The average tuber yields from treatments with chestnut tissue, oregano essential oil, and horseradish were not significantly different from the control ($P < 0.05$) (Table 5.3). Similar results for potato yield were observed at MSU, namely only BAC03 significantly increased the tuber weight ($P < 0.05$) (Table 5.3). In 2012 at MRC, potato tuber yield from the plot treated with BAC03 was higher than those from two other treatments, but not from the control (Table 5.3). On the MSU farm, there were no significant differences in yield observed among treatments with any of the different biomaterials (Table 5.3).

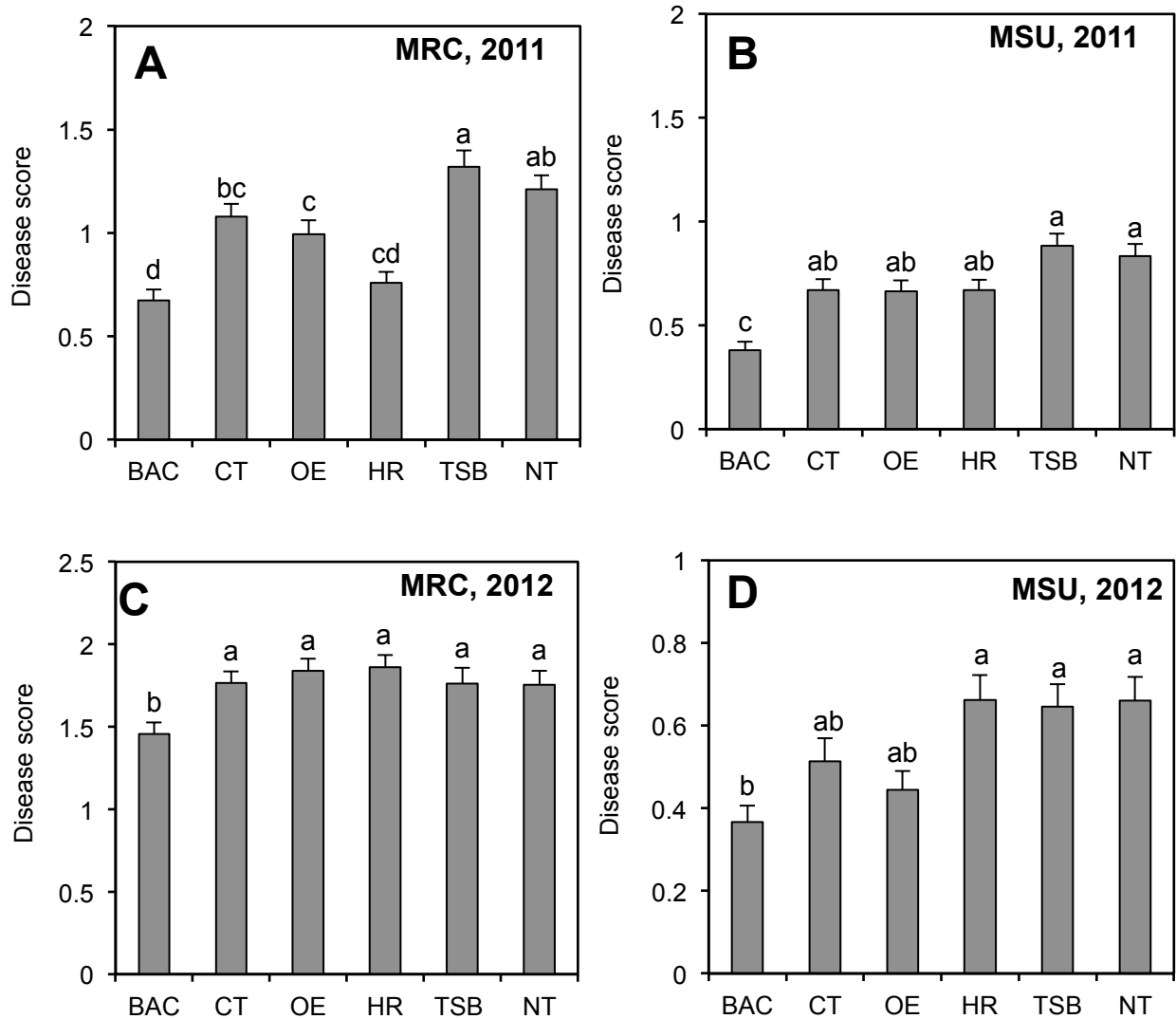


Figure 5.3. Effects of biologically based materials on potato common scab ratings in field trials at the Montcalm Research Center (MRC; A and C) and the Soil Science Research Center on MSU campus (MSU; B+D) in 2011 (A+B) and 2012 (C+D). Treatments included chestnut tissues (CT, 1.15 kg m⁻²), ground horseradish (HR, 0.38 g m⁻²), and oregano essential oil (OE, 1.5 ml m⁻²) mixed into the soil two weeks before potato planting, or a liquid culture of *Bacillus amyloliquefaciens* strain BAC03 [BAC, 1 L m⁻², 10⁶ colony forming units (CFU)] in tryptic soy broth (TSB), TSB only as a control, and a non treated control (NT). Severity of common scab was evaluated using a 0 to 5 scale (10), where 0 = no disease and 5 = 50% surface area of the tuber covered by scab lesions. Bars on each column are standard errors. Values of the bars with the same letters were not significantly different tested by Fisher's least significant difference at significance level $\alpha = 0.05$. Each bar is the average of 200 observations.

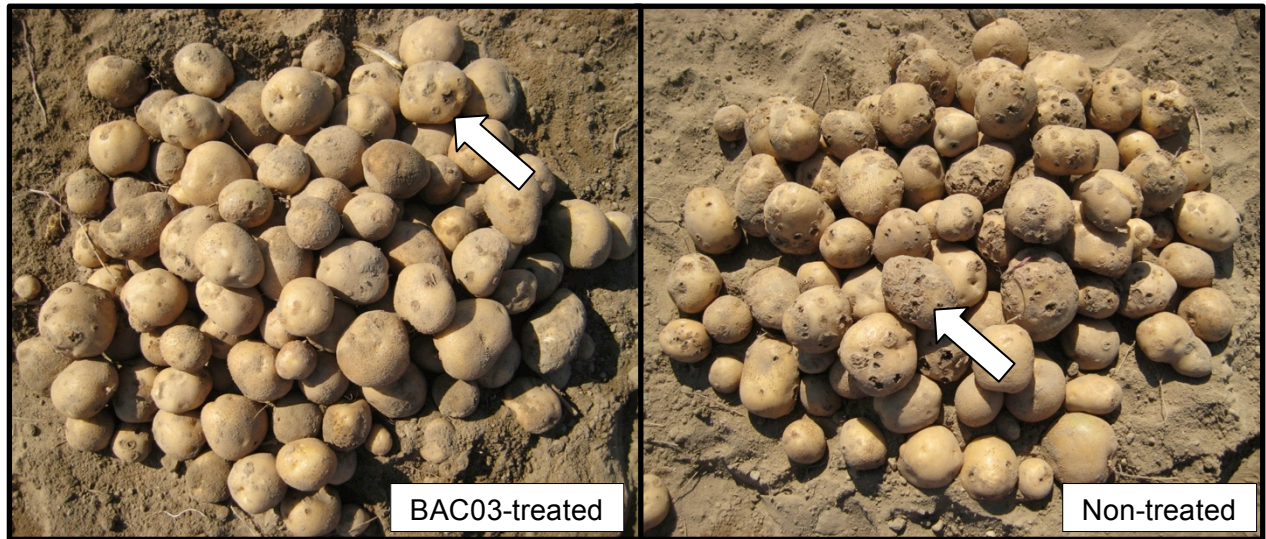


Figure 5.4. Comparison of potato tubers harvested at Montcalm Research Center where soil was naturally infested with *Streptomyces* spp. Left panel: soil treated with *Bacillus amyloliquefaciens* strain BAC03 in tryptic soy broth, and right panel: soil treated with tryptic soy broth only. White arrows indicate scab lesions.

Table 5.3. Effects of *Bacillus amyloliquefaciens* (BAC03), chestnut tissue, ground horseradish, and oregano essential oil on potato yield at Montcalm Research Center (MRC), Lakeview, and Michigan State University Soil Science Research Center (MSU), East Lansing in Michigan in 2011 and 2012

Year	Location	Treatment	Rate of application	Yield (kg m ⁻²)	
2011	MRC	BAC03 (10 ⁶ CFU ml ⁻¹)	1 L m ⁻²	2.7 ± 0.4 a*	
		Chestnut tissue	1.15 kg m ⁻²	1.9 ± 0.2 abc	
		Ground horseradish	38 g m ⁻²	2.6 ± 0.7 ab	
		Oregano essential oil	1.5 ml m ⁻²	2.3 ± 0.3 abc	
		Tryptic soy broth (TSB)	1 L m ⁻²	1.8 ± 0.5 bc	
		Non-treated	-	1.7 ± 0.7 c	
		MSU	BAC03 (10 ⁶ CFU ml ⁻¹)	1 L m ⁻²	7.3 ± 0.4 a
	Chestnut tissue		1.15 kg m ⁻²	5.6 ± 1.2 bc	
	Ground horseradish		38 g m ⁻²	6.2 ± 1.3 abc	
	Oregano essential oil (0.35% v/v)		1.5 ml m ⁻²	7.0 ± 0.6 ab	
	TSB		1 L m ⁻²	5.4 ± 0.7 c	
	Non-treated		-	5.5 ± 1.2 bc	
	2012		MRC	BAC03 (10 ⁶ CFU ml ⁻¹)	1 L m ⁻²
		Chestnut tissue		1.15 kg m ⁻²	3.4 ± 0.2 b
Ground horseradish		38 g m ⁻²		3.6 ± 0.3 b	
Oregano essential oil (0.35% v/v)		1.5 ml m ⁻²		3.8 ± 0.3 ab	
TSB		1 L m ⁻²		4.0 ± 0.5 ab	
Non-treated		-		3.9 ± 0.5 ab	
MSU		BAC03 (10 ⁶ CFU ml ⁻¹)		1 L m ⁻²	2.4 ± 0.2 ^{ns}
		Chestnut tissue	1.15 kg m ⁻²	2.1 ± 0.4	
		Ground horseradish	38 g m ⁻²	2.1 ± 0.3	
		Oregano essential oil (0.35% v/v)	1.5 ml m ⁻²	1.9 ± 0.3	
		TSB	1 L m ⁻²	2.0 ± 0.1	
		Non-treated	-	1.9 ± 0.5	

* Multiple comparisons were performed for each year using Fisher's least significant difference test at significance level $\alpha = 0.05$. Values followed by same letters for a given location and year are not significantly different. ^{ns} no significant differences.

DISCUSSION

In this research, different biologically-based materials were assessed for their efficacies in suppressing scab caused by *Streptomyces* spp. in potato or radish. The results indicated that although application of chestnut tissue, oregano essential oil, and horseradish showed disease inhibition in the greenhouse or growth chamber condition, their efficacies varied in the field depending on years and locations. *Bacillus amyloliquefaciens* BAC03 had a consistent effect on scab reduction in both greenhouse and field condition.

Field soil is a complex environment that contains many factors we cannot completely control (Lazarovits *et al.*, 2007; Dees and Wanner, 2012). Development of potato common scab is not only affected by the pathogen population, but also by physical (Sturz *et al.*, 2004; Lazarovits *et al.*, 2007) and other biological factors (Rosenzweig *et al.*, 2012; Larkin and Tavantzis, 2013), such as soil properties (Lazarovits *et al.*, 2007), plant varieties (Haynes *et al.*, 2010; Weinert *et al.*, 2011), and cultivation strategies (Wiggins and Kinkel, 2005; Larkin, 2008). In the current study, various disease pressures and effects of disease management in different years and locations likely reflect this complexity. For example, the average disease scores at MRC farm were around 1.2 and 1.8 (in the control) in 2011 and 2012, respectively. Also, there was a difference in disease pressure between different locations; for instance, the average disease scores in the control were around 1.8 and 0.6 at MRC and MSU farm in 2012, showing an almost three-fold difference. Furthermore, in comparison to scab only caused by *S. scabies* under the inoculated greenhouse conditions, the backgrounds of *Streptomyces* spp. in the field condition are more complex and could include multiple species of pathogenic *Streptomyces* spp. (Wanner, 2006; Flores-Gonzalez *et al.*, 2008). All above factors could affect the outcomes of disease development under natural conditions and make disease management more difficult (Abbasi *et*

al., 2006; Lazarovits *et al.*, 2007; Dees and Wanner, 2012). Similar results have been observed by other researchers. For example, Abbasi *et al.* (2006) reported treatment with 1% fish emulsion reduced scab severity in the field in 2004, but not in 2005, and concluded that fish emulsion was not effective in soils with high disease pressure (Abbasi *et al.*, 2006). Lazarovits *et al.* (1999) reported amendment with soymeal, as well as meat and bone meal had an ability to inhibit potato scab in the first two years of testing, while no effect was observed in the third year. In addition, application of these biomaterials had a negative impact on potato yield. Therefore, developing a commercial product with consistent efficacy for common scab management is a challenging job and requires testing under diverse conditions.

Treatments with oregano essential oil, chestnut tissues, and horseradish all have demonstrated antimicrobial and biocontrol activities in the laboratory and under controlled environment in this and previous work (Bi *et al.*, 2012; Hao *et al.*, 2012). They could be an addition to currently available strategies for disease management, especially organic agricultural production. Chestnut tissues are by-products of the peeling processing. Using them for disease control can add value to chestnut production, and the economics can be improved (Bounous *et al.*, 2001). As shown in the field results, these three products were less effective compared to BAC03, and they were not as consistent as BAC03 (Fig. 5.3), some efficacy was observed in the greenhouse trial. Further studies are needed to optimize their efficacies possibly by understanding application conditions or other factors on activity.

Among the biomaterials tested in this study, *B. amyloliquefaciens* strain BAC03 has shown a promising result in reducing potato common scab, as well as enhancing the yield of potato and radish either in greenhouse test or field trials. The field results for disease control were significant and consistent in two locations for two years. This is an important step for the

commercialization of BAC03, and important in potato production where effective strategies of common scab management are not satisfied (Wharton *et al.*, 2011; Dees and Wanner, 2012). *Bacillus* spp. are a well known group of bacteria for biological control and plant growth promotion (Gueldner *et al.*, 1988; Turner and Backman, 1991; Lugtenberg and Kamilova, 2009). In a previous study, it has been demonstrated to have antimicrobial activities against *Streptomyces* spp. in the lab (Meng *et al.*, 2012a). Strain *Bacillus* sp. sunhua was reported to inhibit *S. scabies in vitro*, but without field validation for the biological control activity (Han *et al.*, 2005). Schmiedeknecht *et al.* (1998) reported application of several *Bacillus* strains reduced common scab up to 70% in the greenhouse, and up to 67% in the field. In addition, the yields of potato tubers were higher (up to 16 %). Most of *Bacillus* isolates with promising biological control or plant growth promotion activity are still at research stage and need to be further studied and commercialized to apply in the production (McSpadden Gardener and Fravel, 2002b; Borriss, 2011).

In conclusion, this research has indicated BAC03 can be a good biological control agent for potato common scab management. Horseradish, oregano essential oil, and chestnut tissues displayed some biological control activity in the greenhouse. Further investigation of these biomaterials is needed to conduct, such as optimization of application method, determination of spectrum against other soilborne pathogens, and effects assessment on plant growth promotion.

APPENDIX

APPENDIX: CULTURE BASED ASSESSMENT OF MICROBIAL COMMUNITIES IN SOIL SUPPRESSIVE TO POTATO COMMON SCAB

This chapter has been published:

Meng, Q.X., Yin, J.F., Rosenzweig, N., Douches, D., and Hao, J.J. (2012) Culture based assessment of microbial communities in soil suppressive to potato common scab. *Plant Disease*, 96:712-717.

ABSTRACT

A field in East Lansing, Michigan showed a decline of potato common scab compared to an adjacent potato field. To test whether the decline was due to biological factors, the soil was assayed. In the greenhouse, putative common scab suppressive soil (SS) was treated either with various temperatures, or mixed with autoclaved SS at different ratios. Pathogenic *Streptomyces scabies* was incorporated into the treated soil at 10^6 colony forming units g^{-1} soil, followed by planting of either potato or radish. Disease severity was negatively correlated with the percentage of SS in the mixture, and positively correlated with temperature above 60°C. The soil was screened for potential antagonists by pairing isolates from soil in groups of general bacteria, streptomycetes, fluorescent pseudomonads, and bacilli in culture with *S. scabies*. The frequency of antagonistic bacteria in SS was higher than in common scab conducive soil (CS), but only pseudomonads and streptomycetes were significantly higher. The population of pathogenic *Streptomyces* spp. in the rhizosphere of CS was significantly higher than in SS. Dilution plating of CS and SS samples showed no clear trends or differences in populations of total fungi, total bacteria, streptomycetes, fluorescent pseudomonads, and bacilli, but terminal restriction fragment polymorphism analysis revealed two distinct microbial communities were present in SS and CS.

INTRODUCTON

Potato common scab (PCS) is caused by *Streptomyces* spp (Loria *et al.*, 1997). Since it was first reported in North America in the late 19th century, the disease has been found worldwide wherever potatoes are grown (Lindholm *et al.*, 1997; Park *et al.*, 2003b; Wanner, 2006). *Streptomyces* spp. are persistent soil inhabitants that survive saprotrophically on plant debris for long periods (Wharton *et al.*, 2011). Usually the disease affects tuber quality by causing superficial, raised or pitted lesions on the periderm (Loria *et al.*, 1997; Loria *et al.*, 2006). In some cases, potato yield can be reduced due to severe infection (Loria *et al.*, 1997). PCS threatens the \$3.5 billion potato industry in the US (USDA, 2010) due to lack of effective control methods (Hiltunen *et al.*, 2009).

Management of PCS is difficult (Wharton *et al.*, 2011; Dees and Wanner, 2012). Scab resistant cultivars are the ultimate goal, but current commercial varieties have only partial resistance or tolerance (Wanner and Haynes, 2009). The only widely used chemical, pentachloronitrobenzene (Davis *et al.*, 1976) has been withheld in the US recently by the Environmental Protection Agency due to its carcinogenicity and non-degradability in soil (EPA website: <http://www.epa.gov/>). Increasing irrigation intensity (Lapwood *et al.*, 1973) and lowering soil pH (Lambert and Manzer, 1991) are partially effective in reducing the disease severity, but lower pH may also result in yield reduction (Hiltunen *et al.*, 2009). Cultural practices, such as organic soil amendments, green manures, and crop rotation, have been shown to reduce PCS by improving the beneficial soil microbial community (Wiggins and Kinkel, 2005; Larkin, 2008).

Naturally occurring disease suppressive soils provide a valuable resource for studying beneficial microbial communities (Borneman and Becker, 2007). A number of soils have been

reported as suppressive against plant diseases, including Fusarium wilt (*Fusarium oxysporum*), Pythium damping-off (*Pythium* spp.), Rhizoctonia root rot (*Rhizoctonia solani*), take-all (*Gaeumannomyces graminis* var. *tritici*), and PCS (*Streptomyces* species) (Kao and Ko, 1986; Larkin *et al.*, 1996; Weller *et al.*, 2002; Haas and Defago, 2005). In the 1950s, Menzies observed that PCS disease severity was almost zero in a field after many years of potato monoculture in central Washington, and determined the suppressiveness was biological (Menzies, 1959). Decline of common scab with potato monoculture was also identified in fields at Grand Rapids, Minnesota and Becker, Minnesota (Lorang *et al.*, 1989; Liu *et al.*, 1995). In these systems, non-pathogenic *Streptomyces* spp. are believed to play a major role in disease suppressiveness (Lorang *et al.*, 1995; Liu *et al.*, 1997). Elucidating the mechanisms for suppressiveness may help to improve plant health through manipulating soil microbial communities.

In recent years, the authors have observed a decline in PCS in a potato field near the campus of Michigan State University, East Lansing, Michigan and decided to evaluate the soil microbial communities potentially involved in the disease suppressiveness of these fields. Various techniques are available to enumerate, identify, and culture microorganisms, such as selective media-based methods (Tsao, 1970), and molecular approaches surveying the entire microbial community present in a soil (van Elsas *et al.*, 2008). The main objectives of this work were to determine whether the soil was suppressive to PCS, characterize the structure of microbial communities likely contributing to the disease suppressiveness, and culture specific microorganisms or microbial communities that might control common scab. A preliminary report has been published (Meng *et al.*, 2009). A related study using pyrosequencing to characterize soil microbial communities will be reported separately (Rosenzweig *et al.*, 2012).

MATERIALS AND METHODS

Field history and soil properties

A field (N42°43.014'; W84°27.972') near the campus of Michigan State University in East Lansing, MI, has been cultivated with potato consecutively for more than 25 years. This field, designated as the SS field, was used as a scab nursery for potato variety evaluation. Because the disease declined gradually over several years, the varietal test was moved to a new field (N42°42.937'; W84°27.975') in 2005, designated as the CS field, in the same area separated by a distance of 200 meters. This field showed higher disease pressure of PCS according to disease ratings from the past 5 years. Soil samples collected from SS and CS fields were analyzed for physical and chemical properties at the Michigan State University's Soil and Plant Nutrient Laboratories using different methods for each element as recommended for the North Central Region. The soil type from both SS and CS fields is loamy sand (Table S6.1).

Field rating of PCS

The SS and CS fields were divided into 50 quadrants (5 x 10) with 4 x 7 m² per quadrant. Potatoes ('Snowden') were harvested and the tubers scored for common scab disease. Fifty tubers were arbitrarily selected from each plot for disease evaluation. Disease lesions on potato tubers were scored as previously described (Hao et al., 2009) with slight modification: 0 = no symptoms, 1 = 1 to 10% surface area with superficial or raised lesions, 2 = 11 to 25% surface area with superficial or raised lesions, 3 = 26 to 50% surface area with superficial or raised lesions, 4 => 50% surface area with superficial or raised lesions or < 25% pitted lesion area, and 5 => 50% surface area with superficial or raised lesions or > 25% pitted area. The disease severity index was calculated as $\sum(\text{score} \times \text{number of tubers with that score}) / \text{total number of}$

potato tubers evaluated. Scab ratings were conducted for both SS and CS after harvest in 2007 and 2008.

Table S6.1. Physical and chemical properties of disease conducive and suppressive soils

Soil character	Disease suppressive soil	Disease conducive soil
Soil pH	6.8	6.2
P (ppm)	378.0	389.0
K (ppm)	252.0	146.0
Mg (ppm)	129.0	63.0
Ca (ppm)	720.0	383.0
CEC (meq/100g)	5.3	4.0
Exchangeable K (%)	12.1	13.3
Exchangeable Mg (%)	20.2	18.7
Exchangeable Ca (%)	67.7	68.0
Soil type	Loamy sand	Loamy sand

Greenhouse assay of soil suppressive to scab diseases of radish and potato

Pathogen inoculum was prepared following the method described by Wanner (Wanner, 2004) with slight modification. *Streptomyces scabies* strains 1019 from Michigan, and 49173 from the American Type Culture Collection were cultured on yeast malt extract agar (YME, EMB Chemical Inc., Gibbstown, NJ, USA) incubated in the dark at 28°C for 5 days or until spores were produced. A spawn bag (19"× 8"× 5", Fungi Perfecti, LLC, Olympia, Washington, USA) was filled with 2000 cm³ of vermiculite (medium size, premium grade, Sun Gro Horticulture Distribution Inc., Bellevue, Washington, USA) and 200 ml water per bag, and autoclaved for 1 h twice in 24 h. Spore suspensions (10⁷ CFU ml⁻¹), determined by enumerating

colonies on YEM plates of *S. scabies*, were prepared by adding 10 ml sterile distilled water to culture plates and scraping colonies using a sterile scalpel. A mixture (1:1 in volume, 10 ml of each) of *S. scabies* isolates 1019 and 49173 was added to 180 ml of 2x Say solution (Hao *et al.*, 2009), which was then mixed into the sterile vermiculite in the spawn bags. The vermiculite inoculum bags were incubated at 28°C, and mixed every other day by shaking. To determine the inoculum density, 1 cm³ of vermiculite with *S. scabies* was transferred into 9 ml sterile distilled water. Serial dilutions were prepared and plated (100 µl) on YME agar. After 5 days of incubation in the dark at 28°C colonies of *S. scabies* were enumerated.

In a greenhouse, cut seed tubers of potato (‘Atlantic’) were placed in soil infested with *S. scabies* (see below) in 6-liter pots. Fertilizer (N:P:K ratio = 24:8:16, Scotts Miracle Gro Products, Inc., Marysville, OH, USA) was applied once every other week post seedlings were 2.5 cm high. There were four replications (pots) for each treatment. Growth conditions in the greenhouse were 24°C with a 14 h photoperiod, supplemented by 200 µmoles m⁻² s⁻¹ light provided by cool white fluorescent lamps. Plants were watered with 100 to 200 ml per pot every 2 to 3 days. Four months post planting, potato tubers were harvested and evaluated visually for scab symptoms with the method mentioned above.

Radish is another important host of *Streptomyces* spp. and is often used for pathogenicity tests as it has a shorter life cycle (Wanner, 2004). Radish (‘Cherry Belle’) seeds were planted 0.5 cm below the soil surface grown in the greenhouse in one-liter pots at the same conditions as described above for potatoes. After seedlings had germinated, plants were thinned to six plants per pot (evenly spaced) and watered with 100 ml per pot daily. Radish plants were fertilized in the same way as the potato plants 3 weeks post planting and harvested 6 weeks later. There were

four replications (pots) for each treatment. Radish hypocotyls were scored on a scale of 0 to 5, as described by Wanner (2004).

Effect of soil mixtures on disease suppression of SS.

Soil was randomly collected from five locations at the SS field and mixed together. Half of the SS was autoclaved twice (120 min for each) in 24 h, and mixed with non-autoclaved SS at the volume ratios of (SS: autoclaved SS): 100:0, 80:20, 40:60, 20:80, and 0:100 for the first trial; and 100:0, 75:25, 50:50, 25:75, and 0:100 for the second trial. *Streptomyces scabies* inoculum in vermiculite was mixed into the soil thoroughly and the final inoculum level in the soil was adjusted to 10^6 colony forming unit (CFU) cm^{-3} soil. There were four replications (pots) for each treatment. The trials, corresponding to two sets of volume ratios, were tested on both potato and radish.

Effect of heating temperature on disease suppression of SS.

SS was sampled with the same method described above, placed in high-temperature resistant bags (Associate Bag, LLC, WI, 10 liter per bag) and treated at the following temperatures: 40, 60, 70, 80, and 90°C in a water bath for 30 min starting when the center of the soil bag reached the expected temperature, measured with a metal temperature probe (Nova-tech International Inc, TX). An additional bag was autoclaved (121°C) for 30 min. In a second trial, SS was sampled, bagged as before and treated at the following temperatures: 30, 45, 60, 75, and 90°C for 30 min in a water bath, and 121°C (autoclaving) for 30 min. There were four replications (pots) for each treatment. For both trials, after the temperature treatment, the soil was mixed with *S. scabies* inoculum in vermiculite at the final concentration of 10^6 CFU cm^{-3} mixed soil. Non-treated and autoclaved soil without infestation was used as control. The two temperature treatment regimes were tested on both potato and radish.

Microbial communities assayed by dilution plating.

Soil samples were collected from CS and SS fields. Bulk soil was collected in the area between plants. Sample volumes consisted of about two liters, around 5 trowels full, taken from the top 15 cm soil, at 5 random locations. After transporting to the laboratory, the soil was mixed thoroughly in a plastic bag. Ten grams of each soil sample were suspended in 90 ml of sterilized phosphate-buffered saline in 125 ml Erlenmeyer flasks and shaken for 20 min at 180 rpm. For rhizosphere soil sampling, potato roots were dug out and placed in a plastic bag. In the laboratory, the roots were hand shaken loosely, and soil attached to the root surface was used.

Suspensions were serially diluted and 100 μl of each soil dilution spread onto 3 replicate plates of four different semi-selective agar media. The media used and the target microbial communities were as follows: rose bengal agar (EMD Chemicals, Gibbstown, NJ) with chloramphenicol (0.1g L^{-1}) for total fungi, one-tenth-strength tryptic soy agar (TSA, EMD chemicals) for total bacteria, S1 (Tarnawski *et al.*, 2003) for florescent pseudomonads, and *Streptomyces*-selective (Marques *et al.*) medium (Conn *et al.*, 1998) for streptomycetes. TSA was also used to culture *Bacillus* spp. from soil dilutions heated to 80°C for 30 min before plating. All plates were incubated at room temperature ($22 \pm 1^{\circ}\text{C}$) and enumerated after 2 days (total bacteria, *Bacillus*, fluorescent pseudomonads), 3 days (fungi) or 7 days (*Streptomyces* spp.).

Microbial communities assayed by T-RFLP.

Soil was collected as described above from both CS and SS fields, and each mixed thoroughly. Half a gram of soil from each sample was used for total soil genomic DNA extraction, using the FastDNA Spin Kit for soil (MP Biomedicals, Solon, OH, USA) following the manufacturer's instructions. Polymerase chain reaction (PCR) was conducted with primers

FAM-63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') labeled with 6-carboxyfluorescein, and 1387R (5'-GGG CGG WGT GTA CAA GGC-3') (Marchesi *et al.*, 1998), which are universal primers amplifying the 16S rDNA gene of bacteria. Each PCR reaction had a total volume of 25 μ l, containing 5 U Taq DNA polymerase, 5 \times Taq polymerase PCR buffer (Promega, Madison, WI, USA), 200 mM dNTP mixture, 0.2 mM of each primer, and 1 μ l (2 to 25 ng/ μ l) of template DNA. Amplifications were performed on a thermocycler with an initial denaturation step of 5 min at 94°C, followed by 36 cycles of 40 s at 94°C, 40 s at 58°C, and 1.5 min at 72°C, and a final extension of 7 min at 72°C. After confirmation by electrophoresis on a 1.2% (w/v) agarose gel, PCR products were purified using the PCR Purification Kit (Denville Scientific Inc, Metuchen, NJ, USA), and digested with *Rsa* I and *Msp* I enzymes (New England Biolabs Inc, Ipswich, MA) following the manufacturer's instructions and purified by ethanol precipitation. Restriction digests were separated by capillary electrophoresis using an ABI 3130 xl DNA analyzer and data analyzed using GeneScan 3.7 software (Applied Biosystems, Carlsbad, CA) to determine digested fragment sizes.

Frequency of antagonistic bacteria against *S. scabies*.

Isolates of *Bacillus*, fluorescent *Pseudomonas*, *Streptomyces*, and total bacteria, isolated from dilution plating (as described above), were tested for antagonism against *Streptomyces scabies in vitro* using a co-plate assay (Yoshida *et al.*, 2001). A spore suspension of *S. scabies* strain 1019 was prepared as described above. Antibiosis assays were done as follows: a spore suspension (100 μ l) of strain 1019 with the concentration of 10^5 CFU ml⁻¹ (determined by dilution plating) was spread on a YME agar plate and allowed to air-dry in a laminar flow hood. Different isolates growing on the selective media (as described above) were selected to culture with *S. scabies* strain 1019. Ten isolates were tested on each plate, and replicated three times.

Plates were incubated at 28°C for more than 3 days, and the number of antagonists were recorded. This trial was conducted over three years, from 2007 to 2010.

Frequency of pathogenic *Streptomyces* spp.

Streptomyces spp. from SS and CS were isolated by dilution plating on STR media (as described above). From each soil (including bulk and rhizosphere soil) 150 isolates were arbitrarily selected. These isolates were purified and grown on YME agar for 7 to 10 days. Potato tubers were sliced and cut into disks 0.5 cm high, and 2.0 cm in diameter. The disks were placed on moistened filter paper in Petri plates. Mycelial plugs (5 mm) of *Streptomyces* spp. isolates were placed in the center of the tuber disk (Loria *et al.*, 1995). Non-inoculated YME agar plugs were used as controls. Necrotic lesions on potato disks around the *Streptomyces* inoculum were observed and measured at room temperature after 3 to 5 days in the dark.

Statistical analysis

Data were analyzed using SAS software (Version 9, SAS Inc., Cary, NC). Procedure GLM was used for analysis of variance, and Fisher's least significance difference was performed for mean separation. Procedure REG was used for linear regression. The Spearman rank correlation was used to analyze disease and plant growth response to temperatures. T-RFLP Cluster analysis was performed using the R-statistical package (v. 2.10; Revolution Analytics, Palo Alto, CA). If there was no interaction between repeated trials ($P > 0.05$), data were combined from all trials.

RESULTS

Field rating of PCS disease severity

Scab ratings of the SS field were 1.43 ± 0.45 in 2007, and 1.55 ± 0.56 in 2008, respectively, which were significantly lower ($P < 0.05$) than scab ratings from the CS field, which ranged from 2.6 ± 0.62 in 2007 and 2.6 ± 0.54 in 2008, respectively.

Effect of soil mixtures of suppressive and autoclaved soils on PCS disease.

Disease suppressiveness was measured by assessing the disease severity both in potato and radish planted in a soil mixture of untreated natural SS and autoclaved SS. As the percentage of the SS increased, the disease severity decreased in both potato and radish (Fig. S6.1, A1 and B1). No common scab symptoms were observed in potatoes planted in 100% SS (Fig. S6.1, A1). This relationship fit a simple linear regression model with $R^2 = 0.95$ ($P < 0.05$) (Fig. S6.1, A1) and $R^2 = 0.92$ ($P < 0.05$) (Fig. S6.1, B1). Plant fresh weight was positively correlated ($R^2 = 0.93$, $P < 0.05$) with the percentage of SS: higher radish weights were obtained from higher percentages of SS, and vice versa (Fig. S6.1, C1).

Effect of soil temperature treatments on PCS disease suppression.

Temperature treatment impacted disease suppression in both potato and radish. Disease severity showed a positive relationship with the temperature used for soil treatment. Disease suppression was significantly reduced by temperatures above 60°C (Fig. S6.1, A2 and B2). The values of Spearman's rho (ρ) were 0.996 ($P < 0.01$) for potato, and 0.904 ($P < 0.025$) for radish. Plant fresh weight was negatively correlated with temperature treatments: higher radish weights were obtained from soil treated with lower temperatures, and vice versa (Fig. S6.1, C2. $\rho = -0.904$, $0.01 < P < 0.025$).

Microbial communities assayed by dilution plating.

Analysis from eight sampling times gave mixed results (Table S6.2). There were no clear trends in microbial populations among CS and SS. In most trials, SS had higher population numbers of total fungi, total bacteria, streptomycetes, fluorescent pseudomonads, and bacilli in most soil samples tested. However, the majority of these differences were not statistically significant ($P < 0.05$) within the same sampling dates (Table S6.2).

Microbial communities assayed by T-RFLP.

Analysis of T-RFLP fragments digested with *Rsa* I and *Msp* I resulted in two major clusters for SS and CS (Fig. S6.2), indicating two distinct microbial communities inhabited these two types of soils. The variation among samples from the same field was low.

Frequency of antagonistic bacteria against *S. scabies*.

A total of 5,285 single bacterial colonies were obtained from semi-selective media plates and included isolates representing total bacteria, fluorescent pseudomonads, streptomycetes, and bacilli. All together, 961 (18%) isolates including 573 and 388 isolates from SS and CS respectively, exhibited antagonism against *S. scabies* (Fig. S6.3). In all four groups sampled from 2007 to 2010, the frequency of antagonistic bacteria in SS (0.27, 0.19, 0.49, and 0.24 for bacilli, total bacteria, pseudomonads, and streptomycetes, respectively) was higher than CS (0.23, 0.13, 0.30, and 0.018 for bacilli, total bacteria, pseudomonads, and streptomycetes, respectively), but only differences in pseudomonad and streptomycete frequencies were significant ($P < 0.05$) (Fig. S6.3).

Frequency of pathogenic *Streptomyces* spp.

The frequency of pathogenic streptomycetes was higher in the rhizosphere than in the bulk soil. Rhizosphere CS had a significantly higher frequency of pathogenic streptomycetes

than SS. Moreover no difference in the frequency of pathogenic streptomyces from bulk soil was found between SS and CS (Fig. S6.4).

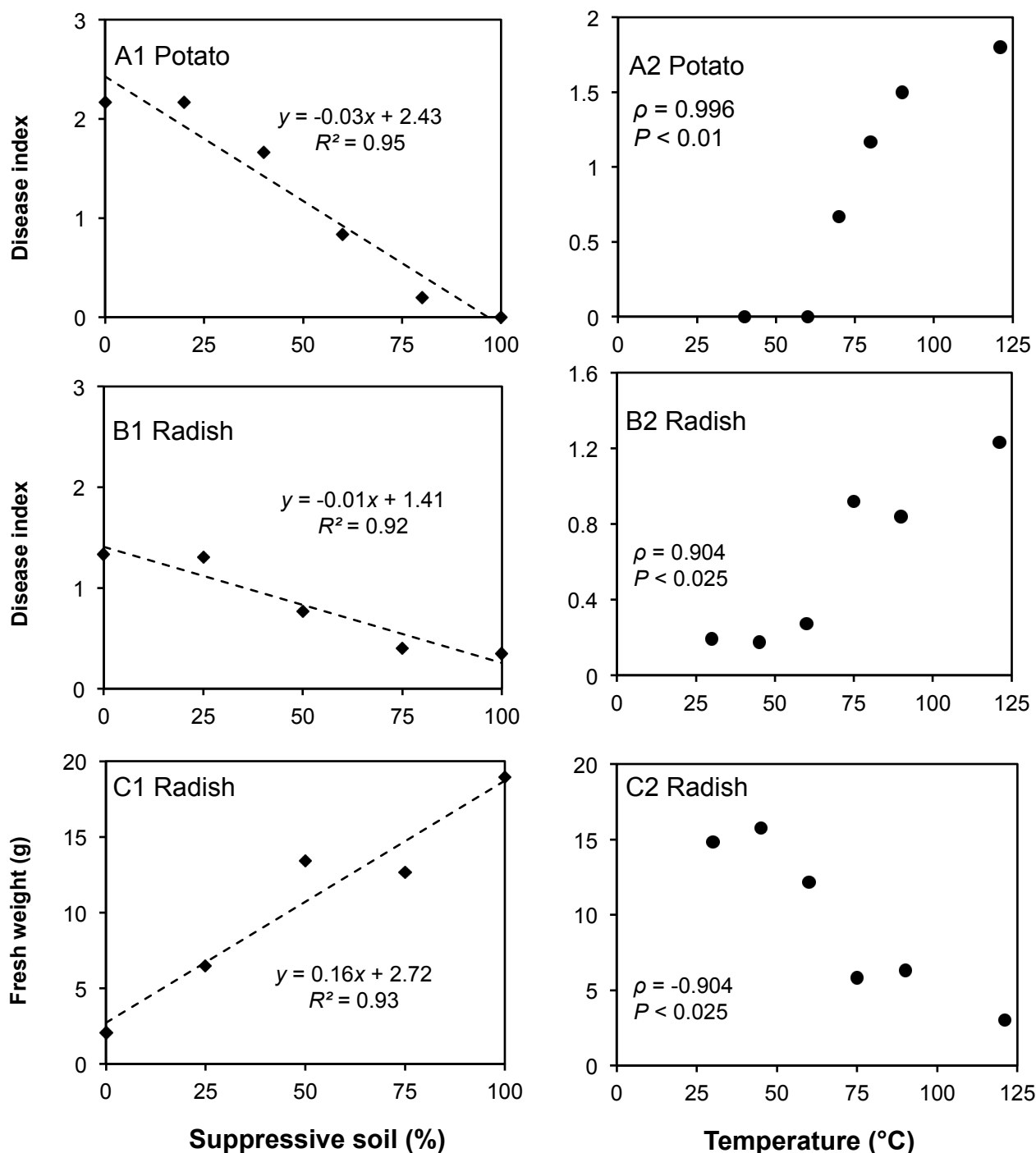


Figure S6.1. Characterization of disease suppressive (SS) soil in potato and radish in the greenhouse. SS was either mixed with autoclaved SS at various portions (A1, B1, C1), or treated with various temperatures for 30 min (A2, B2, C2), followed by infesting the soil with *Streptomyces scabies* at final concentration 10^6 colony forming unit/g soil. Either potato (A1 and A2) or radish (B1 and B2) was seeded in the treated soils in a pot. Disease was rated and PCS index was calculated according to published procedures (Wanner, 2004; Hao *et al.*, 2009). Weight of fresh radish roots was measured in both trials (C1 and C2)

Table S6.2. Microbial populations in disease suppressive (SS) and conducive (CS) soils detected by dilution plating on semi selective media

Microorganism	Soil	Date of soil sampling								Average
		2006	2007	2008			2009	2010		
		1/17	4/10	7/10	4/22	8/6	12/4	7/3	5/13	
Total fungi	SS	4.87 ^w	5.09	6.29b ^x	5.13	4.70b	4.68	5.11	5.59a	5.18
	CS	4.82	5.22	6.63a	5.03	5.04a	4.65	5.26	5.24b	5.24
Total bacteria	SS	6.95	7.5b	7.70	5.57	5.60	5.54	6.79	6.84a	6.56
	CS	6.99	7.84a	7.68	5.44	5.58	5.47	6.86	6.45b	6.54
Streptomyces	SS	5.50	6.08b	6.99	5.42	5.19a	5.40a	5.45	5.76	5.72
	CS	5.24	6.56a	7.03	5.19	4.73b	5.27b	5.49	5.65	5.65
Fluorescent pseudomonads	SS	- ^y	5.52	6.46	4.66	4.52	4.84a	4.61	5.43	5.15
	CS	-	5.62	6.58	3.78	4.37	4.10b	4.07	5.26	4.83
Bacilli	SS	-	5.49a	6.88	5.28	5.28b	5.29b	5.46	5.76	5.63
	CS	-	5.14b	6.71	5.34	5.48a	5.41a	5.47	5.74	5.61

^w Microbial population was expressed as logarithmic transformation of colony formation unit g⁻¹ soil.

^x Multiple comparisons were performed using Fisher's least square difference test at significance level $\alpha = 0.05$. Values followed by same letters have no significant differences in the same column of the table.

^y data were not available.

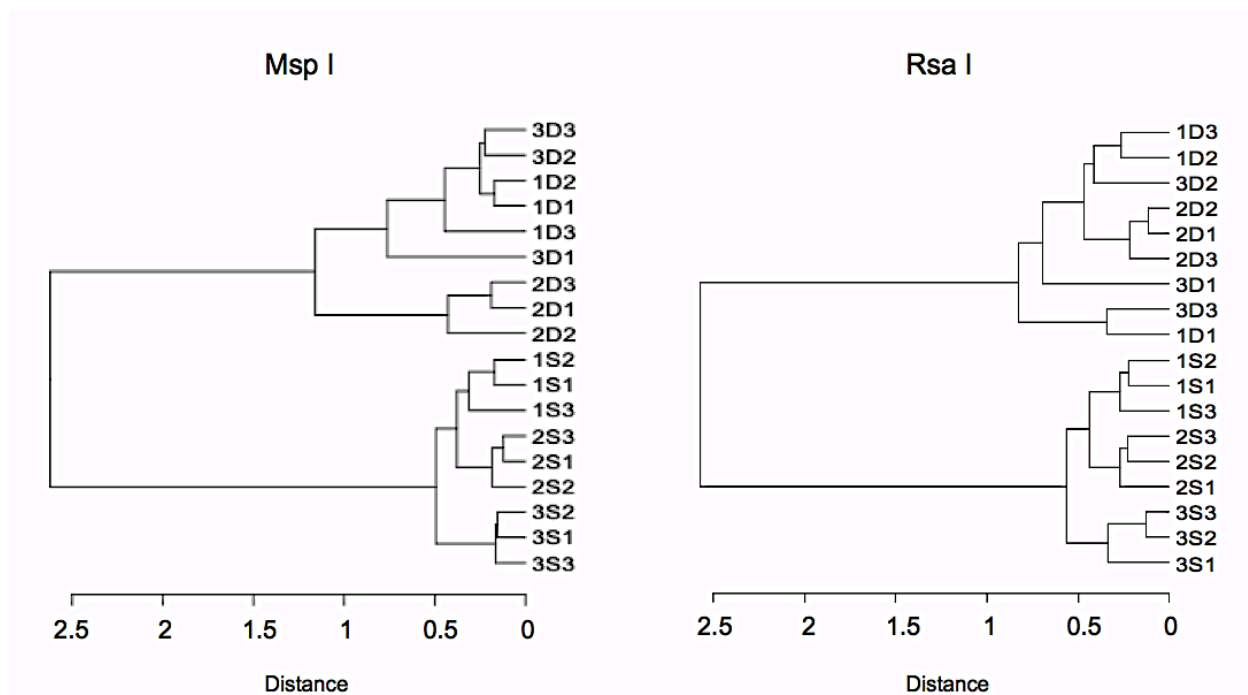


Figure S6.2. Dendrograms of terminal restriction length polymorphs. Soil samples are designated with number-letter-number format, where letter S means disease suppressive soil, and D means disease conducive soil. The number before the letter indicates soil samples and the number after the letter indicates replications of DNA extraction. DNA was digested with either *Msp* I or *Rsa* I restriction enzyme.

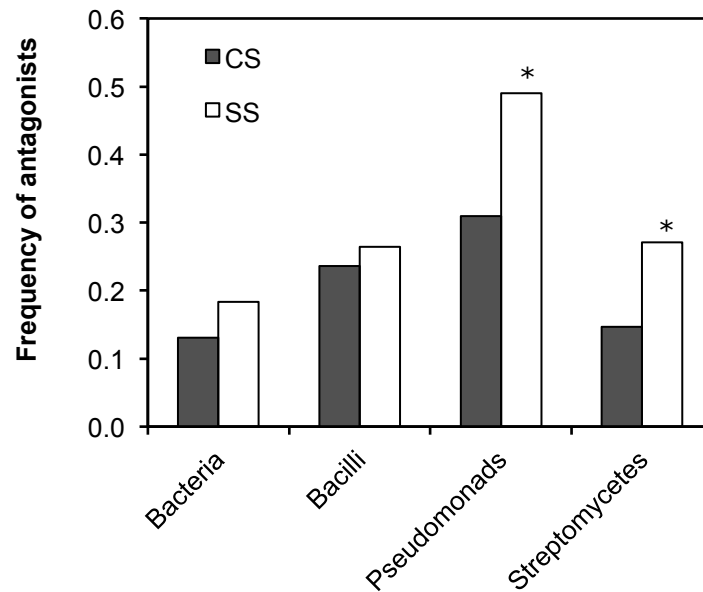


Figure S6.3. Frequency of antagonists from bulk soil (result was derived from combined data sampled on 4/10/07, 11/04/08, 7/03/09, and 5/13/10) in both disease suppressive (SS) and conducive (CS) soils. Bars over the values are standard deviation, and symbol “*” indicates significant differences between SS and CS at $\alpha = 0.05$.

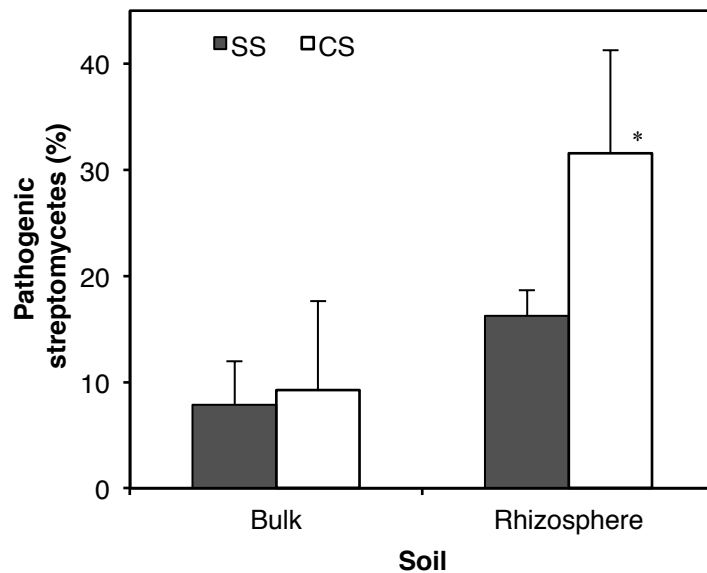


Figure S6.4. Frequency of pathogenic *Streptomyces* strains isolated from bulk and rhizosphere soils in both disease suppressive (SS) and conducive (CS) soils. Bars over the values are standard deviation, and symbol “*” indicates significant differences between SS and CS at $\alpha = 0.05$. Result was derived from combined data sampled on 7/10/07 and 5/13/10.

DISCUSSION

This study has demonstrated that soil suppression of PCS corresponds with differences in microbial community structure. The disease suppression is transferable from soil to soil, and can be reduced or eliminated by high temperature treatments. Continuous crop monoculture could be the driving force for the development of the observed disease suppression, as demonstrated previously with common scab and other disease systems (Lorang *et al.*, 1995; Weller *et al.*, 2002; Postma *et al.*, 2008).

Disease suppression was affected by temperatures higher than 60°C. The corresponding organisms for suppressiveness could be elucidated based on the maximum temperatures they can tolerate. In this study suppression does not decrease, or decreases little below 60°C, indicating that the contribution of unicellular bacteria, such as pseudomonads, to disease suppression may be limited. Loss of suppressiveness around 80°C supports the role of streptomycetes, and loss of suppressiveness at higher temperatures supports the idea that spore-forming organisms such as bacilli or other fungi may be important (Yoshida *et al.*, 2001; Weller *et al.*, 2002; Mendes *et al.*, 2011).

After narrowing the scope of organisms of interest responsible for the suppressiveness, further investigation was conducted based on the microbial community comparison between SS and CS. The successive planting of potato may have enhanced the soil microbial community and resulted in the accumulation of host specific beneficial organisms, including antagonists against plant pathogens (Borneman and Becker, 2007). In this study, the frequency of antagonistic bacteria from SS was significantly higher than from CS, particularly for streptomycetes. *Streptomyces* spp. produce a range of antibiotics (Loria *et al.*, 2006) that may contribute to disease suppression. The frequency of pathogenic *Streptomyces* in the SS in this study was less

than that in the CS, but total streptomycetes were higher in the SS. Non-pathogenic streptomycetes, such as *S. diastatochromogenes* and *S. albogriseolus*, can produce antibiotic-like compounds active against *S. scabies*, and are considered important biological components of disease suppression (Lorang *et al.*, 1995; Bowers *et al.*, 1996). Greater numbers of nonpathogenic *Streptomyces* spp. are associated with less severe common scab, suggesting an interaction between host plant and *Streptomyces* microbial communities affecting disease severity of PCS (Bowers *et al.*, 1996; Schottel *et al.*, 2001). Disease-causing strains of *S. scabies* can be controlled by nonpathogenic *Streptomyces* strains when the pathogenic and nonpathogenic isolates co-exist at a certain ratio (Hiltunen *et al.*, 2009; Postma *et al.*, 2010). This inhibition is especially important if nonpathogenic streptomycetes compete for nutrition and colonization, and can produce inhibitory compounds towards pathogens (Menzies, 1959; Schottel *et al.*, 2001).

Based on the results from treating soil at different temperatures, other possible organisms responsible for the suppressive mechanism are *Bacillus* spp., which have been shown to act as pathogen antagonists in nature and can be used as biological control agents, such as *B. subtilis* (Kim *et al.*, 2010) and *B. amyloliquefaciens* (Arguelles-Arias *et al.*, 2009). In this study, the population of bacilli in suppressive soil was higher than that in CS, while not significantly different. This is in agreement with results of pyrosequencing analysis in our companion study (Rosenzweig *et al.*, 2012). However, if the relative abundance of bacilli did not play an important role, it is possible that the species or population composition of this group is different between the two soils.

There may be other candidate microbes that could be involved in disease suppressiveness. Results from pyrosequencing analysis showed that *Lysobacter* was significantly

higher in disease suppressive soil than in the CS (Rosenzweig *et al.*, 2012). Several isolates within this genus display potential biocontrol activities and inhibit disease-causing bacterial, fungal and oomycetes (Folman *et al.*, 2003; Park *et al.*, 2008). The role of *Lysobacter* spp. in this suppressive soil remains unclear and needs further study.

Based on this study, disease suppression in the SS field is apparently due to a group of microorganisms including bacilli, fluorescent pseudomonads, and non-pathogenic streptomycetes. Results from culture-based and molecular studies taken together suggest distinct communities made up of different functional groups are capable of PCS disease suppression. Using alternative methods on the same soil, it was possible to identify a greater number of groups of microorganisms potentially contributing to disease suppressiveness (Rosenzweig *et al.*, 2012).

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